The Structural Features Determining the Affinity of Haptens for an Antibody Binding Acetylcholine

H. F. MARLOW,[1] J. C. METCALFE,[2] AND A. S. V. BURGEN

Medical Research Council, Molecular Pharmacology Research Unit, Cambridge, England

(Received August 27, 1968)

SUMMARY

An antibody prepared against albumin-azophenoxycholine binds tetramethylammonium and other simple aliphatic cations. In choline and acetylcholine, about half the binding is due to ion-ion interaction, and the rest to dispersion interaction. The ionic contribution is reduced in benzilylcholine and probably in the larger alkylammonium ions.

In the alkyltrimethylammonium series, binding energy increases with chain length. Similar binding energy is found with aromatic or alicyclic side chains with equivalent numbers of carbon atoms. The results are compatible with a cation-binding site lying in a pit or groove in the antibody, with the side chain associating with a superficial area.

INTRODUCTION

We have shown previously that an antibody directed against albumin-azophenoxycholine reacts with a wide range of drugs which also interact with pharmacological receptors for acetylcholine (1). Among these drugs were the quaternary cations tetramethylammonium and tetraethylammonium, which, because of their symmetry and simplicity, provide a good starting point for a systematic study of the relationship between structure and affinity.

It is generally agreed that a total description of a molecular interaction should be possible in terms of a restricted number of interatomic forces which can be defined on the basis of the three-dimensional disposition of the atoms concerned. In the absence of detailed three-dimensional information about the binding site in the antibody (or receptor sites), one is reduced to making inferences about the nature of the site from the effect on the free energy of interaction of homologous and other alterations in the structure of the interacting small molecule. There are a number of difficulties inherent in this approach. First, it is very difficult to proceed without the assumption that a linear addition of force components at point source(s) is an allowable approximation for interactions that are really occurring in a complex space-time contour. Indeed, it is not unlikely that it is precisely the spatial distribution of forces in the complex which may be of great significance in determining subtleties of drug-receptor interactions. It is also assumed that only one unique atomic arrangement in a complex is possible, whereas it is likely in many cases that more than one type of complex can occur and that the over-all free energy is a mean due to time sharing between the component types. Rather similar considerations apply to the availability of multiple conformational states of the interacting molecule.

It is particularly attractive for these reasons to use as a starting point a molecule

We are grateful to the Smith Kline & French Foundation for generous support during part of this study.

[1] Present address, Department of Pharmacology, University of Stellenbosch Medical School, Karl Bremer Hospital, Bellville, South Africa.

[2] Imperial Chemical Industries Research Fellow.

such as tetramethylammonium, which offers the minimum of ambiguities. It is small and spherically symmetrical, with four chemically equivalent methyl groups disposed around a central nitrogen bearing a unit charge. No significant conformational variants are present. From this starting point we can consider the effects of symmetrical and unsymmetrical substitutions.

METHODS

The preparation of antibody and the determination of affinity constants by fluorescence titration have been described previously (1). In all cases affinities were determined competitively against the quenching hapten 4-hydroxyphenylazophenoxycholine. In the case of tetramethylammonium, tetraethylammonium, and decamethonium, affinities were also determined by equilibrium dialysis with ^{14}C-labeled drugs. There was close agreement between the results obtained by this method and by fluorescence titration.

RESULTS

Comparison of cations with uncharged species. In Table 1 the affinities of choline and two choline esters are shown in comparison with their uncharged analogues in which the quaternary nitrogen has been replaced by carbon. These are 3,3-dimethylbutan-1-ol and its corresponding esters. The affinities of choline and acetylcholine are close to 100 times greater than those of the uncharged analogues. Between benzilylcholine and its uncharged analogue the affinity ratio is only 11. The comparison of charged with uncharged pairs has been used previously to obtain an estimate of the interaction distance between the quaternary nitrogen and a presumed negatively charged oxygen (probably that of an aliphatic carboxylate) (2, 3) in the antibody against phenyltrimethylammonium, in acetylcholinesterase (4, 5), and in muscarinic receptors of smooth muscle (6).

The assumption made is that in the charged analogue the free energy of complex formation is derived from the linear sum of a component due to ion pair formation, and to other forces which are mainly dispersive in character, whereas the residual free energy of the complex formed by the uncharged analogue is due to the unperturbed operation of the remaining forces. The difference in free energy in the two cases is therefore the free energy of ion pair formation. For choline, the results in Table 1 show that the contribution of the ion interaction is −2.81 kcal/mole. The assumption that the residual forces remain unperturbed cannot be accepted without examination. If we set up an equation of the Lennard-Jones type, in which three components contributing to the total free energy, i.e., ion-ion attraction and Van der Waals attraction and repulsion, are included, one can examine the effect of withdrawal of the ionic component on (a) the equilibrium internuclear distance and (b) the free energy of interaction due to dispersion. Using realistic parameters, i.e., that the total free energy is −6 kcal/mole and the equilibrium internuclear distance for choline is 4 A (see below), we find that

TABLE 1
A comparison of affinity of charged and uncharged analogues

Compound	K	$-\Delta F$	Difference in ΔF ($a - b$)
	$10^{-3}M^{-1}$	*kcals/mole*	
Choline	25.2	6.00	2.81
3,3-Dimethylbutan-1-ol	0.22	3.19	
Acetylcholine	46.8	6.36	2.71
3,3-Dimethylbutan-1-acetate	0.48	3.65	
Benzilylcholine	84.2	6.71	1.42
3,3-Dimethylbutan-1-benzilate	7.6	5.29	

if the real value of the ionic component is −3 kcal/mole, on its removal the internuclear distance increases by 0.13 A and the net dispersion free energy increases by −0.12 kcal/mole to −3.12 kcal/mole. This effect therefore leads to a small underestimation of the ionic component. We can correct the value for choline by addition of −0.12 kcal/mole to give a corrected value of the ionic component of −2.93 kcal/mole, and a corresponding correction brings the ionic component of acetylcholine to −2.83 kcal/mole. The correction decreases as the fraction of the total free energy due to ionic interaction decreases and, in the case of benzilylcholine, is only 0.03 kcal/mole, giving a corrected value of −1.45 kcal/mole. These values are summarized in Table 2.

over-all interaction, compared with that which would be due to the purely local interaction centered on the charged nitrogen atom. In this case the likelihood that the removal of the charge will cause a greater translation of the quaternary carbon, taking it beyond its equilibrium distance, must be considered. One cannot be certain whether this will lead to a greater or lesser correction to the free energy value, but, as pointed out above, at a 5-A separation such a correction is likely to be small.

A second estimate of the magnitude of the ionic interaction is obtained from the affinity of the ammonium ion with a ΔF of −2.73 kcal/mole. The dispersion interaction of the ammonium protons is likely to be very small, probably not more than

TABLE 2
Assessment of the ionic component of complex formations

Compound	$-\Delta F$ total	$-\Delta F$ ionic	$-\Delta F$ "dispersion"	d_e[a]
		kcals/mole		*A*
Choline	6.00	2.93	3.17	3.97
Acetylcholine	6.36	2.80	3.56	4.06
Benzilylcholine	6.71	1.45	5.26	5.07

[a] Equilibrium internuclear distance between ionic centers.

Estimates of internuclear distance can be made by substitution into a Coulomb equation, in which allowance is made for the Debye-Hückel effect and for dielectric polarization (3). The correction for the latter is approximate, so that we can only regard the results obtained as being useful for comparative purposes. The calculated values for choline and acetylcholine are close to 4 A, but the value for benzilylcholine is nearly 5 A (Table 2). A similarly large charge separation was calculated for benzilylcholine when interacting with the muscarinic receptor as an acetylcholine antagonist (4). It was suggested in that case that the extra binding forces exerted by the acyl end of the molecule were in competition with the forces interacting in the neighborhood of the charged group, and that the result of this balance of forces was a withdrawal of the charged group to an equilibrium distance determined by the

−0.2 kcal/mole, so that we can estimate the ionic interaction to be of the order of −2.5 kcal/mole, which is similar in magnitude to that for choline and acetylcholine.

Small quaternary cations. The results obtained with substitutions in tetramethylammonium are shown in Figs. 1 and 2. They can be interpreted more readily if one takes into account the effect on (a) the distance separating charge centers and (b) the change in potential dispersion contribution, which may be considered to be roughly proportional to the molecular weight of the cation.

The progressive loss of affinity with increasing proton substitution is thus due mainly to the loss of dispersion contributions. On the other hand, we can expect with larger alkyl groups that the bulk of the group will prevent close approach of the charges and hence reduce the ionic contribution, but at the same time will

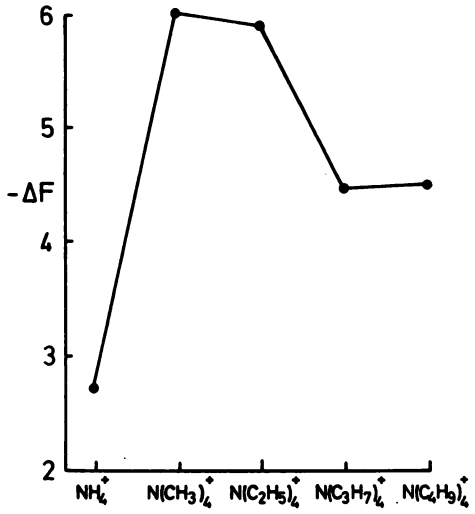


FIG. 1. *Free energy of binding of symmetrical ammonium ions with the antibody (pH 7.2; 25°)*

increase the potential dispersion interaction. The equilibrium distance between charge centers is essentially limited by repulsive encroachment on the Van der Waals envelopes of the interacting molecules. We have estimated earlier that for choline this

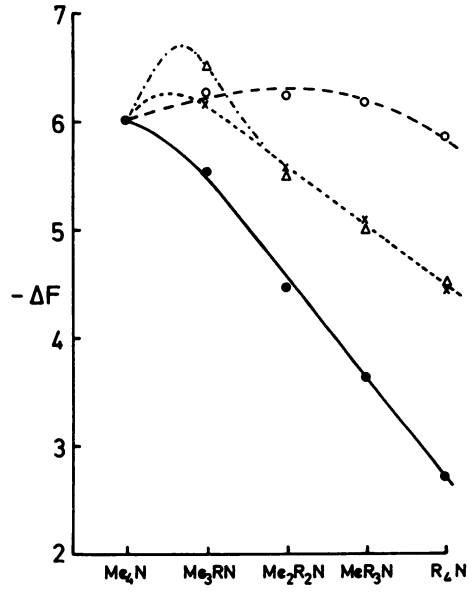


FIG. 2. *Free energy of binding of partially substituted methylammonium cations with the antibody* ●——●, R = H; O- - -O, R = C_2H_5; × ----- ×, R = *n* C_3H_7; Δ- · - ·Δ, R = *n* C_4H_9.

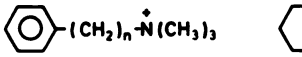
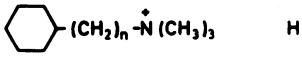
distance is about 4.0 A. With a similar trimethylammonium grouping, it is likely that the spacing of tetramethylammonium would be very similar. As measured on Corey-Pauling-Koltun molecular models, the radius of tetramethylammonium is 3.0 A. By comparison, the tetraethylammonium cation has a radius of 3.62 A, and this increased internuclear spacing causes a reduction of the free energy due to charge interaction from −3.0 kcal/mole in tetramethylammonium to about −1.9 kcal/mole in tetraethylammonium, with a corresponding increase in the dispersion interaction to 4.0 kcal/mole. In the case of tetrapropylammonium, the measured ionic radius was 4.60 A, and the calculated ionic free energy was −1.1 kcal/mole. The estimated dispersion free energy is thus −3.4 kcal/mole and is smaller than that of tetraethylammonium. This does not agree with the original proposition that the dispersion energy ought to be approximately proportional to the mass of the cation. It seems probable that we must take into account a further factor, namely, the precision with which the cation can fit into the binding site. If the size of the larger cations leads to some measure of misfit, the distance of approach will be increased and neither ionic nor dispersion energies will reach their potential values. This is what appears to be happening with tetrapropyl- and tetrabutylammonium.

It is interesting that the monosubstitution of tetramethylammonium by a larger alkyl group increases affinity, apparently as a result of asymmetry of the binding site, which may be conveniently subdivided into a site for the onium group and for a molecular chain. This feature is explored in detail in the next section.

The alkyltrimethylammoniums. With increasing length of the alkyl chain, the affinity generally increases (Fig. 3). Dodecyltrimethylammonium has 10 times the affinity of tetramethylammonium. The results presented in Fig. 3 show that this is not a simple response to increasing chain length, since there appear to be breaks between C_2 and C_3 and between C_5 and C_6.

In the initial portion of the three seg-

TABLE 3
A comparison of the effects of aromatic, alicyclic, and aliphatic groups on affinity

n			
	-ΔF	-ΔF	-ΔF
0	6·50	6·76	6·78
1	7·14	6·89	6·95
2	6·92	7·13	7·13
3	7·13	7·19	7·22

ments, the increments of ΔF per CH_2 group are respectively −0.36, −0.30, and −0.18 kcal/mole. These are relatively small compared with the contribution of the methyl groups around the cation (−0.88 kcal/methyl), and it is clear that there is no significant specificity as to length of the chain. If the contribution of the alkyl chain is to be regarded as largely "hydrophobic," that is, a free energy consequence of transfer of the chain from an aqueous to an anhydrous environment, it will be appreciated that the methylene increment is smaller than is found in partition between water and a nonpolar solvent (5, 7). In this case the free energy change is usually −0.6 to −0.8 kcal/mole/CH_2. This suggests that the alkyl chain associates with a surface feature of the antibody in such a way that it lies in the interface between the aqueous phase and a partially hydrophobic surface. The discontinuities in the chain length series are possibly due to the nature of the fit between the chain and the protein surface.

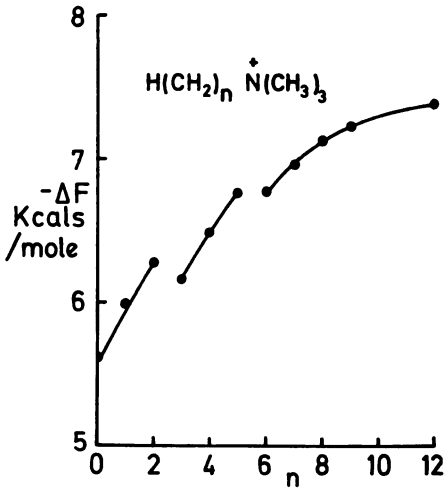


FIG. 3. *Free energy of binding of alkyltrimethylammonium ions with the antibody*

Alicyclic and aromatic oniums. In Table 3 are presented results with cyclohexyl- and phenylalkyltrimethylammoniums. For comparison results are also shown for the *n*-alkyltrimethylammoniums with the same number of carbon atoms. The differences are insignificant and reinforce the conclusion in the last section that the detailed nature of the hydrocarbon side chain is not important. This is further emphasized by the results in Table 4. The only compound

TABLE 4
The effect of various ring structures on affinity

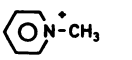
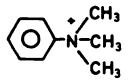
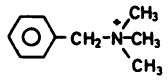
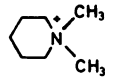
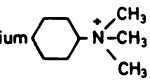
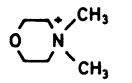
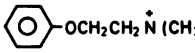
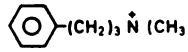
		-ΔF
N-methylpyridinium		5·37
Phenyltrimethylammonium		6·50
Benzyltrimethylammonium		7·14
NN Dimethylpiperidinium		6·71
Cyclohexyltrimethylammonium		6·76
NN Dimethylmorpholinium		6·27

TABLE 5
A comparison of paraffin chains with oxy groups

		-ΔF		-ΔF
Choline	$HO\,CH_2CH_2\overset{+}{N}(CH_3)_3$	6·00	$C_2H_5\overset{+}{N}(CH_3)_3$	6·28
Homocholine	$HO\,CH_2CH_2CH_2\overset{+}{N}(CH_3)_3$	5·84	$C_3H_7\overset{+}{N}(CH_3)_3$	6·16
4-Hydroxybutyl TMA	$HO(CH_2)_4\overset{+}{N}(CH_3)_3$	6·04	$C_4H_9\overset{+}{N}(CH_3)_3$	6·49
Acetylcholine	$CH_3COO\,CH_2CH_2\overset{+}{N}(CH_3)_3$	6·36	$C_5H_{11}\overset{+}{N}(CH_3)_3$	6·75
Butyrylcholine	$C_3H_7COO\,CH_2CH_2\overset{+}{N}(CH_3)_3$	6·62	$C_6H_{13}\overset{+}{N}(CH_3)_3$	6·78
Cholinephenylether		7·25		7·13

deserving special mention is *N*-methylpyridinium, whose $-\Delta F$ is 1.13 kcal/mole less than phenyltrimethylammonium and 1.34 kcal/mole less than *N*,*N*-dimethylpiperidinium. This probably is due to the presence of only one methyl group attached to the nitrogen and to the essentially planar triradiate structure of the pyridinium. It is noteworthy that there is no significant difference in affinity between *N*,*N*-dimethylpiperidinium and cyclohexyltrimethylammonium. Although the former has only two methyl groups, it nevertheless retains a tetrahedral arrangement of the carbons attached to the nitrogen that does not deviate very far from that in tetramethylammonium.

Choline and acetylcholine derivatives. In Table 5 results obtained for choline and acetylcholine derivatives are compared with equivalent alkyltrimethylammoniums. With the single exception of choline phenyl ether, their affinities are significantly lower than the simple alkyl compounds. This is probably to be attributed to the more hydrophilic nature of these chains. The position of choline phenyl ether becomes less anomalous when butyrylcholine is considered; it has only a marginally lower affinity than hexyltrimethylammonium. It seems not unlikely that a large nonpolar terminal portion of the side chain can compensate for a hydrophilic intervening segment.

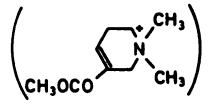
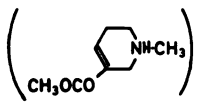
Bisquaternary compounds. As can be seen in Table 6, the three alkyl-bisquaternary compounds examined had free energies of the order expected for alkyltrimethylammoniums of comparable chain length. The second quaternary group did not appear to have any specific effect. It is noteworthy that suxamethonium, which has a more polar chain than decamethonium, has a lower affinity, and this is in accord with the results presented in the preceding section.

Quaternary and tertiary compounds. It has already been pointed out that trimethylammonium had a somewhat lower affinity than tetramethylammonium; the differences were greater with two other tertiary-quaternary pairs examined (Table

TABLE 6
A comparison of mono and bisoniums of comparable chain lengths

		-ΔF		-ΔF
Pentamethonium	$(CH_3)_3\overset{+}{N}(CH_2)_5\overset{+}{N}(CH_3)_3$	6·87	Heptyl TMA	6·95
Hexamethonium	$(CH_3)_3\overset{+}{N}(CH_2)_6\overset{+}{N}(CH_3)_3$	6·92	Octyl TMA	7·13
Decamethonium	$(CH_3)_3\overset{+}{N}(CH_2)_{10}\overset{+}{N}(CH_3)_3$	7·51	Dodecyl TMA	7·38
Suxamethonium	$\left[(CH_3)_3\overset{+}{N}\,CH_2CH_2OCOCH_2\right]_2$	6·89		

TABLE 7
A comparison of quaternary-tertiary pairs differing only in one methyl group

Quaternary	-ΔF	Tertiary	-ΔF	δ (Quart.-Tert.)
Tetramethylammonium	6·00	Trimethylammonium	5·56	0·44
Phenoxyethyltrimethyl ammonium	7·25	Phenoxyethyldimethyl ammonium	5·83	1·42
Arecoline methiodide	6·51	Arecoline	5·42	1·09
				

7). These results become consistent if it is assumed that in tetramethylammonium three methyl groups interact relatively strongly with the site, and the fourth, which interacts to a lesser extent, is oriented in the direction occupied by a longer molecular chain when present. In the other two compounds this position is pre-empted by the side chain, so that in these tertiary compounds it is one of the strongly reacting sites that must be vacated.

DISCUSSION

The results presented in this paper permit us to draw a preliminary view of the topography of the antibody-binding site. The site evidently contains one or more anionic charges which are responsible for the large ion pair contribution to the total binding energy. In this respect, as well as others, the antibody resembles the antibody against azophenyltrimethylammonium, for which more direct evidence has been produced of the participation of one or more free carboxyl groups in the binding site (2, 3). It is clear from the affinities of the series tetra-, tri-, and dimethylammonium, methylammonium, and ammonium, and also from the comparisons of tertiary and quaternary derivatives, that all four methyl groups in tetramethylammonium contribute significantly to the binding. This suggests that the site must largely enclose this part of the molecule, and one might speculate that this part of the site is a pit or groove in the tertiary structure of the protein, perhaps comparable to those becoming familiar from X-ray diffraction studies of crystalline proteins (8–12). On the other hand, the lack of stringent structural requirements associated with side chain interaction suggests that this is accommodated in a nonspatially constricted part of the protein, possibly on the surface. This would also be compatible with the rather low affinity increment per methylene group.

This study illustrates that a useful but nevertheless restricted amount of information can be obtained from structure-activity series when the chemical nature of the interacting small molecules is very simple. The real difficulty is that a molecular landscape arrived at by inference is very schematized, and the analysis of the contributory forces very oversimplified—how much so may be apprehended from the interaction between xenon and metmyoglobin, which involves no less than 32 significant interatomic force components (13). Since there is little likelihood that X-ray diffraction will be available for more than a few studies of complexes for some time, structure-activity series will retain some usefulness in deducing information about binding sites, particularly if supplemented by additional spectroscopic data. We have previously shown that nuclear magnetic resonance spectroscopy is a useful way of studying the molecular dynamics of complexes, and we are at present studying this antibody system further by this method (14, 15).

REFERENCES

1. H. F. Marlow, J. C. Metcalfe and A. S. V. Burgen, *Mol. Pharmacol.* **5**, 156 (1969).
2. D. Pressman, A. L. Grossberg, L. H. Pence and L. Pauling, *J. Amer. Chem. Soc.* **68**, 250 (1946).
3. A. L. Grossberg and D. Pressman, *J. Amer. Chem. Soc.* **82**, 5478 (1960).
4. D. H. Adams and V. P. Whittaker, *Biochim. Biophys. Acta* **4**, 543 (1950).
5. J. L. Webb, "Enzyme and Metabolic Inhibitors," Vol. 1. Academic Press, New York, 1963.
6. A. S. V. Burgen, *Brit. J. Pharmacol. Chemother.* **25**, 4 (1965).
7. W. Kauzman, *Advan. Protein Chem.* **14**, 1 (1959).
8. C. L. Nobbs, H. C. Watson and J. C. Kendrew, *Nature* **209**, 339 (1966).
9. K. Fridborg, K. K. Kannan, A. Liljas, J. Lundin, B. Strandberg, R. Strandberg, B. Tilander and G. Wiren, *J. Mol. Biol.* **25**, 505 (1967).
10. C. C. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips and V. R. Sarma, *Nature* **206**, 757 (1965).
11. H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson and F. M. Richards, *J. Biol. Chem.* **242**, 3984 (1967).
12. M. L. Ludwig, J. A. Hartsuck, T. A. Steitz, H. Muirhead, J. C. Coppola, G. N. Reeke and W. N. Lipscomb. *Proc. Nat. Acad. Sci. U. S. A.* **57**, 511 (1967).
13. B. P. Schoenborn, H. C. Watson and J. C. Kendrew, *Nature* **207**, 28 (1965).
14. A. S. V. Burgen, O. Jardetzky, J. C. Metcalfe and N. Wade-Jardetzky, *Proc. Nat. Acad. Sci. U. S. A.* **58**, 447 (1967).
15. J. C. Metcalfe, A. S. V. Burgen and O. Jardetzky, *in* "Molecular Associations in Biology" (B. Pullman, ed.), p. 487. Academic Press, New York, 1968.