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# SPECIFIC VERSUS NON-SPECIFIC INTERACTIONS OF MAMMALIAN AND INSECT ACETYLCHOLINESTERASE WITH SUBSTRATES AND REVERSIBLE INHIBITORS

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## SUMMARY

The affinities of various cationic inhibitors and substrates for acetylcholinesterase from bovine erythrocytes and house fly heads have been determined, as well as the maximum velocities of hydrolysis of the substrates. As the bulk and hydrophobic bonding ability of inhibitors increases they become more strongly bound. By contrast, in substrates of increasing size affinity declines, and at the same time there is a drop in the maximum rate of substrate hydrolysis. The decline in affinity is more marked in insect than in erythrocyte acetylcholinesterase, but the decline in activity is far less severe. Weakened substrate binding is shown to be due to weaker adsorption of both the acyl residue at the esteratic site (which directly results in lowered catalytic efficiency) and the onium group at the anionic site.

These findings argue that there are two distinct modes of interaction at the active center. Substrates apparently fit into a crevice or its equivalent that tends to exclude larger molecules and also limits hydrophobic bonding of large substituents not present in the normal substrate, acetylcholine. Reversible cationic inhibitors, on the other hand, adhere by means of hydrophobic bonds to non-polar regions surrounding the active center, and consequently increased size strengthens their affinity. If bulky substrates form bonds of the latter type, disorientation with respect to the active center and reduced catalytic efficiency result. Such bonding may therefore be described as unproductive and non-specific, in contrast to attachments formed by good substrates in the active center proper.

## INTRODUCTION

The addition of ligands to acetylcholinesterase can undoubtedly involve several different interaction sites. Many experimental studies of the past half dozen years reflect this diversity [1–4], though the nature of the binding sites remains obscure. Even the question of whether they are parts of a single complex active center interacting with one substrate molecule, or whether separate allosteric sites are

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involved, has not yet been decided with certainty. In this report it is shown that the active center interacts with acetylcholine and reversible cationic inhibitors in fundamentally different ways. The studies have been carried out with acetylcholinesterase from two sources —bovine erythrocytes and the heads of house flies— since it is found that comparisons of ligand interactions on these two closely related enzymes often lead to a clearer understanding of binding mechanisms.

## MATERIALS AND METHODS

Partially purified acetylcholinesterase from bovine erythrocytes was obtained from Sigma Chemical Co., and that from house flies was prepared by the method described before [5]. Techniques for determining rates of substrate hydrolysis and for calculating values of $K_m$, $V$ and dissociation constants for the complex of reversible inhibitors and the free enzyme, $K_i$, have been described [6]. All measurements were carried out at pH 7.5 and 26 °C in solutions containing 0.1 M NaCl and 0.04 M $MgCl_2$ by automatic titration with 0.01 M NaOH of acid released in substrate hydrolysis. Inhibitions were measured over a range of acetylcholine concentrations. All constants were evaluated using a computer program based on a least squares analysis of plots of $[S]/v$ against $[S]$. The inhibition constants are derived from apparent $V/K_m$ values in the presence or absence of inhibitor, a procedure which may be shown to give the constant for association of enzyme and inhibitor, uninfluenced by association of the inhibitor with any enzyme–substrate intermediate that may occur in the course of the hydrolysis reaction. The appropriate equation is as follows:

$$K_i = [I] \Big/ \left\{ \frac{(K_m/V)_I}{(K_m/V)_0} - 1 \right\}$$

where $[I]$ is the inhibitor concentration, $(K_m/V)_I$ is the intercept in an $[S]/v \cdot [S]$ plot in the presence of inhibitor, and $(K_m/V_0)$ is the corresponding value in the absence of inhibitor. $K_m$ in the absence of inhibitor was not actually determined in every experiment. Instead uninhibited rates were measured in triplicate at high substrate concentration (usually 1 or 2 mM) and a value for $K_m/V$, with its standard error, was then calculated from a predetermined $K_m$, shown in Table I. In this way all measurements required on a given inhibitor could be made in one day which facilitates the precise estimation of constants. A second advantage is that any error in the determination of $K_m$ affects all inhibition constants in the same way and should not alter their relative values, it is these rather than absolute values that are of chief importance in the present study. It may also be noted that precise determination of $K_m$ values for the reference substrate acetylcholine requires rate estimations at low substrate concentrations (down to $5 \cdot 10^{-5}$ M). These measurements were made using a reaction volume of 20 ml and a double syringe technique, in which equal volumes of 0.01 M NaOH and acetylcholine were delivered into the reaction mixture, the former to neutralize acetic acid released in acetylcholine hydrolysis, and the latter to maintain a steady concentration of substrate. Under these conditions the recorder tracings of base added to maintain a constant pH in the reaction vessel were linear with time over a sufficient period (about 10 min) for initial rates to be determined with reasonable precision, as is indicated by the magnitude of the standard error of $K_m$. A further

point regarding the technique is that the enzyme concentration was varied in some runs in order to produce a rate of acid formation which previous work has shown to be optimal for precise measurement. That is very low measured rates were avoided because the control rate in the absence of enzyme would then be comparatively large, whereas reproducability is better when the control rate is negligible by comparison with the experiment. High measured rates were also avoided since accumulation of the product of the reaction, as well as depletion of substrate in cases where the double syringe technique was not used, lead to diminishing rates of substrate hydrolysis with time. It had also been shown in previous work that the measured rate is proportional to the enzyme concentration over a wide range of concentrations.

## RESULTS

### *Binding of substrates and other quaternary ammonium ions*

Tables I and II list $K_m$ values for various substrates and inhibition constants for a number of reversible inhibitors. All the substrates, with the possible exception of butyrylcholine, have a higher affinity for insect than mammalian acetylcholin-

TABLE I

RELATIVE MAXIMUM VELOCITIES ($V$) AND $K_m$ VALUES FOR FLY HEAD AND BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE WITH VARIOUS SUBSTRATES

| | Substrate | Relative $V$ | | $K_m$ (M) | |
|---|---|---|---|---|---|
| | | Fly head | Erythrocyte | Fly head | Erythrocyte |
| I | $CH_3C(O)O(CH_2)_2\overset{+}{N}(CH_3)_3$ | 1.00 | 1.00 | $(4.87\pm0.47)\cdot10^{-5}$ | $(2.35\pm0.17)\cdot10^{-4}$ |
| II* | $CH_3C(O)O(CH_2)_2\overset{+}{N}H(CH_3)_2$ | | 0.36 | | $1.50\cdot10^{-3}$ |
| III | $CH_3C(O)O(CH_2)_2\overset{+}{N}(CH_3)_2C_3H_7$ | 1.00 | 0.63 | $(4.56\pm0.69)\cdot10^{-5}$ | $(5.31\pm0.62)\cdot10^{-4}$ |
| IV | $CH_3C(O)O(CH_2)_2\overset{+}{N}(CH_3)_2C_4H_9$ | 0.66 | 0.54 | $(9.90\pm1.39)\cdot10^{-5}$ | $(6.16\pm0.17)\cdot10^{-4}$ |
| V | $CH_3C(O)O(CH_2)_2\overset{+}{N}(C_2H_5)_2C_4H_9$ | 0.30 | 0.16 | $(2.53\pm0.18)\cdot10^{-4}$ | $(8.30\pm0.57)\cdot10^{-4}$ |
| VI | $CH_3C(O)O(CH_2)_2\overset{+}{N}CH_3(CH_2CH_2CH_3)_2$ | | 0.08 | | $(1.27\pm0.01)\cdot10^{-3}$ |
| VII | $CH_3C(O)O(CH_2)_2\overset{+}{N}C_2H_5(CH_2CH_2CH_3)_2$ | | 0.05 | | $(5.22\pm0.33)\cdot10^{-4}$ |
| VIII | $CH_3C(O)O$–C$_6$H$_4$–$(CH_2)_2N(CH_3)_3$ | 0.79 | 0.27 | $(7.15\pm1.37)\cdot10^{-5}$ | $(1.31\pm0.23)\cdot10^{-3}$ |
| IX** | $C_2H_5C(O)O(CH_2)_2\overset{+}{N}(CH_3)_3$ | 0.78 | 0.83 | $(4.41\pm1.01)\cdot10^{-5}$ | $4\cdot10^{-4}$ |
| X*** | $C_3H_7C(O)O(CH_2)_2\overset{+}{N}(CH_3)_3$ | 0.50 | ~0 | $(9.64\pm0.89)\cdot10^{-5}$ | $(1.19\pm0.10)\cdot10^{-4}$ |

* $K_m$ value calculated from the measured value at pH 6.5 (see text).

** Data for human erythrocyte acetylcholinesterase, ref. 7.

*** With erythrocyte acetylcholinesterase, $K_i$ was determined by inhibition of acetylcholine hydrolysis: $K_m = K_i$.

TABLE II

DISSOCIATION CONSTANTS FOR COMPLEX FORMATION BETWEEN REVERSIBLE CATIONIC INHIBITORS AND FLY HEAD OR BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE

| Cation | $K_i$ (M) | |
|---|---|---|
| | Fly acetylcholinesterase | Bovine acetylcholinesterase |
| Tetramethylammonium | $(3.07 \pm 0.32) \cdot 10^{-3}$ | $(2.58 \pm 0.22) \cdot 10^{-3}$ |
| Tetraethylammonium | $(8.60 \pm 0.11) \cdot 10^{-4}$ | $(9.90 \pm 0.92) \cdot 10^{-4}$ |
| Triethyl-*n*-propylammonium | | $(5.30 \pm 0.72) \cdot 10^{-4}$ |
| Tetra-*n*-propylammonium | | $(7.55 \pm 1.30) \cdot 10^{-5}$ |
| Tetra-*n*-butylammonium | $(1.22 \pm 0.19) \cdot 10^{-5}$ | $(5.71 \pm 0.96) \cdot 10^{-5}$ |
| Tetra-*n*-pentylammonium | $(9.50 \pm 1.05) \cdot 10^{-6}$ | $(2.25 \pm 0.31) \cdot 10^{-4}$ |
| Choline | $(3.58 \pm 0.37) \cdot 10^{-4}$ | $(1.03 \pm 0.11) \cdot 10^{-3}$ |
| *N,N*-Dimethylaminoethanol | | $(2.68 \pm 0.97) \cdot 10^{-3}$ |
| *N,N,N*-Dimethyl-*n*-butylaminoethanol | $(2.39 \pm 0.36) \cdot 10^{-4}$ | $(3.17 \pm 0.53) \cdot 10^{-4}$ |
| *N,N,N*-Diethyl-*n*-propylaminoethanol | $(1.92 \pm 0.38) \cdot 10^{-4}$ | $(6.01 \pm 0.85) \cdot 10^{-4}$ |
| *N,N,N*-Diethyl-*n*-butylaminoethanol | $(9.01 \pm 1.35) \cdot 10^{-5}$ | $(6.01 \pm 0.14) \cdot 10^{-4}$ |
| Phenyl-trimethylammonium | $(2.23 \pm 0.05) \cdot 10^{-4}$ | $(8.39 \pm 0.79) \cdot 10^{-4}$ |
| *N*-Methylhordenine | $(2.55 \pm 0.53) \cdot 10^{-4}$ | $(2.33 \pm 0.97) \cdot 10^{-3}$ |

esterase. The binding strengths are similar with tetramethylammonium ion, showing that the enzymes have a similar affinity for methyl groups at the anionic site. However, larger groups are more strongly bound by the insect enzyme.

*Rates of substrate hydrolysis*

Adaptability of insect acetylcholinesterase to substrates more bulky than acetylcholine is illustrated by the maximum velocity measurements listed in Table I. When groups attached to the quaternary nitrogen atom are larger than the methyl groups of acetylcholine (Compounds III, IV and V) or when the distance between the ester carbonyl carbon atom and the quarternary nitrogen is lengthened by interposition of an additional phenyl ring, in *O*-acetyl hordenine methiodide, (VIII) or when the acyl group is enlarged as in butyrylcholine (X), the slowing of the hydrolysis rate is more marked with erythrocyte than with fly head enzyme.

The ability of both enzymes to hydrolyze substrates with bulky acyl groups is definitely limited, as shown by their observed failure to catalyse hydrolysis of benzoylcholine at a detectable rate.

## DISCUSSION

A fundamental distinction between the binding of substrates and reversible inhibitors emerges from these observations on the affinities of acetylcholine and choline analogs. Regarding the interpretation of affinity constants it is to be noted that competitive inhibition constants ($K_i$) which are derived from $K_m/V$ ratios, are always directly related to equilibrium binding energies. However experimental $K_m$ values [8, 9] include a term for the ratio of acylation and deacylation rates ($k_2$ and $k_3$) as follows: $K_{m\,(apparent)} = K_s/(1 + k_2/k_3)$. Hence they are simple measures of the

reversible association of enzyme and substrate only if acylation is rate-limiting (i.e. when $k_2/k_3 \ll 1$). Acetylation must be at least partly rate-limiting with all acetyl esters having a lower $V$ than acetylcholine, and if $V$ is sufficiently small, say half that of acetylcholine or less, the $k_2/k_3$ ratio should be small enough relative to unity to have a negligible effect on measured affinity. With acetylcholine itself, and other substrates with a similar $V$, deacetylation may be rate-limiting, and in fact has been shown to be rate-limiting in the case of erythrocyte acetylcholinesterase. For this enzyme therefore, another substrate, *N,N*-dimethyl-aminoethyl acetate may serve at a point of comparison for affinities, since its $V$ is relatively low. Its $K_m$ at pH 7.5 is found to be $1.50 \cdot 10^{-3}$ M*.

With erythrocyte acetylcholinesterase the substrates *N,N,N*-dimethyl-*n*-propyl- and *N,N,N*-dimethyl-*n*-butyl-*O*-acetyl ethanolamine (III and IV) have $K_m$ values of $5.31 \cdot 10^{-4}$ and $6.16 \cdot 10^{-4}$ M, respectively, and as seen above these should be true measures of reversible binding. *n*-Propyl and *n*-butyl groups therefore contribute a factor of about 2.5 to binding strength. The $K_m$ values for three other substrates, the *N,N,N*-methyl-di-*n*-propyl, *N,N,N*-ethyl-di-*n*-propyl and *N,N,N*-diethyl-*n*-butyl analogs of acetylcholine, are $1.27 \cdot 10^{-3}$, $5.22 \cdot 10^{-4}$ and $8.30 \cdot 10^{-4}$ M (VI, VII and V), and here the additional methylene groups contribute factors calculated to be 1.2, 2.9 and 1.8.

The contribution of a methyl group can be roughly estimated by comparison of choline and dimethylaminoethanol, the ratio of whose $K_i$ values is $2.6 \pm 1.0$, and this agrees with a factor of 3 reported by Wilson for eel acetylcholinesterase [10]. The significant conclusion, then, derived from these observations is that alkyl substituents in substrates contribute no more to binding than a methyl group, and possibly less.

By contrast alkyl substituents in reversible cationic inhibitors substantially increase affinity. Among choline analogs the *N,N,N*-diethyl-*n*-propyl, *N,N,N*-diethyl-*n*-butyl and *N,N,N*-dimethyl-*n*-butyl compounds are held 2 to 3 times more tightly than the parent molecule (Table II). The ratios in these cases, with their calculated standard errors, are as follows: $1.71 \pm 0.30$, $1.71 \pm 0.19$ and $3.25 \pm 0.64$, respectively. Though these changes are not large they clearly denote tighter rather than looser binding. The trend to higher affinities is more marked among symmetrical ammonium ions. Thus tetraethyl, tetrapropyl, tetrabutyl and tetrapentyl ammonium ions are bound 2.6, 34 and 45 and 11 times more strongly than tetramethylammonium, respectively. The addition of one methylene group to tetraethylammonium, giving triethyl-*n*-propyl ammonium ion, improves binding by a factor of nearly 2 ($1.87 \pm 0.29$). Replacement of all four ethyl by *n*-propyl groups increases affinity 13-fold, indicating that at least 3 and possibly 4 of the added groups contribute to binding. Similarly the affinity of *N*-alkyl trimethylammonium ion inhibitors rises with increasing chain length [11]; the ethyl, propyl and butyl analogs are reported to be bound $1.46 \pm 0.01$, $1.74 \pm 0.01$ and $2.77 \pm 0.02$ times, respectively, more firmly than tetra-

* The experimental $K_m$ for dimethylaminoethyl acetate at pH 6.5 with erythrocyte acetylcholeinasterase is $2.06 \cdot 10^{-3} \pm 0.22 \cdot 10^{-3}$ M. $K_m$ at pH 7.5 is found [9] from the formula $K_{m\,(apparent)} = K_m (1 + [H^+]/K)/(1 + H^+/K')$ where $K$ and $K'$, the acid dissociation constants for ionizing groups that determine catalytic activity in the free enzyme and enzyme substrate complex, are $10^{-6.3}$ and $10^{-5.5}$ respectively [8].

methylammonium. In general affinity continues to increase as the chain grows beyond this length.

The same trends are seen in fly head acetylcholinesterase. Since acetylation is probably rate limiting with all the substrates, including acetylcholine, the latter may serve as a reference point [6]. Its *N,N,N*-dimethyl-*n*-propyl analog has the same affinity, but the *N,N,N*-dimethyl-*n*-butyl and *N,N,N*-diethyl-*n*-butyl analogs are more weakly bound by factors of 2.03 $\pm$ 0.35 and 5.20 $\pm$ 0.62. With inhibitors related to choline affinity rises instead of falling: the *N,N,N*-dimethyl-*n*-butyl, *N,N,N*-diethyl-*n*-propyl and *N,N,N*-diethyl-*n*-butyl derivatives of ethanolamine are bound more tightly than choline by factors of 1.50 $\pm$ 0.27, 1.86 $\pm$ 0.41 and 3.97 $\pm$ 0.72. The affinities of tetraethyl, tetrabutyl and tetrapentyl ammonium ions are 3.6, 250 and 330 times higher than that of tetramethylammonium.

Affinity for *O*-acetyl hordenine methiodide, an acetylcholine analog having added bulk between the ester bond and nitrogen atom is reduced 2-fold with both enzymes, while increased size in the acyl group, in butyrylcholine, reduces binding strength 2-fold in insect acetylcholinesterase but increases it 4-fold in erythrocyte acetylcholinesterase. The possible significance of this increase is considered below.

Though the reductions in binding strengths of acetylcholine analogs appear to be slightly larger in fly than erythrocyte acetylcholinesterase, the reductions in maximum velocity are less marked*: the catalytic activity of fly acetylcholinesterase is found to be far less sensitive to variations in structure at the quaternary nitrogen atom, or between the latter and the ester bond, or in the acyl residue (Table I). These opposite trends suggest that insofar as bulky groups contribute to binding they disorient the complex and reduce the rate of hydrolysis, and also that non-productive binding, i.e. formation of unreactive complexes, is far more likely in the mammalian enzyme. This is most obviously true of butyrylcholine. With insect acetylcholinesterase both the affinity and maximum velocity for this substrate are reduced (by a factor of 2) below the values for acetylcholine, which is in accord with the trends already noted. However with erythrocyte acetylcholinesterase affinity rises (by a factor of 4) and consequently hydrolysis falls to a barely detectable level.

Substrates are bound at the active center by means of a two-point attachment, involving first the ammonium and then the acyl group. Lower affinity could therefore be due to diminished attraction at one or both these points. Weakened interaction of the acyl group in cases of substrates with bulky substituents on the nitrogen atom was demonstrated by observations on enzyme inactivation by methanesulfonyl fluoride [12]. From comparisons of acetylcholinesterase from two sources it was apparent that when enlargement of the substrate's onium group lowers the maximum velocity it also causes the substrate to accelerate reaction of the active center with this reagent; when enlargement fails to alter the maximum velocity, the same substrate protects against inactivation. This is understandable since hindrance of substrate absorption at the esteratic site has two effects: it reduces the catalytic rate, and it allows the

* The reductions in $V$ with erythrocyte acetylcholinesterase actually represent minimal declines in the rate of reaction in the enzyme–substrate complex. The rate-limiting step in acetylcholine hydrolysis is deacetylation [8]; hence acetylation rates have little affect on the velocity until they become about as slow deacetylation. In fly head acetylcholinesterase on the other hand, acetylation appears to be rate-limiting even with acetylcholine, so that declining acetylation rates should directly lower $V$ [6].

unobstructed site to react with methane sulfonyl fluoride. If interaction with the esteratic site persists the velocity is normal and the enzyme is protected.

Besides weakening interaction at the esteratic site, N-alkyl substituents in substrates are apparently themselves weakly bound, as shown by the fact that choline analogs sometimes have a higher affinity than the corresponding acetylcholine analogs; e.g. the dimethylbutyl derivatives with erythrocyte acetylcholinesterase (ratio $1.94 \pm 0.33$) and the diethylbutyl derivatives with both enzymes (the ratios for the erythrocyte and fly enzymes are $1.38 \pm 0.10$ and $2.81 \pm 0.47$, respectively). The acetyl group itself should contribute positively to binding, as in acetylcholine, which is more strongly bound than choline by a factor of roughly 7. The ester may therefore experience greater difficulty in penetrating into the binding site than the corresponding alcohol, on account of its larger size, i.e. through steric repulsion. The active center must then have the form of a crevice. Acetylcholine and closely similar molecules tend to occupy this crevice, but larger cations should fail to penetrate it deeply. The high affinities often seen in large molecules therefore argue that they are bound at least partly in non-polar regions adjacent to the active center crevice. More direct evidence for this was obtained from studies of the effects of large symmetrical cations on enzyme inactivation by methanesulfonyl fluoride [12]. It appears to follow that in a series of compounds of gradually changing size and shape there should be a progression from those bound within the active center to others bound mainly outside.

An important question arises as to the nature and function of the external sites involved in inhibitor attachment. The present evidence favors the view that such interactions are essentially accidental. Earlier experiments on sulfonylation rates had suggested, on the contrary, some kind of functional role. *n*-Alkyl trimethylammonium ion inhibitors had been shown to accelerate reaction with methanesulfonyl fluoride; their alkyl chains must therefore have been bound in a different region than acetylcholine, and the activation phenomenon suggested that binding was specific and possibly related to regulation of enzyme activity by small molecules [13]. Opposing this view stands the following. These binding modes are characteristic of inhibitors, in that they are highly unspecific: no special molecular size or shape is required [12], and affinity generally increases with the non-polar character of substituents, just as in non-specific partitioning between polar and non-polar phases [14]. Such binding is also counter-productive from the point of view of substrates, and leads to greatly reduced catalytic rates.

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