The Specificity of Drug Receptors
An Immunochemical Model for Cholinergic Receptors

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SUMMARY

Precipitating antibodies were prepared against the determinant phenoxycholine, a nicotinic agonist. The affinity of the antibody for a wide range of agonists and antagonists related to acetylcholine was tested. The affinity constants were found to range between 3.2×10^5 and 6.9×10^3 M^{-1}. The antibody did not discriminate between agonists and antagonists, or between drugs acting at muscarinic and nicotinic receptors.

INTRODUCTION

The idea that a specific combining site is the basis of the interaction between small molecules and their biological sites of action was originally proposed by Emil Fischer to explain his discovery that the enzymatic hydrolysis of glycosides is stereospecific (1). He suggested that "the chemical reaction is possible only when certain geometrical considerations are met; the enzyme and glucoside must fit together like a lock and key." This analogue has continued to be popular. Essentially similar ideas were developed by Ehrlich (2) to explain the specificity of antigen-antibody reactions, and by Langley (3, 4), who postulated receptor sites with which a drug combines. In contemporary terms these ideas are taken to mean that the atomic groups in the combining site are in a fixed arrangement in space and interact with atomic groups on the combining molecules, through distance-dependent forces which range from simple ion pairing to dispersion forces. In recent years this concept has been refined to include effects due to the solvent in which the reaction takes place, and conformational selection and alteration of the interacting species.

Starting from a reference molecule, structure-activity studies have usually proceeded by carbon addition or subtraction from aliphatic chains, nuclear substitution in aromatics, and attempts to find isosteric groups. Many examples of this type of study can be found in the investigation of hapten-antibody combinations by Landsteiner (5) and by Pauling and his associates (6, 7). Although this procedure is also commonly used in the development of drugs, the pharmacological ideas of what constitutes a set of related species has been greatly complicated by the fact that in the investigation of the biological activity of new chemical structures it has been found that a new drug may have biological properties homologous with those of an existing drug whether or not there is a close chemical relationship. This is usually demonstrated by quantitative resemblances in activity on a variety of biological preparations as well as through common antagonism by antagonists.

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In an attempt to rationalize these findings, common chemical features have been sought in the drugs belonging to a pharmacological series, and the structure has often been dissected into those elements regarded as essential for the generation of the pharmacological response and those regarded as inessential. A simple example of this type relevant to the present paper is shown in Fig. 1. While the discovery of what has been called the "pharmacophore" may appear obvious in some cases, in others the selection of groups seems arbitrary and indeed fanciful.

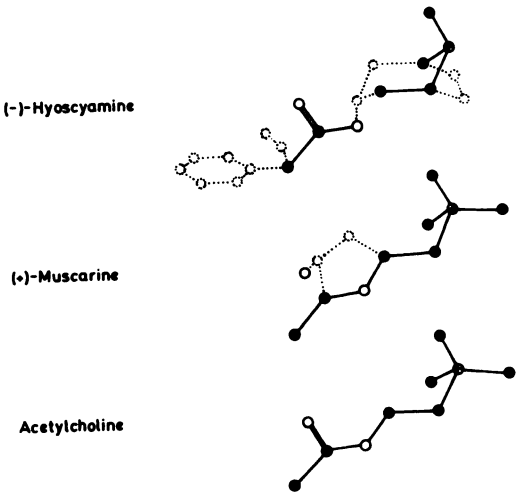


FIG. 1. *Three drugs believed to combine with the "muscarinic" receptor*

The common "essential" structural features are shown as full lines; the "inessential" ones, as dotted lines.

There is an additional problem of very great importance, namely, that drugs in a functional group divide themselves into agonists, or drugs producing an overt action, and antagonists, or drugs that usually have no overt action but can reduce the action of agonists. In many instances a smooth transition from one type of action to the other can be shown to occur in a homologous series, which is compatible with combination with a common receptor site but with a progressive change in the consequences of the reaction. However, there are situations (for instance, the catecholamine α-receptor) in which homologous alteration does not produce active antagonists, but antagonists nevertheless do exist which bear little chemical resemblance to the typical agonist for this receptor, noradrenaline. The problem raised by such observations, therefore, is the extent to which chemical resemblances exist among these apparently dissimilar substances and the degree by which they differ from drugs not having a similar pharmacological action, or whether the attempt to define a single set of structural parameters which determine the interrelation of a drug and receptor is not inherently useful.

In this paper we have tested the basic problem of whether apparently unrelated chemical structures can combine with the same site on a biological macromolecule, by preparing antibodies against a determinant belonging to the acetylcholine group of drugs and then determining its ability to combine with other members of this group.

METHODS

The determinant chosen was the phenyl ether of choline, a powerful acetylcholine-like agonist of the nicotine group. For coupling to a protein carrier, the 4-amino derivative was prepared. 4-Acetaminophenoxyethyl bromide was prepared by allowing the sodium salt of 4-acetaminophenol in ethanol to react with a 6-fold excess of ethylene dibromide (yield, 40%; m.p. 131–133°). 4-Acetaminophenoxyethyl bromide (0.1 mole) was dissolved in 200 ml of ethyl methyl ketone, and 1.0 mole of anhydrous trimethylamine was added. After standing for 4 days, the quaternary bromide was filtered off and recrystallized from an ethanol–diethyl ether mixture (yield, 75%; m.p. 246–247°). One gram of the 4-acetaminophenoxycholine bromide was refluxed with 10 ml of 5 N HCl for 30 min. The hydrochloride of 4-aminophenoxycholine chloride crystallized on cooling and was recrystallized from water (yield, 85%; m.p. 196–198°).

$C_{11}H_{20}Cl_2N_2O$

Calculated:	C 49.44,	H 7.54,	N 10.48,	Cl 26.62
Found:	C 49.34,	H 7.47,	N 10.51,	Cl 26.58

The amino compound was diazotized and allowed to react with bovine serum albumin (Fraction V) in the usual way at 5° for 6 hr; the pH of the reaction mixture was kept at 8.5–9 by addition of potassium carbonate. The pH was then brought to 5.0 by the slow addition of 20% acetic acid with stirring. An equal volume of saturated ammonium sulfate was then added, and after standing at 5° for 2 hr the precipitated protein was removed by centrifugation, redissolved in water, and brought to pH 7.2 with 0.05 N NaOH. The solution was then passed twice through a column of Sephadex G-75 equilibrated with 0.15 M NaCl–0.01 M phosphate, pH 7.2. Various preparations of antigen were made, but the most useful had a labeling density of 6–10 groups/mole as determined from the absorption at 365 nm.

Preparation of the insoluble polymer of the choline phenyl ether antigen was carried out as described by Metcalfe, Marlow, and Burgen (8).

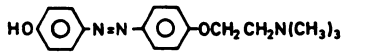
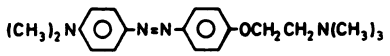
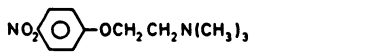
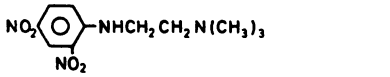
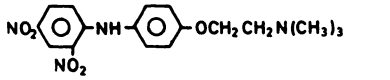
Of several schedules for preparing antibodies to bovine serum albumin-azophenoxycholine, the most successful was the following. New Zealand white rabbits were treated with 20 mg of soluble antigen intravenously and 50 mg of the formol polymer intraperitoneally. After 4 days a further 20 mg of soluble antigen were given intravenously, and this dose was repeated every 3 days for a further five doses. With the last dose a second 50-mg amount of the formol polymer was injected intraperitoneally. Six days after the last dose of antigen the rabbits were bled. After ring-testing, all the active sera were pooled and the antibody concentration was determined by a quantitative precipitin test. In a number of preparations the antibody concentration fell in the range of 10–20 mg/g of serum proteins.

Purification of antibody. After the trial of a number of methods of purification, two methods using insoluble immunoadsorbents were found satisfactory. In the first, bovine serum albumin was conjugated to carboxymethyl Sephadex by a variant of the method of Weliky, Weetall, Gilden, and Campbell (9) using an aqueous solution of 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)-carbodiimide methosulfate as the coupling agent in place of a two-phase aqueous tetrahydrofuran system with dicyclohexylcarbodiimide. After thorough washing, the serum albumin-carboxymethyl Sephadex was then treated with diazophenoxycholine to form the complete immunoadsorbent, which was then washed exhaustively. The capacity of the adsorbent was about 20 mg of antibody per gram. The second immunoadsorbent used was the insoluble formol polymer of serum albumin-azophenoxycholine referred to previously. This had a much higher adsorbing capacity (approximately 300 mg of antibody per gram).

The procedure used in purifying antibody with the formol polymer can be illustrated by a sample preparation. Antiserum (600 ml) containing 330 mg of antibody protein was added to 1 g of the polymer and stirred at 25° for 30 min. The polymer was removed by centrifugation and washed with 0.15 M NaCl–0.01 M sodium phosphate, pH 7.2, until the washings contained less than 10 μg of protein per milliliter. The antibody was then specifically desorbed by addition of 50 ml of 2 M choline chloride adjusted to pH 7.2, which was stirred with the polymer for 15 min. The elution was repeated with two further portions of 2 M choline chloride. The eluates were combined, and the choline was removed by passage twice through a column of Sephadex G-75 equilibrated with 0.015 M ammonium acetate. The antibody was then freeze-dried, yielding 225 mg of dry antibody. In the analytical ultracentrifuge the antibody showed only a 7 S peak. Determination of precipitability at optimal antigen concentration gave a value of 96.1%. The effective combining weight of the antibody determined by equilibrium dialysis with ^{14}C-labeled decamethonium was 70,000. The molecular weight of IgG globulins is 145,000–150,000. Since the combining weight is half this value, the antibody is bivalent, as is usual with IgG.

The combination of haptens with the antibody was measured by the fluorescence quenching method described by Velick, Parker, and Eisen (10). This depends on

TABLE 1
Haptens which quench antibody fluorescence

Hapten	Affinity (M^{-1})	% Quenching
	7.6×10^5	52
	2.3×10^5	49
	1.7×10^5	45
	1.2×10^5	34
	2.8×10^5	43

the use of a hapten whose absorption spectrum shows a considerable overlap with the emission spectrum of the tryptophan groups in the protein.

Five haptens were prepared that had adequate affinities for the antibody and showed usable degrees of quenching (Table 1). The first of these, 4-hydroxyphenylazophenoxycholine, was the most satisfactory for both affinity and quenching capacity and was used in all subsequent work. Fluorescence titrations were carried out in a Zeiss PMQ III spectrofluorometer with the cell compartment maintained at 25°.

Antibody concentration was normally 100 μg/ml, excitation was at 290 nm, and emission was measured at 355 nm. The standard contained 100 μg/ml of nonspecific rabbit γ-globulin not reactive with the choline phenyl ether antigen or the quenching haptens. All titrations were carried out in 0.14 M NaCl–0.01 M sodium phosphate, pH 7.2. All additions of titrants were made to a buffer blank, to the control γ-globulin solution, and to duplicate cuvettes containing antibody. Additions were made with an Agla micrometer syringe, and the solutions were thoroughly stirred before readings of fluorescence were made.

RESULTS

Titration of antibody with 4-hydroxyphenylazophenoxycholine. A titration of antibody with PACPE[3] is shown in Fig. 2. After correction for the amount of PACPE bound by the antibody, the data can be replotted as percentage of maximum

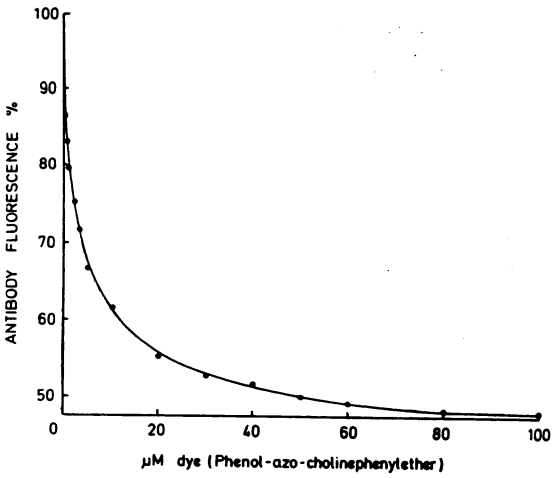


FIG. 2. *Quenching of the fluorescence of antibody to choline phenyl ether by 4-hydroxyphenylazophenoxycholine*

The antibody concentration was 100 μg/ml, or approximately 0.7×10^{-6} M. Volume, 1 ml; temperature, 25°.

quenching against the concentration of free dye (c). The mean affinity constant of the antibody for PACPE is given by the reciprocal of the concentration of PACPE caus-

[3] The abbreviation used is: PACPE, 4-hydroxyphenylazophenoxycholine.

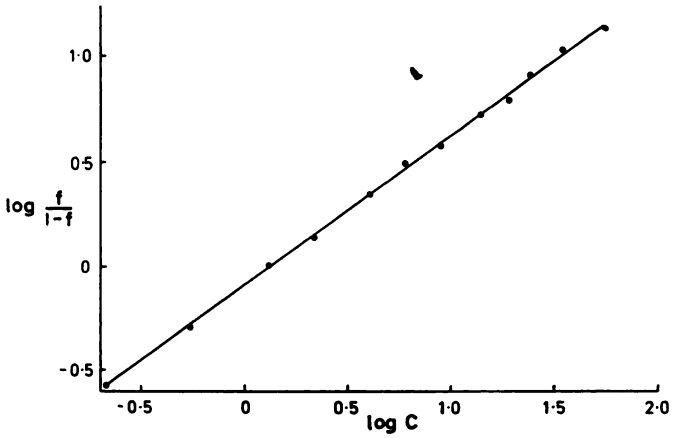


FIG. 3. *Data illustrated in Fig. 2, after correction for bound dye, presented in the form suitable for evaluation of the Sips coefficient a*

ing half-maximum quenching, in this instance, 7.6×10^5 M^{-1}

Since antibodies are usually inhomogeneous, it was desirable to obtain an assessment of this, which may be done by the method of Sips (11, 12). The usual mass action equation for a bimolecular complex is modified by raising the product KC to the power a; thus

$$f = \frac{(KC)^a}{(KC)^a + 1} \qquad (1)$$

where f is the fractional saturation of the antibody-binding sites and a is the Sips heterogeneity index. This equation may be rearranged in a suitable form for graphical determination of a:

$$\log \frac{f}{1-f} = a \log C + a \log K \qquad (2)$$

The data of Fig. 2 are replotted in this form in Fig. 3. The value of a found was 0.72. The antibody is thus moderately heterogeneous, 90% of the antibody molecules having affinities in the range of $0.70\text{–}84 \times 10^5$ M^{-1}.

Titration of other haptens. The aliphatic agonist drugs, for example, acetylcholine, muscarine, and tetramethylammonium, do not absorb light significantly in the overlap region of 300–400 nm and therefore were not able to quench the antibody fluorescence by the optical dipole effect. Their affinities could, however, be deter-

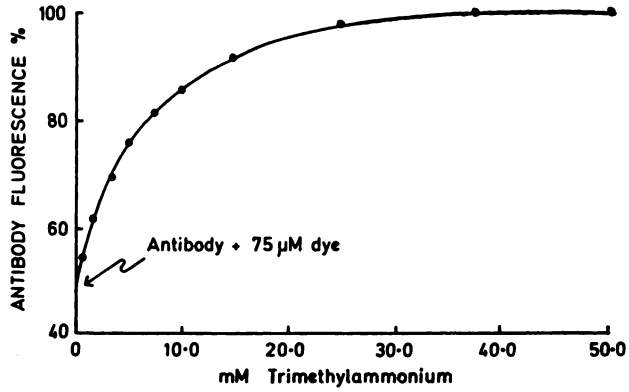


FIG. 4. *Titration of antibody with PACPE and trimethylammonium*

Antibody was titrated with PACPE to a final concentration of 75 μM. It was then back-titrated with trimethylammonium chloride, and the original fluorescence was restored.

mined by competitive titration with PACPE. Our usual procedure was first to titrate the antibody with PACPE and then to back-titrate with the second hapten. A titration curve obtained in this way with trimethylammonium is seen in Fig. 4. It can be seen that the fluorescence was restored to the original level existing before addition of titrants.

Many haptens were themselves able to increase the fluorescence yield of the antibody; an example is the determinant for this antibody, choline phenyl ether, which enhances fluorescence by about 20%. Back-titration of PACPE-quenched antibody with this hapten tended to an asymptotic value of fluorescence 20% higher than the initial value. We have also encountered a number of instances in which haptens absorbing below 300 nm, i.e., in the nonoverlap region, nevertheless quench fluorescence; an example of this is benzyltrimethylammonium (Fig. 5). The processes involved in the changed quantum efficiency of fluorescence in these last two situations will be discussed elsewhere.

In a few instances when haptens of both low affinity and low solubility were examined, it was not possible to obtain satisfactory titrations by the back-titration

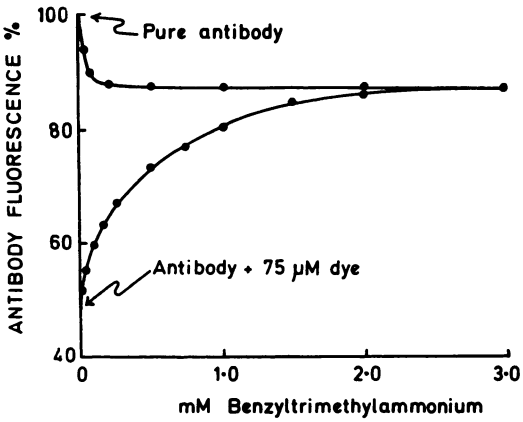


FIG. 5. *Titration of antibody with PACPE and benzyltrimethylammonium*

Back-titration with benzyltrimethylammonium does not lead to complete restoration of the initial fluorescence. This is due to quenching by benzyltrimethylammonium, as is found when the antibody is directly titrated with this hapten.

method. An alternative procedure was used, in which the hapten was added directly to the antibody and the mixture was titrated with PACPE. The affinity was derived from the change in titration curve compared with that of antibody alone. These methods of competitive titration have the merit that they provide an intrinsic check that binding to the same molecular site on the antibody is being studied.

In the mixture of antibody, PACPE, and back-titrant, the over-all fluorescence relative to that of the antibody alone is given by

$$f = \frac{100 + P \cdot K_1 f_p + B \cdot K_2 f_b}{1 + P \cdot K_1 + B \cdot K_2} \quad (3)$$

where P and B are the concentrations of free PACPE and free back-titrant, respectively, K_1 and K_2 are the equilibrium constants for the reaction of antibody with PACPE and the back-titrant, and f_p and f_b are the fluorescences when antibody is completely combined with PACPE and the back-titrant, expressed as a percentage of the initial fluorescence.

Calculation of K_2 is most conveniently made for the amount of free back-titrant (B_m) necessary to bring fluorescence to the midpoint between f_p and f_b, that is, where

$$f = \frac{f_p + f_b}{2}$$

For this point

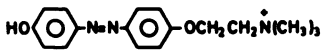
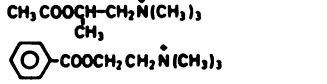
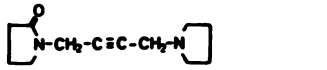
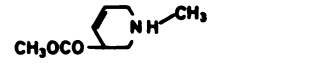
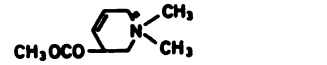
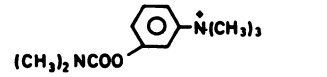
$$K_2 = \frac{1}{B_m}\left(PK_1 + \frac{f_b + f_p - 200}{f_b - f_p}\right) \quad (4)$$

If the back-titrant produces no direct change in fluorescence, the expression is simplified to

$$K_2 = \frac{1}{B_m}(PK_1 - 1)$$

Affinities for the antibody of agonist drugs belonging to the acetylcholine group. Choline phenyl ether, the nicotinic drug used as the antibody determinant, had a high affinity for the antibody (Table 2) although not as high as PACPE. This is compatible with the site also being reactive to the azobenzene linkage present

TABLE 2
Affinities of muscarinic and nicotinic agonists for the antibody

Compound	Structure	Affinity ($M^{-1} \times 10^{-4}$)	$-\Delta F$ (kcals/mole)
PACPE		750	8·01
Cholinephenylether		210	7·25
Acetylcholine	$CH_3COOCH_2CH_2\overset{+}{N}(CH_3)_3$	47	6·37
Choline	$HOCH_2CH_2\overset{+}{N}(CH_3)_3$	25	6·00
Butyrylcholine	$C_3H_7COOCH_2CH_2\overset{+}{N}(CH_3)_3$	73	6·63
(±) Methacholine	$CH_3COOCH(CH_3)CH_2\overset{+}{N}(CH_3)_3$	75	6·64
Benzoylcholine		126	6·95
Tetramethylammonium	$\overset{+}{N}(CH_3)_4$	25	6·00
n-Pentyltrimethylammonium	$CH_3CH_2CH_2CH_2CH_2\overset{+}{N}(CH_3)_3$	90	6·75
(±) Muscarine		33	6·14
Oxotremorine		6·9	5·23
Arecoline		9·5	5·42
N-methylarecolinium		60	6·51
Nicotine		21	5·89
Benzyltrimethylammonium		175	7·14
Neostigmine		67	6·57

in serum albumin-choline phenyl ether. This was also found to be the case by Pauling and Pressman (6, 7) in antibodies against the determinant phenyltrimethylammonium. Acetylcholine itself is less strongly bound than choline phenyl ether, while butyrylcholine, benzoylcholine, and methacholine have the same order of affinity as do also muscarine, nicotine, and *N*-methylarecolinium. Arecoline and oxotremorine are less strongly bound. The simple quaternary tetramethylammonium and the substituted derivatives benzyltrimethylammonium and *n*-pentyltrimethylammonium are also bound. There is no noticeable discrimination between those agents primarily active on muscarine receptors (methacholine, muscarine, oxotremorine) and those mainly active against nicotine receptors (choline phenyl ether, butyrylcholine, benzoylcholine, nicotine, and benzyltrimethylammonium); indeed, the antibody showed a remarkable lack of specificity and it is disturbing that choline, a substance with very weak pharmacological activity, had a higher affinity than the pharmacologically

very active substances oxotremorine and arecoline. Neostigmine, and anticholinesterase with stimulant actions on the nicotinic receptor, also showed fairly high affinity.

Affinities for the antibody of antagonist drugs belonging to the acetylcholine group. The results in Table 3 show the relatively high order of affinity of all the antagonists examined, whether their pharmacological sites of action are at the muscarine receptor, or the ganglionic or neuromuscular types of nicotine receptors. It is noteworthy that the highest activities are present with the dibasic methoniums and the tribasic drug gallamine.

Affinities for the antibody of drugs not

TABLE 3

Affinities of various acetylcholine antagonists

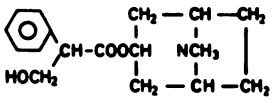
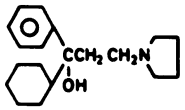
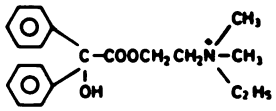
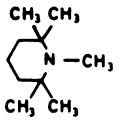
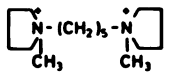
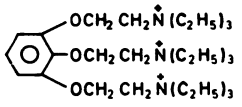
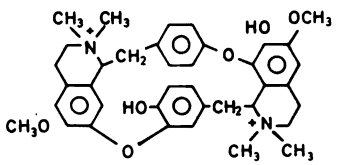
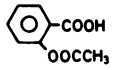
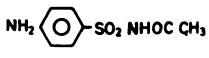
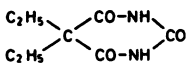
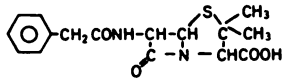
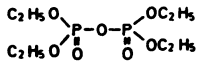
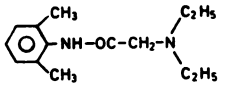
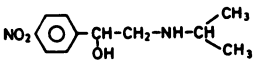
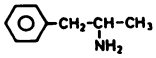
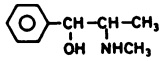
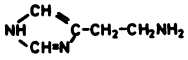
Compound	Structure	Affinity ($M^{-1} \times 10^8$)	$-\Delta F$ (Kcals/mole)
Atropine		42	6·30
Tricyclamol		78	6·67
Lachesine		81	6·69
Tetraethylammonium	$\overset{+}{N}(C_2H_5)_4$	21	5·89
Pempidine		18	5·80
Pentamethonium	$(CH_3)_3\overset{+}{N}(CH_2)_5\overset{+}{N}(CH_3)_3$	111	6·88
Hexamethonium	$(CH_3)_3\overset{+}{N}(CH_2)_6\overset{+}{N}(CH_3)_3$	119	6·92
Pentolinium		109	6·86
Decamethonium	$(CH_3)\overset{+}{N}(CH_2)_{10}\overset{+}{N}(CH_3)_3$	324	7·52
Suxamethonium	$\left[(CH_3)_3\overset{+}{N}CH_2CH_2OOCCH_2^-\right]_2$	113	6·89
Gallamine		100	6·81
d-Tubocurarine		42	6·29

TABLE 4

Affinities of compounds not closely related to choline phenyl ether

Compound	Structure	Affinity ($M^{-1} \times 10^3$)	$-\Delta F$ (Kcals/mole)
Acetylsalicylic acid		< 0·1	< 3·0
Sulphacetamide		< 0·1	< 3·0
Barbitone		< 0·1	< 3·0
Benzylpencillin		< 0·1	< 3·0
Tetraethylpyrophosphate		< 0·1	< 3·0
Xylocaine		17·5	5·78
Inpea		19·2	5·84
Amphetamine		2·5	4·63
Ephedrine		2·1	4·52
Histamine		3·2	4·78

belonging to the acetylcholine group. Acetylsalicylic acid, sulfacetamide, barbital, and benzylpenicillin (Table 4) were without measurable affinity for the antibody. These are all anions at the experimental pH. Tetraethyl pyrophosphate was also ineffective. On the other hand, Xylocaine and Inpea had a rather strong affinity, whereas amphetamine, ephedrine, and histamine were much weaker haptens.

DISCUSSION

The results obtained in this study show that this antibody combines with a surprisingly wide range of basic drugs. Indeed, the specificity is sufficiently low not to differentiate between the agonists and antagonists or between the muscarine and nicotine agonists.

From the immunological point of view this antibody would be regarded as of low specificity. However, such a statement necessarily implies that the range of haptens examined was adequate to explore the specificity fully. In the present instance the range of substances studied has been guided by knowledge of pharmacological activity. This principle has not been used previously, and it is therefore possible that other antibodies might not be as specific as supposed if an appropriate range of haptens were examined.

We have found that all the cationic drugs examined showed some affinity for the antibody, while none of the anionic drugs had

a detectable affinity. This suggests that a positive charge is an important element in determining affinity. It is shown in the following paper (13) that the positive charge alone accounts for some —2.5 to —3.0 kcal/mole of the binding free energy, and this is the dominating element in the lower-affinity haptens. It seems reasonable to attribute the low order of specificity mainly to the magnitude of the binding energy due to ion pairing.

It is most interesting to compare the role of the cationic charge in pharmacological activity. Among agonists, loss of the charge reduces activity to a very low order; indeed, there is serious doubt whether the uncharged species are directly active (14, 15). In the case of antagonists, a moderate reduction in activity is found. These findings are quite in character with the antibody results.

As a model for the cholinergic receptor, the choline phenyl ether antibody has distinct limitations. First, the high affinity for phenylazo haptens suggests that part of the combining site is directed against the tyrosylazo linkage present in the antigen, and this is irrelevant to the supposed determinant phenoxycholine. Second, the antibody differs from the receptor in the small distinction that it makes between choline and acetylcholine, whereas the pharmacological activity of the latter is very much (1000–100,000-fold) greater. This suggests that the antibody is relatively undemanding in the characteristics of the side chain, and indeed must lack a binding site of the kind presumably present in the acetylcholine receptor and known to be present in acetylcholinesterase (the "esteratic site").

Although our present observations have met our initial objective and demonstrated that it is indeed possible for an antibody to have a specificity as broad as that seen in pharmacological series, we are left with the uncertainty whether this is only a feature of a system in which ion pairing is so significant a contributor to interaction energy. It is highly desirable that the philisophy underlying this study should be extended to other drug antibodies for which there is evidence from pharmacological structure-activity series that ion pairing is not important.

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