Brit. J. Pharmacol. (1965), **25**, 4–17.

THE ROLE OF IONIC INTERACTION AT THE MUSCARINIC RECEPTOR

BY

A. S. V. BURGEN

From the University Department of Pharmacology, Cambridge

(*Received August* 27, 1964)

It is commonly assumed by pharmacologists that the positively charged cationic group in such agonists as acetylcholine, noradrenaline and histamine is essential for their agonist action. The evidence in favour of this view is far from conclusive. Barlow & Hamilton (1962) examined the effect of *p*H on the activity of nicotine in producing synaptic block of the rat phrenic nerve-diaphragm preparation. When compared with the quaternary nicotine *N*-methiodide the activity of nicotine was related to the *p*H in the way to be expected if the nicotinium ion were more active than the non-protonated form, but neither the range of *p*H values studied nor the precision of the results were sufficient to establish the activity (if any) of the non-protonated nicotine relative to the nicotinium ion. Hamilton (1963) has shown a similar effect of *p*H in the action of nicotine on the frog rectus abdominus muscle, but in this case also the activity of the non-protonated form was not established. Ariens, Simonis & van Rossum (1964) were unable to discover any clear relationship between *p*H and the action of arecoline on the frog rectus, but did not extend their study over a wide enough *p*H range to obtain decisive results. Rocha e Silva (1961) examined the effect of *p*H on the action of histamine. There was a clear *p*H-dependence below *p*H 7 but the action was independent of *p*H above 7. These results are compatible with the response to histamine being dependent on a group with a $pKa \sim 7.0$. However, since the dissociation constants of the ionizable groups in histamine are pKa_1, 5.9; pKa_2, 9.7, the *p*H-dependence cannot be attributed to the change in histamine ionization and is probably due to a change in the charge on the receptors.

An alternative approach to the problem is to study the activity of analogues of the agonist in which the basic nitrogen atom has been replaced by an atom with a different electron shell so that a cation is not formed. Banister & Whittaker (1951) examined the action of 3,3-dimethylbutyl acetate, the analogue of acetylcholine in which the basic nitrogen atom has been replaced by a carbon atom. On the frog rectus, this drug had about 1/12,000th the activity of acetylcholine and, on the guinea-pig ileum, the activity was small and maximal responses were not obtained. The analogue of histamine in which the primary amino-group is replaced by a hydroxyl group, H-(2-hydroxyethyl)-imidazole, is reported to have little or no histamine-like activity (Vartiainen, 1935; Schneedorf & Ivy, 1935; Grossman, Robertson & Rosiere, 1952). These latter pieces of evidence do suggest that uncharged analogues may have some activity and it seemed that the problem merited further examination in view of its importance for the theoretical interpretation of drug-receptor interactions.

METHODS

Experiments with arecoline

Guinea-pig ileum was set up under isotonic conditions in an isolated organ-bath at 35° C in modified Krebs-Henseleit solution whose *p*H was in the range 6.05 to 9.36. In order to avoid difficulties with calcium and magnesium precipitation at the more alkaline *p*H values the concentration of these ions was reduced to one-fifth of the usual. This produced little change in the response to the reference substances, carbachol and acetylcholine. The basic solution consisted therefore of (mM): NaCl 118, KCl 4.5, $CaCl_2$ 0.5, $MgCl_2$ 0.25 and glucose 5, to which were added the following buffers [tris=2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride]:

*p*H				
6·05	NaH_2PO_4	20 mM	Na_2HPO_4	5 mM
6·70	NaH_2PO_4	12·5 mM	Na_2HPO_4	12·5 mM
7·44	Tris	30 mM	HCl	25 mM
7·58	Tris	30 mM	HCl	20 mM
7·97	Tris	30 mM	HCl	15 mM
8·23	Tris	30 mM	HCl	10 mM
8·56	Tris	30 mM	HCl	7 mM
8·72	Tris	30 mM	HCl	5 mM
8·88	Glycine	50 mM	NaOH	12·5 mM
9·36	Glycine	50 mM	NaOH	25 mM

To all solutions hexamethonium bromide (1 to 10 mg/l.) was added to block ganglionic responses.

The *p*H values of the solutions were measured at 35° C with a Pye *p*H-meter, the temperature of the solutions being maintained by circulating water from a bath at constant temperature through a water-jacket cup; all solutions were allowed adequate time to come to complete temperature equilibrium before measurements were made. Standard buffers made from B.D.H. buffer tablets were used for calibration of the *p*H-meter and the *p*H values were corrected to 35° C.

The *pK*a of arecoline was determined by titrating arecoline hydrobromide (B.D.H.) with 0.2 N-sodium hydroxide in a water-jacketed cup stirred by a magnetic stirrer. Duplicate determinations gave a value of *pK*a (35° C)=7.61.

3,3-Dimethylbutyl acetate was prepared from 3,3-dimethylbutanol (generously given by Dr V. P. Whittaker) by refluxing with an equimolar amount of acetic anhydride, washing with dilute sodium bicarbonate solution until carbon dioxide evolution had ceased, drying over anhydrous Na_2SO_4 and distilling. 3,3-Dimethylbutyl acetate distilled at 154° C and had a strong fruity odour (Birch, 1949). Dr E. W. Gill kindly confirmed the identity and purity of the ester by gas chromatography and infrared spectroscopy.

2-Dimethylaminoethyl acetate and isopentyl acetate were obtained from Kodak Ltd.

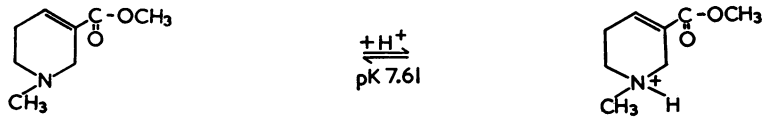
In all *p*H experiments the activity of arecoline was compared with the activity of carbachol chloride (Merck). This was used rather than acetylcholine so that hydrolysis by cholinesterase should not affect the result.

Comparisons between carbachol, acetylcholine perchlorate (Koch-Light Ltd.), 3,3-dimethylbutyl acetate, isopentyl acetate and 2-dimethylaminoethyl acetate were made either in phosphate-buffered modified Krebs solution, *p*H 6.70, containing 10 mg/l. of hexamethonium or in ordinary bicarbonate-Krebs solution, *p*H 7.25. No differences in relative potency were noted in the two solutions.

RESULTS

The activity of arecoline as a function of p*H*

In these experiments, dose/response curves were obtained on the guinea-pig ileum immersed in salt solutions ranging in *p*H from 6.05 to 9.36. In order to make allowance for any change in receptor sensitivity as a function of *p*H or the buffer salts used, a dose/response curve for carbachol was also done in each case and the activity of arecoline was expressed as a ratio to the activity of carbachol. Carbachol, being a stable quaternary



ammonium ion, can be considered to be fully ionized over the whole *p*H range studied. The results from a total of thirteen such determinations are shown in Fig. 1. It can be seen that the activity of arecoline fell off as the *p*H increased, as would be expected if the non-protonated arecoline were either inactive or very much less active than the protonated arecolinium ion. The continuous line has been drawn for the theoretical relationship expected for a substance with the *pK*a of arecoline (7.61) and in which the protonated ion had 1.42-times the molar activity of carbachol and the non-protonated form was completely

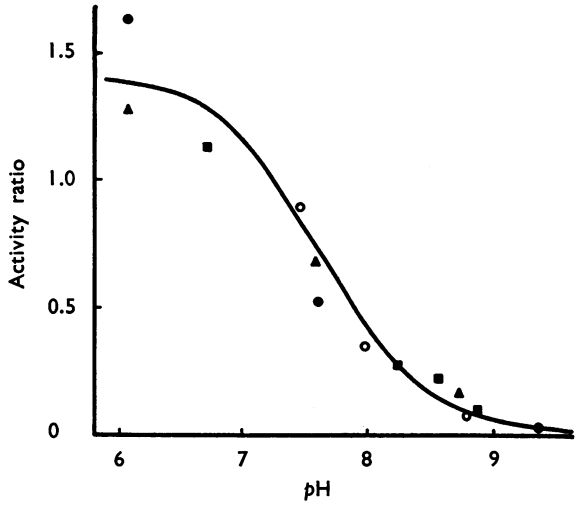


Fig. 1. The activity of arecoline in the guinea-pig ileum relative to the activity of carbachol. The line is the calculated relationship expected if the arecolinium ion had an activity 1.42-times that of carbachol and if non-protonated arecoline were inert. Ordinate: molar ratio of the activity of arecoline to that of carbachol; abscissa: *p*H of the modified Krebs solution.

inactive. It can be seen that the agreement is reasonable. In Fig. 2 the values have been plotted as activity against the calculated fraction of the arecoline which is protonated at the *p*H of the test. The data fit a linear relationship between the two parameters very satisfactorily (r=0.914). This kind of experiment does not, however, establish accurately the activity of the non-protonated arecoline because of the difficulty of working at a sufficiently high *p*H. In only one experiment was the ileum found to tolerate a *p*H higher than 9 and even in this case the variability was high so that the precision of the comparison was very poor. To establish that the activity of the non-protonated form was less 0.5% that of the protonated form it would be necessary to study the tissue at *p*H 9.61, and that it was less than 0.1% at *p*H 10.31. All we can say from the present experiment is that the activity of the non-protonated form is probably less than 2% of that of the protonated arecoline.

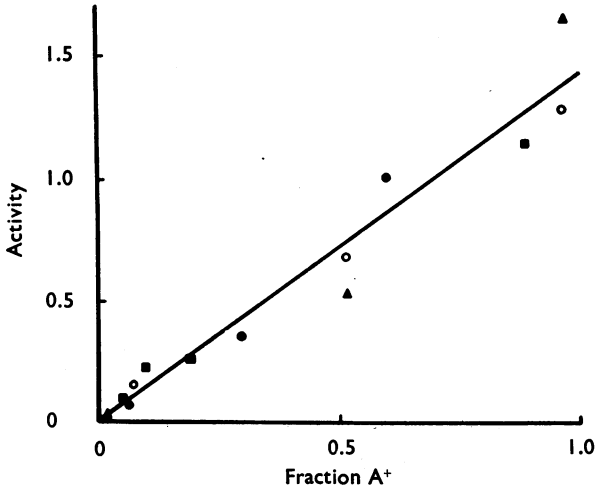
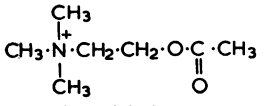


Fig. 2. The activity of arecoline related to the fraction of the arecoline that is protonated (A^+). The line is the regression expected if the activity of $A^+=1.42$ and $A=0$. The correlation coefficient, $r=0.914$. Abscissa: fraction of arecoline molecules that are protonated.

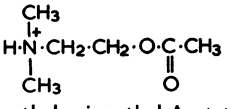
A striking feature of these experiments is the finding that the arecolinium ion has such a high activity, being 1.42-times as active as carbachol. Since acetylcholine itself was only slightly more active than carbachol in our experiments, arecoline was about 30% more active than acetylcholine. van Rossum (1962) has reported a similar activity on the rat ileum.

The activity of 3,3-*dimethylbutyl acetate*

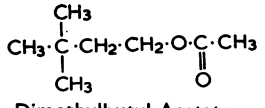
The activity of this ester was studied in a total of fifteen preparations. On one preparation, which was rather insensitive to acetylcholine, little response to the ester was obtained. In all others responses were obtained that were very similar to those produced by acetyl-



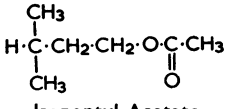
Acetylcholine



Dimethylaminoethyl Acetate



Dimethylbutyl Acetate



Isopentyl Acetate

choline (Fig. 3), although both contraction and relaxation were slightly slower. In one preparation, dimethylbutyl acetate was a partial agonist and the maximum response was only 60% of that of acetylcholine; in the remainder the maximum response was equal to, or even slightly exceeded, that of acetylcholine. The dose/response curve obtained for dimethylbutyl acetate was parallel to that of acetylcholine in all the experiments. An example of a comparison of the two substances is seen in Fig. 4. It is clear that dimethylbutyl acetate is far less active than acetylcholine and the equimolar potency ratio was 3,170 (95% range, 1,780 to 5,650). The responses to dimethylbutyl acetate were reduced

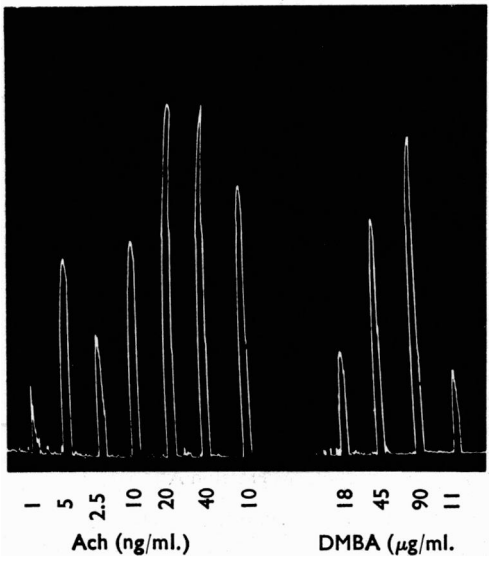


Fig. 3. Response of the guinea-pig ileum to acetylcholine perchlorate (Ach) and 3,3-dimethylbutyl acetate (DMBA). Bath volume, 10 ml.

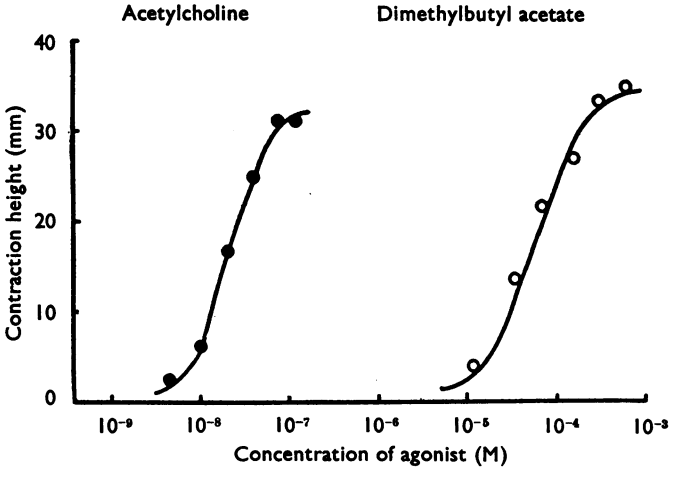


Fig. 4. Dose/response curves for acetylcholine and 3,3-dimethylbutyl acetate in guinea-pig ileum. Ordinate: height of contraction (mm); abscissa: molar concentration of agonist.

by more than 75% by exposure to 10^{-8} M-atropine for 2 min; the responses to acetylcholine were reduced to a slightly greater extent. Exposure to 10^{-7} M-atropine for 2 min completely blocked the responses to the usual doses of acetylcholine and dimethylbutyl acetate. It was not practical to determine the pA_2 of atropine against dimethylbutyl acetate because of the low potency of the ester and its limited solubility.

Activity of 2-dimethylaminoethyl acetate and isopentyl acetate

In view of the importance of the finding that dimethylbutyl acetate is an active muscarinic agent, it was felt desirable to confirm this result with another non-basic ester. We have therefore compared 2-dimethylaminoethyl acetate (the tertiary analogue of acetylcholine) and its non-basic carbon analogue isopentyl acetate. These substances were tested in phosphate Krebs solution at *p*H 6.70, at which *p*H dimethylaminoethyl acetate is nearly completely ionized (pK_a=8.63; 99% ionized). Dimethylaminoethyl acetate was found to have an equimolar potency ratio relative to acetylcholine of 120, a figure comparable to that found by Holton & Ing (1949) on the rabbit intestine. Isopentyl acetate contracted the intestine but its activity was considerably lower than that of dimethylbutyl acetate and it was not possible to give a large enough dose to obtain a maximum response owing to the low solubility of the ester in Krebs solution. A comparison at about 25% of the maximum response for acetylcholine gave an equimolar potency ratio to acetylcholine of 160,000. The effect of replacing the nitrogen atom by a carbon atom in this case is to reduce potency by a factor of 1,300. This can be regarded as only an approximate result in view of our inability to study complete dose/response curves but may be taken as confirmatory evidence that the effect of the charged group is of the same order in this case too.

Estimation of receptor reserve

Further interpretation of the results obtained with dimethylbutyl acetate depend on whether the lowered potency in the non-basic esters is to be attributed mainly to a reduction

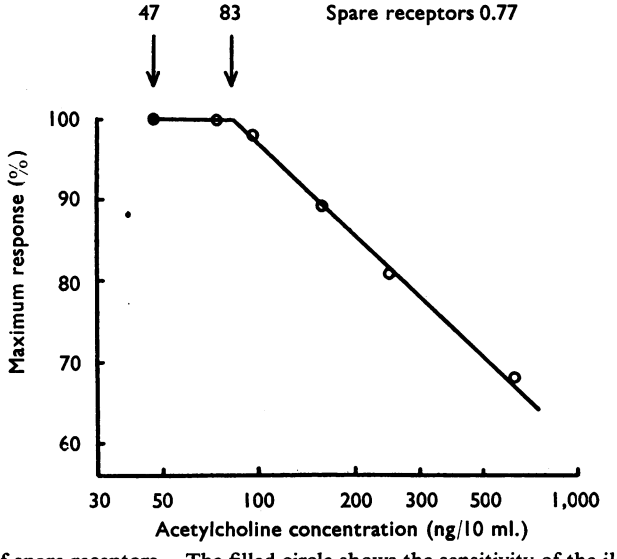


Fig. 5. Estimation of spare receptors. The filled circle shows the sensitivity of the ileum to acetylcholine before exposure to dibenamine. The empty circles show the sensitivity of the ileum after exposure to 2×10^{-5} M-dibenamine for successive periods of 4 min. Each point is derived from a complete dose/response curve. On the ordinate is plotted the maximum response obtained as a percentage of that found before treatment with dibenamine. On the abscissa is plotted the concentration of acetylcholine perchlorate (ng/10 ml.) at which half maximal responses were obtained. Spare receptors are estimated from the point at which the maximal response starts to decline which is the point where the two linear segments intersect. In this case, the spare receptors were only 77% of those occupied in the untreated ileum.

in the affinity of the ester for the receptors or to a reduced efficacy of the ester in producing agonist activity after it has combined with the receptors. Stephenson (1956) has pointed out that if there is a receptor reserve a lowered activity may be due to either of these causes. The most satisfactory method yet described for evaluating the receptor reserve is the use of the irreversible antagonist dibenamine, introduced by Nickerson (1956) for studying receptor reserves of catechol amine receptors and applied by van Rossum & Ariens (1962) and others to assessment of the reserves of acetylcholine and histamine receptors. We have used the method of these authors, with acetylcholine as the agonist. An example of the results is shown in Fig. 5. In three such studies the spare receptor ratio was 1.7, 3.0 and 3.3 respectively. These values are similar to those found by van Rossum & Ariens on the rat intestine. Since the spare receptor reserve is so small in the case of acetylcholine on the guinea-pig ileum, the low potency of the full agonist dimethylbutyl acetate must be attributable mainly to low affinity for the receptors rather than to a low efficacy.

DISCUSSION

The activity of arecoline at different *p*Hs agreed well with the assumption that the activity of the uncharged species is small in comparison with the arecolinium cation. However, it was not possible to show that the uncharged species was totally without activity. This is a limitation of the technique of using weak proton acceptors for investigating the quantitative role of ionic forces in drug-receptor interaction. The use of the carbon analogue of acetylcholine is free of this limitation and appears to indicate that while the absence of a cationic group greatly reduces the activity in muscarinic agents it is nevertheless compatible with the retention of full agonist activity. Furthermore, the fact that the guinea-pig ileum has only a modest spare receptor reserve when examined by dibenamine inhibition leads to the conclusion that the lower activity of dimethylbutyl acetate is almost wholly due to a reduced affinity for the receptors rather than to any substantial impairment in efficacy in comparison with acetylcholine. For simplicity we will assume in the rest of this discussion that the reduced activity is wholly due to a reduced affinity. We may therefore calculate the contribution of ionic association to the total free energy of formation of the drug receptor complex as:

$$\triangle F = -RT \ln \rho \qquad (1)$$

where ρ is the equimolar potency ratio of dimethylbutyl acetate and acetylcholine. Inserting the value 3,170 for ρ gives $\triangle F = -5.0$ kcals/mole.

It was pointed out by Pressman, Grossberg, Pence & Pauling (1946) that knowledge of the free energy of association of ions permits the calculation of the equilibrium distance separating the charge centres. The reasoning behind this calculation is that the work done in removing a unit charge from its equilibrium distance from another unit charge to a position infinitely distant is equal to the free energy of association. The work done is defined by the Coulomb equation:

$$\triangle F = \frac{z_1 z_2 e^2}{d_e} \qquad (2)$$

where z_1 and z_2 are ion valencies, e is the electron charge and d_e is the equilibrium distance.

Equation (2) applies to the work done in vacuum. In an aqueous electrolyte solution allowance must be made for the shielding effect of the ionic atmosphere (Debye-Hückel

effect) and also for the dielectric constant of the medium (Robinson & Stokes, 1959; Webb, 1963; Mcelwyn-Hughes, 1961). The dielectric constant of the bulk solution is not applicable here because the dielectric constant is depressed in the neighbourhood of the ion owing to restricted rotation of water molecules due to the high local field strength. Several relationships have been proposed for arriving at a correction for this effect. The most generally accepted for the range of d=3 to 10 Å is that of Conway, Bockris & Ammer (1951), in which $D=6d-7$.

Finally, some allowance needs to be made for the effect of repulsion which reduces the energy of interaction when ions approach. Correction can be made by Mie's equation (Moelwyn-Hughes, 1961). In the range we will be concerned with this effect is small and reduces the interaction energy by less than 8%. The full equation is therefore:

$$\triangle F = \frac{z_1 z_2 e^2 f_r}{d_e(6d_e-7)} \cdot \left(\frac{\exp\ [x(r_0-d_e)]}{1-xr_0}\right) \qquad (3)$$

where f_r=repulsion correction, x=Debye-Hückel constant and r_0=distance of closest approach of an atmosphere ion.

Inserting a value of $\triangle F=-5.0$ kcals/mole in this equation we arrive at a value for the equilibrium distance separating the ions d_e=3.29 Å.

The closest approach of a negative oxygen atom to the charge centre of the trimethylammonium group is possible when the oxygen atom is symmetrically in contact with the three methyl groups. Measurements on Courtauld models give a value of 3.25±0.066 Å for the distance of closest approach in this plane. The closeness of this value to that calculated from the Coulomb equation is satisfactory and suggests that the arrangement at the receptor is such that the negative oxygen atom can fit nearly perfectly into the space formed between the envelopes of the three methyl groups. That the orientation of the combination is relatively critical can be seen from measurements made of the closest approach possible when the nitrogen atom, one methyl group and the negative oxygen atom are in line. The minimum distance is found to be 4.30±0.075 Å. This extra separation between the charge centres of 1.05 Å would weaken the coulombic attraction by about 2.3 kcals/mole, and would be equivalent to lowering potency by a factor of 40. The requirement for precise orientation may explain in part why incorporating the nitrogen atom into a ring structure so infrequently leads to a molecule of high muscarinic potency; this is due to the rigidity of the structure compared with a more compliant non-cyclic side-chain. It may also partly explain the effect of substituting ethyl groups for methyl groups in the cationic head of acetylcholine. It would be expected that replacement of a single methyl group might have little effect, since it could be orientated normal to the receptor surface without interfering with attraction by the anionic group, and the only effect in this instance would be due to the lowered probability of the molecule being presented to the receptor in the acceptable orientation.

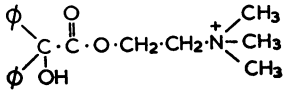
Replacement of one of the remaining methyl groups would force the cationic centre away from the anionic group by about 1.0 Å and this would reduce affinity by about 80-fold. The reduction in activity found experimentally (600-fold) (Holton & Ing, 1949) is greater than this and presumably points to other factors in addition to reduced electrostatic interaction contributing to the lowered affinity when methyl groups are replaced by ethyl groups.

In our calculations thus far we have made the assumption that the charge on the cation is a point charge localized to the centre of the nitrogen atom. However, atomic orbital theory permits the calculation of the delocalization of the charge from the nitrogen atom on to the covalently attached carbon atoms. The calculations of Inouye, Shinagawa & Takaishi (1963) ascribe 85% of the formal charge to the nitrogen atom and 3.7% of the charge to each methyl carbon atom. If we estimate the free energy of ionic attraction by summing the interactions of these four partial charges with a unit negative point charge, and calculate the equilibrium distance for a free energy of interaction of 5.0 kcals/mole, the value found is d_e=3.33 Å. The increase of d_e by 0.04 Å is too small to make any important differences to our conclusions and it appears adequate therefore to adhere to the simpler point-charge model.

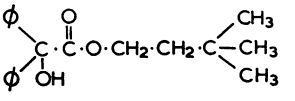
Similar calculations of the distance of approach of dimethylaminoethyl acetate based on the relative effectiveness of this drug and isopentyl acetate give an equilibrium distance of 3.40 Å. It is doubtful if any significance should be attached to this, since complete dose/response curves were not obtained.

It should be pointed out that the calculation of equilibrium distance from the free energy of ionic interaction does not carry with it the necessary implication that the equilibrium distance is a *result* of the ionic interaction and hence that the separation of the isosteric carbon atom in dimethylbutyl acetate from the receptor is necessarily greater than the separation of the nitrogen atom in acetylcholine. It is, however, likely that this is the case.

The effect of charge on association between acetylcholine and acetylcholinesterase has been estimated by Wilson (1952) and by Cohen & Oosterbaan (1963). The mean effect of the charge was to increase affinity by a factor of 17, corresponding to a coulombic free energy of −1.75 kcals/mole. Pressman *et al.* (1946) compared the affinity of trimethylphenylammonium and its uncharged homologue t-butylbenzene for antibodies against azophenyltrimethylammonium. The affinity ratio was 8 and $\triangle F$=−1.15 kcals/mole. However, the most relevant report on the effect of molecular charge to the present study is that of Funcke, Rekker, Ernsting, Tersteege & Nauta (1959). These authors prepared the



Benzoylcholine



Benziloyldimethylbutanol

benzilic ester of 3,3-dimethylbutanol and found that it was a potent atropine-like compound with 32% of the activity of atropine. From the published data on the relative potency of atropine and (2-benziloyloxyethyl)trimethylammonium (benzoylcholine) (Ing, Dawes & Wajda, 1945; Barlow, Scott & Stephenson, 1963; Ariens, 1964) it can be estimated that the carbon-for-nitrogen replacement reduced affinity by a factor of about 14. The free energy due to ionic interaction in this case is $\triangle F$=−1.6 kcals/mole. This suggests that ionic interaction is considerably less important in the antagonists than in the agonists.

Inserting this free-energy value in equation (3) we find that the equilibrium distance between the charge centre in benzoylcholine and that in the receptor is 4.96 Å, which is some 1.67 Å greater than that for acetylcholine. This extra distance is greater even than that expected from interaction with the nitrogen atom, methyl-carbon atom and the receptor ion in line (see above).

It was pointed out above that the reduction of agonist activity when ethyl groups are substituted for methyl groups on the cationic end of acetylcholine could in part be attributed to a reduced ionic interaction imposed by the interposition of an extra carbon atom between the charge centres. This would not be the case with the antagonist benziloylcholine if the charged groups are not in contact, and we would therefore expect that ethyl replacement would either be without effect or, indeed, that the potency might be slightly increased if the extra carbon atom were able to add to affinity through Van der Waal's or hydrophobic interaction with the receptor site.

Ing *et al.* (1945) found that replacement of one methyl group of benziloylcholine by an ethyl group increased the potency by a factor of 2.5 but replacement of the other methyl groups had little effect. Barlow *et al.* (1963), in a very careful study of five series of cholinolytic substance, found an effect of similar magnitude to this in all cases. Furthermore, it is well known that in cholinolytics replacement of choline-like structures by piperidine, pyrrolidine or tropane is compatible with high activity (compare with Barlow, 1964). This contrasts strikingly with the inactive products that result when the corresponding substitution is made in muscarinic agonists. This difference in response to substitution at the cationic end of the molecule in agonists and antagonists is an expected consequence of the different equilibrium distance of approach to the receptor for the two groups of compounds.

A further observation suggesting a less critical apposition between drug and receptor at the choline end of the molecule in antagonists as compared to agonists is the comparison of the activity of the optical enantiomers of methacholine (acetyl-β-methylcholine) and benziloyl-β-methylcholine (Ellenbroek & van Rossum, 1960; Ellenbroek, 1963). (+)-Methacholine is 300-times as active a muscarinic substance as (−)-methacholine; however, there is no difference in potency between the antagonists (+)- and (−)-benziloyl-β-methylcholine. This suggests that, whereas the extra bulk of a methyl group compared with a hydrogen atom (a protusion of ~0.82 Å) can seriously interfere with the combination of the agonist with the receptor, the same group can be accommodated without appreciable change in activity in the antagonist.

On the other hand, it is known that in the acyl cholines there is a rapid attenuation of muscarinic potency as the carbon chain on the carboxyl group is lengthened, so that butyrylcholine has less than 1% of the activity of acetylcholine on the guinea-pig intestine (Wurzel, 1959), but, with further lengthening of the acyl group, agonist activity becomes replaced by increasingly potent antagonist action (Schneider & Timms, 1957). A further substantial increase in antagonist potency results when a phenyl group is introduced into the acyl methyl group, as in diphenylacetylcholine and benziloylcholine (Barlow *et al.*, 1963).

This dual effect of chain lengthening at the acyl end of the molecule can be explained plausibly if the reduced agonist action is due to the larger bulk of the acyl group preventing good apposition of the acyl end of the molecule with the receptor. The development of antagonist action could be due to the increasing role of Van der Waal's and hydrophobic bonding of the acyl end of the molecule to the receptor. It is clear that in such a strong antagonist as benziloylcholine the contribution of the charged group to the total affinity of the molecule is relatively small, being only −1.6 kcals/mole out of a total free energy of −12.1 kcals/mole (or 13%). Much of the remaining energy must be derived from the

acyl part of the molecule. Strong association at the acyl end suggests that in order to generate such strong forces the groups at the acyl end of the molecule must come into close approximation with the receptor surface and that, therefore, steric effects at this end of the molecule should be important. That this is the case is shown by the activity of (−)-hyoscyamine, which has 110-times the affinity of (+)-hyoscyamine, while (−)-benzhexol has 160-times the affinity of (+)-benzhexol when tested on the rabbit ileum (Long, Luduena, Tullar & Lands, 1956). The latter case is especially remarkable because the optical asymmetry in benzhexol is due to the possession of a phenyl group and a cyclohexyl group which differ rather little in size and in their capacity to form dispersion interactions but the hydrophobic bond-forming capacity of cyclohexane is appreciably greater than that of benzene. These differences are sufficiently small to make it likely that the difference of affinity of the enantiomers of benzhexol must be dependent on a very close fit of (−)-benzhexol to the receptor.

The sum total of the evidence presented suggests that, whereas the cationic end of acetylcholine plays a dominating role in the production of agonist activity, this is less so for antagonists where the dominating role is taken over by the large substituents in the acyl end and indeed that the preponderance of the latter effect prevents the cationic group coming into an optimal relationship with the anionic group in the receptor. In Fig. 6 the way in which this might happen is shown diagrammatically. One of the abiding problems of pharmacology is the subtle distinction in drug structure which determines whether a drug shows activity as an agonist or as an antagonist. It is tempting to believe that the difference in intimacy of association discussed in this paper provides a clue to understanding

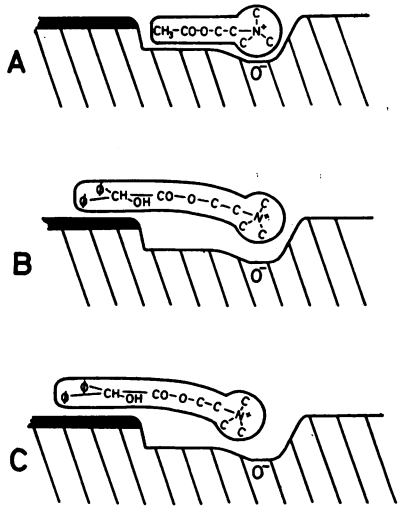


Fig. 6. Diagrammatic representation of interaction between drug and receptor. The dark area at the left is assumed to be capable of giving strong hydrophobic interactions. **A**: Relation of acetylcholine to the receptors. The molecule forms a close association. Note the close approximation of the trimethylammonium group to the anionic site on the receptor. **B**: One possible mode of interaction with benziloylcholine. The benzene rings interact strongly with the " hydrophobic " area and elevate the chain above the normal equilibrium position seen in **A**. **C**: A second possibility is that the strong hydrophobic association leads to translation of the molecule to the left of the figure, so withdrawing the quaternary group out of close contact with the anionic site in the receptor.

this mystery. If we accept as likely that agonist action results from a conformation change in the receptor induced by combination with the drug it does not seem unreasonable to postulate that this conformation change is only likely to occur when the association with the relevant part of the drug (that is, that equivalent to acetylcholine) is intimate enough so that the available energy of interaction is also large and can reach a value sufficient to cause a redistribution of tertiary structure in the receptor. In antagonists it appears that most of the interaction energy occurs between a part of the molecule not present in the agonists and a part of the receptor surface not participating in interaction with the agonists. This part of the receptor may not be susceptible to a conformational change and the association with the responsive part of the receptor may be inadequate to induce a conformation change.

One of the most stimulating contemporary theories of drug antagonism is that of Paton (1961). The two necessary parts of his theory are firstly that agonists are substances which, after association with the receptors, dissociate rapidly so that a high frequency of associations between agonists and receptors is possible, and secondly that antagonists are postulated as being substances which dissociate slowly from the receptors, and hence have a low turnover rate. It is postulated that pharmacological agonist activity is dependent on the rate of turnover. It can be seen that the theory discussed in this paper is perfectly compatible with Paton's; it does not absolutely require that dissociation of antagonists should be slow, but, if this were not so, considerably more partial agonist action would be expected with atropine-like drugs than is commonly seen. Otherwise this theory does not discriminate between the turnover and occupation theories, although the many factors adduced by Paton in favour of the turnover theory make it seem a very plausible one at the present time. However antagonists combine with the receptor it will be necessary to explain why the activation energy of dissociation is so high as to make it a rather improbable event.

We have pointed out above that Barlow and his colleagues (Barlow *et al.*, 1963) have made a thorough study of the effect of ethyl substitution in choline esters and, in addition to studying the activity as cholinolytics of the benzilic, diphenylacetic and other series, they have also studied the effect of ethyl substitution in the agonists acetylcholine and (2-ethoxyethyl)trimethylammonium. Their experimental work confirms much other work in the literature and puts these relationships on an excellently quantitative level. Briefly summarized, their findings amount to: (1) in agonists, ethyl groups reduce activity, the first group substituted having a small effect, the second a large effect and the third being practically ineffective; and (2) in antagonists, a single ethyl group increases affinity by two- to threefold but substitution of further ethyl groups has little effect but if anything decreases affinity.

They interpret these findings in the following way: the combining forces at the cationic end of the molecule are the same in both agonists and antagonists but the result of combination is modified by the " efficacy," that is the probability of an agonist type of activity being produced after a drug-receptor interaction. Pure antagonists by definition have an efficacy of zero and therefore the effect of ethyl substitution in these substances is a pure measure of the effect of substitution in the molecule on affinity. They postulate that these changes in affinity are also found in the agonists and the different order of agonist activity found is due to an effect of ethyl substitution on efficacy. It can be seen that the logical basis of this approach rests entirely on the assumption that the combining force at the

cationic end of the molecule is the same in the agonists and antagonists. We have argued in this paper that this premise is probably unsound and that the affinity of the cationic end of the molecules in antagonists is far smaller than in the agonists. It is, however, true that our argument does depend on the validity of evaluating spare receptors by dibenamine. This appears to be the best procedure at present available but there is undoubtedly need for other methods for settling this point. Even if the reliability of the dibenamine method is questioned, the pragmatic value of the ideas presented in this paper depend on the reasonable way in which they explain several intractable problems concerning structure-activity relationships of the muscarinic agonist-antagonist group. Further experimental evaluation of this theory must obviously be carried out both in relationship to the muscarinic and other receptors before it can find acceptance as a general theory of drug action.

In conclusion it should be pointed out that a carbon atom is a rather satisfactory volume isostere for a nitrogen atom. The tetrahedral bond arrangement is identical in the two atoms and the covalent radius of the carbon atom is only 0.07 Å greater than that of the nitrogen atom.

SUMMARY

1. The activity of arecoline was compared with that of carbachol on the guinea-pig ileum at *p*H 6.05 to 9.36. The results are consistent with non-protonated arecoline having an activity less than 2% of the protonated arecolinium ion.

2. The uncharged carbon analogue of acetylcholine, 3,3-dimethylbutyl acetate, had typical acetylcholine-like activity on the ileum but its potency was 3,170-times less than acetylcholine itself. Isopentyl acetate was also active and was 1,300-times less active than its charged relative 2-dimethylaminoethyl acetate.

3. Measurement of receptor reserve in the guinea-pig ileum with dibenamine shows it to be small. The low activity of dimethylbutyl acetate is mainly due to a low affinity for the receptors.

4. Calculations have been made of the equilibrium distance of the quaternary nitrogen atom from a negatively charged group in the receptor based on the difference of free energy of interaction of acetylcholine and dimethylbutyl acetate and, using the Coulomb equation, give a value of 3.29 Å, which is similar to the distance of closest approach measured on models.

5. Experimental results available in the literature were used to calculate the equilibrium distance of the charged group in benziloylcholine from the negative group in the receptor. The value found was 4.96 Å.

6. These values are compatible with the assumption that in antagonists the strong association of the massive acyl end of the molecule with the receptor prevents the optimal fitting of the trimethylammonium end of the antagonists with the anionic site of the receptor.

6. It is suggested that the distinction between agonists and antagonists may lie in the greater intimacy of association with the receptor of the agonists and their consequent ability to induce conformation changes in the receptor.

REFERENCES

ARIENS, E. J. (1964). Ed. of *Molecular Pharmacology*. New York: Academic Press.

ARIENS, E. J., SIMONIS, A. M. & VAN ROSSUM, J. M. (1964). In *Molecular Pharmacology*, ed. ARIENS, E. J., p. 382. New York: Academic Press.

BANISTER, J. & WHITTAKER, V. P. (1951). Pharmacological activity of the carbon analogue of acetylcholine. *Nature (Lond.)*, **167**, 605.

BARLOW, R. B. (1964). *Introduction to Chemical Pharmacology*. London: Methuen.

BARLOW, R. B. & HAMILTON, J. T. (1962). Effects of *p*H on the activity of nicotine and nicotine methiodide on the rat diaphragm preparation. *Brit. J. Pharmacol.*, **18**, 543–549.

BARLOW, R. B., SCOTT, K. A. & STEPHENSON, R. P. (1963). An attempt to study the effects of chemical structure on the affinity and efficacy of compounds related to acetylcholine. *Brit. J. Pharmacol.*, **21**, 509–522.

BIRCH, A. J. (1949). The preparation of some esters of 3:3 dimethylbutanol and 3:3 dimethylpentanol. *J. chem. Soc.*, 2721–2722.

COHEN, J. A. & OOSTERBAAN, R. A. (1963). The active site of acetylcholinesterase and related esterases and its relativity towards substrates and inhibitors. In *Heffter's Hb. der. exp. Pharm.*, vol. 15, pp. 299–373. Berlin: Springer.

CONWAY, B. E., BOCKRIS, J. O. & AMMER, I. A. (1951). The dielectric constant of the solution in the diffuse and Helmholtz double layers at a charged interface in aqueous solution. *Trans. Faraday Soc.*, **47**, 756–766.

ELLENBROEK, B. W. J. (1963). Ph.D. thesis quoted in VAN ROSSUM, J. M. (1963). The relation between chemical structure and biological activity. *J. Pharm. Pharmacol.*, **15**, 285–316.

ELLENBROEK, B. W. J. & VAN ROSSUM, J. M. (1960). Absolute configuration and parasympathomimetic activity. *Arch. int. Pharmacodyn.*, **125**, 216–220.

FUNCKE, A. B. H., REKKER, R. F., ERNSTING, M. J. E., TERSTEEGE, H. M. & NAUTA, W. T. (1959). Untersuchungen über Spasmolytica; V Benzilsäureester. *Arzneimittel-Forsch.*, **9**, 573–576.

GROSSMAN, M. I., ROBERTSON, C. & ROSIERE, C. E. (1952). The effect of some compounds related to histamine on gastric acid secretion. *J. Pharmacol. exp. Ther.*, **104**, 277–283.

HAMILTON, J. T. (1963). The influence of pH on the activity of nicotine at the neuromuscular junction. *Canad. J. Biochem.*, **41**, 283–289.

HOLTON, P. & ING, H. R. (1949). The specificity of the trimethylammonium group in acetylcholine. *Brit. J. Pharmacol.*, **4**, 190–196.

ING, H. R., DAWES, G. S. & WAJDA, I. (1945). Synthetic substitutes for atropine. *J. Pharmacol. exp. Ther.*, **85**, 85–102.

INOUYE, A., SHINAGAWAI Y. & TAKAISHI, Y. (1963). Note on the pharmacological role of "cationic head" of acetylcholine and its congeners in the light of its electronic structure. *Arch. int. Pharmacodyn.*, **145**, 546–552.

LONG, J. P., LUDUENA, F. P., TULLAR, B. F. & LANDS, A. M. (1956). Stereochemical factors involved in cholinolytic activity. *J. Pharmacol. exp. Ther.*, **117**, 29–37.

MOELWYN-HUGHES, E. A. (1961). *Physical Chemistry*, 2nd ed. Oxford: Pergamon.

NICKERSON, M. (1956). Receptor occupancy and tissue response. *Nature (Lond.)*, **178**, 697–698.

PATON, W. D. M. (1961). A theory of drug action based on the rate of drug receptor combination. *Proc. roy. Soc.* B, **154**, 21–69.

PRESSMAN, D., GROSSBERG, A. L., PENCE, L. H. & PAULING, L. (1946). The reactions of antiserum homologous to the p-azophenyltri-methylammonium group. *J. Amer. chem. Soc.*, **68**, 250–255.

ROBINSON, R. A. & STOKES, R. H. (1959). *Electrolyte Solutions*, 2nd ed. London: Butterworth.

ROCHA E SILVA, M. (1961). On the nature of the receptors for histamine. *Chemotherapia (Basel)*, **3**, 544–559.

SCHNEEDORF, J. G. & IVY, A. C. (1935). Effect of methyl histamine and hydroxyethylglyoxaline on gastric secretion and blood pressure in the dog. *Proc. Soc. exp. Biol. (N.Y.)*, **32**, 777–778.

SCHNEIDER, R. & TIMMS, A. R. (1957). Some aspects of the pharmacology of an homologous series of choline esters of fatty acids. *Brit. J. Pharmacol.*, **12**, 30–38.

STEPHENSON, R. P. (1956). A modification of receptor theory. *Brit. J. Pharmacol.*, **11**, 379–393.

VAN ROSSUM, J. M. (1962). Mechanism of action and structure-action relationship of tertiary ammonium salts. *Arch. int. Pharmacodyn.*, **140**, 592–605.

VAN ROSSUM, J. M. & ARIENS, E. J. (1962). Theories on drug action and a quantitative approach to spare receptors and threshold values. *Arch. int. Pharmacodyn.*, **136**, 385–413.

VARTIAINEN, A. (1935). The action of certain new histamine derivatives. *J. Pharmacol. exp. Ther.*, **54**, 265–282.

WEBB, J. L. (1963). *Enzyme and Metabolic Inhibitors*, vol. 1. New York: Academic Press.

WILSON, I. B. (1952). Further studies of binding forces. *J. biol. Chem.*, **197**, 215–225.

WURZEL, M. (1959). A suggested mechanism for the action of choline esters on animal organs inferred from a study of the effect of choline, β-methylcholine and thiocholine esters. *Experientia (Basel)*, **15**, 430–433.