Drug–Biomolecule Interactions: Binding Study of Substrate and Inhibitors to Acetylcholinesterase Using NMR

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Abstract □ NMR was used to study the binding of acetylcholine, atropine, and physostigmine to acetylcholinesterase. Changes in the linewidth of the *N*-methyl resonance of acetylcholine, resulting from association with the enzyme during hydrolysis, were utilized to study the enzyme–substrate interaction. Physostigmine inhibited the binding of the substrate while atropine accelerated substrate hydrolysis without interfering with its binding. The dissociation constant, K_D, and the linewidth of the acetylcholinesterase–inhibitor complex, $\Delta\nu_{bound}$, for atropine and physostigmine can be estimated from the linewidth changes of the *N*-methyl and phenyl group resonances of atropine and from the *N*-methyl and C-methyl group resonances of physostigmine resulting from association with the enzyme. The results indicate that there is at least one binding site on the enzyme surface for atropine and one for physostigmine. Further evidence that the two sites are distinct is indicated by the fact that gallamine displaces atropine from its site without competing with physostigmine.

Keyphrases □ Acetylcholinesterase—binding of substrate and inhibitors, NMR □ Binding—substrate and inhibitors to acetylcholinesterase, studied by NMR □ NMR—evaluation, binding of substrate and inhibitors to acetylcholinesterase □ Drug–bimolecule interactions—binding of substrate and inhibitors to acetylcholinesterase, NMR □ Interactions—drugs with biomolecules, symposium

Since drugs and the complex biological entities on which they act are composed of molecules, their effects can accordingly be interpreted on the basis of molecular events, *i.e.*, by the interaction of drug with receptor molecules. The receptors are conceived as being special structures within the molecular framework of the cell membranes and cellular organelles. Such structures are capable of direct binding with specific, biologically active substances, and it is this binding that results in an observable physiological, pharmacological, or biochemical response.

It is widely believed that the receptors are macromolecules—probably proteins—with structures resembling those of enzymes or antibodies (1). They almost certainly include structures sensitive to acetylcholine, epinephrine, histamine, serotonin, and the peptide hormones. But while the chemical structure of most drugs is known, the structure of receptors is still unknown. The binding of a number of drugs to the enzyme acetylcholinesterase was studied with the hope of using this system as a possible model for other drug–receptor interactions (2–5).

DISCUSSION

Acetylcholinesterase catalyzes the hydrolysis of acetylcholine into choline and acetate. This reaction involves the active center of the enzyme which consists of two subsites, the anionic site and the esteratic site (6). The anionic site is responsible for binding, while the esteratic site is responsible for cleavage of the substrate molecule.

Apart from the active site, acetylcholinesterase contains noncatalytic binding sites. For example, Changeux (7) demonstrated the binding of gallamine triethiodide and tubocurarine, two receptor inhibitors that only partially inhibited the enzyme. Belleau and DiTullio (8, 9) showed that at least two anionic sites are present on acetylcholinesterase; site I is part of the active site, and site II is at the periphery and binds tubocurarine. Their results imply certain similarities between the anionic sites of acetylcholinesterase with the acetylcholine receptors of excitable membranes. Several other investigators (10–13) also showed the presence of allosteric sites on acetylcholinesterase which bind certain cholinergic ligands with more or less high affinity.

The aim of the present work was to investigate the binding of acetylcholine, atropine, and physostigmine to purified squid enzyme using a high-resolution NMR method. The binding of inhibitors to enzymes is usually studied by kinetic methods or by equilibrium dialysis. Both techniques, however, have inherent limitations. The nature of the interaction can be studied in more detail by NMR methods (14).

With NMR spectroscopy, two types of change are commonly observed in the spectrum of a small molecule when it binds to a macromolecule. The linewidths of the protons of a small molecule give information about the degree of restriction of motion of specific parts of small molecules, the degree of saturation of binding sites, and the rates of exchange. In addition, chemical shift changes for some protons of the small molecule give information on the magnetic environment of the small molecule when it is bound. Such an effect could result from nearby aromatic systems or paramagnetic ions. A recent review summarized the application of NMR spectroscopy to biochemical problems (14).

THEORETICAL

All elementary particles of the atom, such as the proton or the electron, constantly spin around their axis with a certain momentum. In NMR spectroscopy, the H atom, which has a single proton with spin $I = \frac{1}{2}$ and a magnetic moment μ is of primary interest. If the spinning proton is placed in a strong magnetic field, H_0, it will act to tilt its axis either in the direction of the field or in opposition to the field. Thus, a proton will be able to assume only one of two possible orientations, corresponding to energy levels of $-\mu H_0$ in the lower state and $+\mu H_0$ in the upper state.

The lower level corresponds to the situation in which the magnetic field, H_0, and the nuclear moment are parallel; in the upper level, they are antiparallel. To induce transitions between the two states, a discrete amount of energy, ΔE, is applied to the system. This is the energy difference between the two states and is given by $\Delta E = 2\mu H_0 = h\nu$, where h is Planck's constant and ν is the frequency of the electromagnetic radiation necessary for such a transition. For protons in a magnetic field of 14,000 gauss, the frequency of such energy is about 60 MHz, which is in the radiofrequency region.

Under ordinary conditions in a magnetic field, there is a very small difference in the population of the two levels given by a Boltzmann distribution. This small excess of protons in the lower energy state is important. The small, but finite, excess of nuclei in the lower energy state gives rise to an absorption of energy in the radiofrequency region.

The information indicates that the resonance condition can be obtained by varying either the magnetic field, H_0, or the frequency, ν. In practice, one usually works with a fixed frequency and sweeps the magnetic field through the resonance value, obtaining a spectrum of the compound. The sample is placed in a tall glass cy-

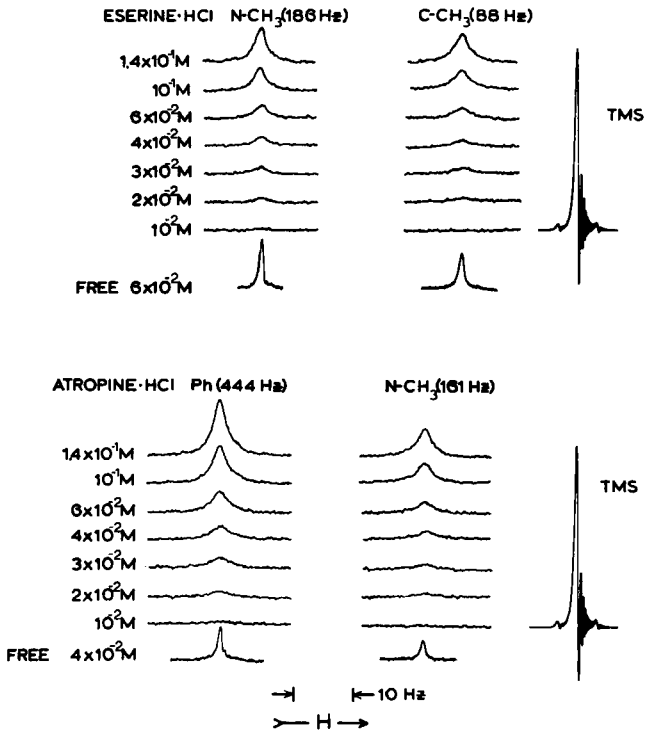

Figure 1—*(Top) The 60-MHz NMR spectra of the* N*-methyl and C-methyl resonances of physostigmine hydrochloride (eserine hydrochloride), free (lower trace) and in the presence of acetylcholinesterase (1.7 × 10⁻⁵* M*) (upper traces). (Bottom) The 60-MHz NMR spectra of the phenyl and* N*-methyl resonances of atropine hydrochloride, free (lower trace) and in the presence of acetylcholinesterase (1.7 × 10⁻⁵* M*) (upper traces). The intense resonances at right are those of protons of an external tetramethylsilane standard. Samples were dissolved in 0.1* M *phosphate buffer, pH 7.4, in D_2O, and spectra were recorded at 39°. The specific activity of squid acetylcholinesterase was 200 mmoles of acetylthiocholine/hr/mg.*

lindrical tube, which is inserted between the poles of a magnet surrounded with a coil which produces the radiofrequency field. If the field of the magnet is scanned at some fixed radiofrequency field, the protons absorb energy which can be recorded by a radio receiver. When the signal is fed directly into a recorder, a sharp peak is obtained in the spectrum.

Each different chemical group of an organic compound containing hydrogens gives rise to a separate absorption peak at different values of the applied magnetic field. Hydrogen atoms in the various chemical groups absorb at slightly different frequencies because the applied magnetic field becomes modified by the movements of the electrons and nuclei in adjacent atoms and neighboring molecules. The absolute position of the absorption cannot be obtained from the instrument. The exact position of the resonance frequencies of protons in different environments is measured from some arbitrary standard (*e.g.*, tetramethylsilane). The position of the peak is termed the chemical shift.

Upon removal of the exciting field at resonance, the Boltzmann distribution of the sample is regained by a relaxation process. This is described by two relaxation times, T_1 and T_2. T_1 is termed the spin–lattice relaxation time, and T_2 is the spin–spin relaxation time. The spin–spin relaxation rates, $1/T_2$, can be obtained from $1/T_2 = \pi \Delta\nu_{1/2}$, where $\Delta\nu_{1/2}$ is the linewidth at one-half maximum peak height. For rapid translational or rotational motion, T_2 is long and $1/T_2$ is small; hence, narrow peaks are obtained. As the molecular motions are reduced by viscosity or by interaction with a larger rigid molecule, T_2 becomes shorter and the peaks become broader.

In solution, small molecules have rapid translational and rotational molecular motion (*i.e.*, they have short correlation times) so narrow peaks are obtained. In solids, molecules occupy fixed positions and thus give rise to very broad peaks. Macromolecules such as proteins in solution give rise to complex NMR spectra due to the overlapping of resonance peaks from similar chemical groups. The longer correlation times due to slow rotational and translational motion also result in broad resonance peaks.

In certain conditions, the protein often shows no spectrum, while the interacting species shows a typical narrow line NMR spectrum. When a freely moving molecule interacts with a protein, the rotational correlation time of the small molecule is increased. As the correlation times are increased, line broadening occurs. It is the changes in the spectra of the small molecules resulting from the binding to high molecular weight macromolecules that are of interest. The width of the resonance peaks in particular are extremely sensitive to variations in the molecular environment and provide the most informative measurements for the study of molecular interaction.

For the equilibrium $E + S \rightleftharpoons ES$, describing enzyme–small molecule association:

$$K_D = \frac{[E][S]}{[ES]} \qquad \text{(Eq. 1)}$$

where $[E] = E_0 - [ES]$ and $[S] = S_0 - [ES]$. The term $[ES]$ is the concentration of the enzyme–small molecule complex, $[S_0]$ is the total concentration of the small molecule, and $[E_0]$ is the total concentration of the enzyme.

For the situation in which a small molecule binds reversibly to an enzyme, the observed linewidth of a proton on the small molecule is given by:

$$\Delta\nu_{obs} = P_{ES}\Delta\nu_{ES} + P_S\Delta\nu_S + \left(\frac{1}{\pi T_2}\right)_{ex} \qquad \text{(Eq. 2)}$$

where P_{ES} and P_S are the fractions of the small molecule bound and free in solution, respectively; $\Delta\nu_{ES}$ and $\Delta\nu_S$ are the linewidths of the bound and free species, respectively; and $(1/\pi T_2)_{ex}$ is the contribution to the observed linewidth due to exchange (15).

If the exchange lifetime is much less than $1/\Delta\nu_{ES}$, then:

$$\Delta\nu_{obs} = P_{ES}\Delta\nu_{ES} + P_S\Delta\nu_S \qquad \text{(Eq. 3)}$$

or:

$$\Delta\nu_{obs} = \left(\frac{[ES]}{[S_0]}\right)\Delta\nu_{ES} + \left(\frac{S_0 - [ES]}{S_0}\right)\Delta\nu_S \qquad \text{(Eq. 4)}$$

In all experiments, $S_0 \gg [E_0]$ so $(S_0 - [ES])/S_0 = 1$ and:

$$\Delta\nu_{obs} = \frac{[ES]}{[S_0]}\Delta\nu_{ES} + \Delta\nu_S \qquad \text{(Eq. 5)}$$

For inhibitor binding studies, the values of $\Delta\nu$ were corrected by

Table I—Linewidth Data for *N*-Methyl and Phenyl Protons of Atropine and *N*-Methyl Protons of Physostigmine Obtained from Association of Atropine and Physostigmine with Acetylcholinesterase, in Absence and Presence of Gallamine Triethiodide[a]

Inhibitor (Protons)	Concentration: Inhibitor, *M*	Concentration: Acetylcholinesterase, mg/ml	$\Delta\nu_{obs}$[b], Hz	$\Delta\Delta\nu$, Hz
Atropine (NCH_3)	0.02	0	1.5	—
Atropine (NCH_3)	0.02	4	3.9	2.4
Atropine (NCH_3) Plus gallamine	0.02 0.04	4	2.8	1.3
Atropine (phenyl)	0.02	0	1.0	
Atropine (phenyl)	0.02	4	3.1	2.1
Atropine (phenyl) Plus gallamine	0.02 0.04	4	2.2	1.2
Physostigmine (NCH_3)	0.02	0	1.2	
Physostigmine (NCH_3)	0.02	4	2.8	1.6
Physostigmine (NCH_3) Plus gallamine	0.02 0.04	4	3.0	1.8

[a] Measurements were made in 0.1 *M* phosphate buffer, pH 7.4, at 39° in a Varian A-60D spectrometer. [b] Values given are observed measured linewidths corrected for instrumental broadening (0.4 Hz).

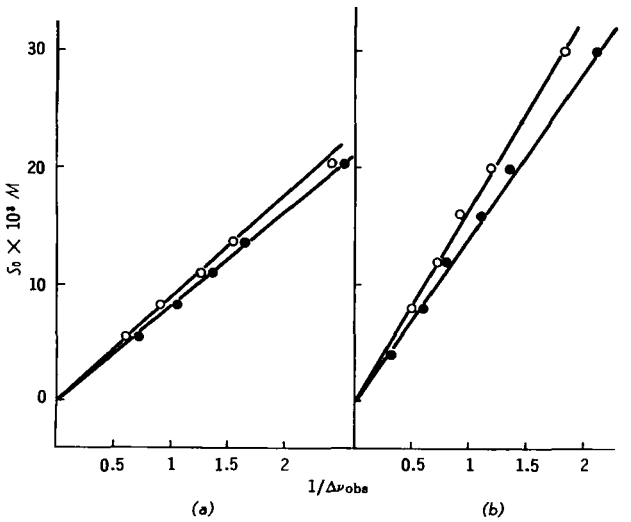

Figure 2—(a) *Plot of the reciprocal of the observed linewidth* ($\Delta\nu_{obs}$) *for the* N-*methyl* (O) *and C-methyl* (●) *group resonances of physostigmine* versus *varying concentrations of physostigmine sulfate* (S_0) *in the presence of constant concentrations of acetylcholinesterase* (1.7×10^{-5} M). (*b*) *Plot of the reciprocal of the observed linewidth* ($\Delta\nu_{obs}$) *for the phenyl* (●) *and* N-*methyl* (O) *group resonances of atropine* versus *varying concentrations of atropine sulfate* (S_0) *in the presence of a constant concentration of acetylcholinesterase* (1.7×10^{-5} M). *Samples were dissolved in 0.1* M *phosphate buffer, pH 7.4, in* D_2O, *and spectra were recorded at 39°. In these experiments the linewidth of the free inhibitor,* $\Delta\nu_S$, *was subtracted from the observed linewidth,* $\Delta\nu_{obs}$.

subtracting the values of $\Delta\nu_S$ from the measured values of $\Delta\nu$. Therefore:

$$\Delta\nu_{obs} = \frac{[ES]}{[S_0]}\Delta\nu_{ES} \qquad \text{(Eq. 6}a\text{)}$$

$$[ES] = \frac{\Delta\nu_{obs}}{\Delta\nu_{ES}}[S_0] \qquad \text{(Eq. 6}b\text{)}$$

Substitution of Eq. 1 leads to Eq. 7:

$$K_D = E_0\left[\frac{\Delta\nu_{ES} - \Delta\nu_{obs}}{\Delta\nu_{obs}}\right] - \frac{\Delta\nu_{obs}}{\Delta\nu_{ES}}S_0\left[\frac{\Delta\nu_{ES} - \Delta\nu_{obs}}{\Delta\nu_{obs}}\right] \qquad \text{(Eq. 7)}$$

If $\Delta\nu_{obs} \ll \Delta\nu_{ES}$:

$$S_0 = \frac{E_0\Delta\nu_{ES}}{\Delta\nu_{obs}} - K_D \qquad \text{(Eq. 8)}$$

A plot of S_0 *versus* $1/\Delta\nu_{obs}$ gives a line whose intercept is $-K_D$ and whose slope is used to calculate $\Delta\nu_{ES}$ (16).

EXPERIMENTAL

Binding of Atropine and Physostigmine to Acetylcholinesterase—The NMR spectrum of atropine hydrochloride in D_2O exhibits resonances at 444 and 161 Hz, which can be assigned to the phenyl and *N*-methyl group protons, respectively. The spectrum of physostigmine hydrochloride consists of a resonance at 186 Hz due to the *N*-methyl group and a resonance at 88 Hz due to the C-methyl group in the fused ring system. Because these were the most intense resonances in the spectra, they were used for the present study.

Figure 1 shows these resonances at 60 MHz, with tetramethylsilane as an external standard. The addition of highly purified acetylcholinesterase (specific activity = 200 mmoles acetylthiocholine/hr/mg; 1.7×10^{-5} *M* final concentration) to a sample of physostigmine or atropine hydrochloride at various concentrations (10^{-2}–1.4×10^{-1} *M*) resulted in the spectra shown in Fig. 1. Both resonances of atropine and physostigmine were extensively broadened without a change in their shifts.

Figure 1 illustrates that the *N*-methyl and aromatic resonances of atropine and the *N*-methyl and C-methyl resonances of physostigmine become progressively broader as the concentration of inhibitor is decreased. It is evident from these observations that the chemical exchange of both inhibitors between free and enzyme-bound species is rapid.

As described under *Theoretical*, if the enzyme–inhibitor binding is studied by varying the inhibitor concentration at a fixed concentration of enzyme, a plot of S_0 with respect to $1/\Delta\nu_{obs}$ should yield a straight line with a slope of $E_0\,\Delta\nu_{ES}$ and an intercept equal to $-K_D$, provided the described conditions are met.

The linewidth data obtained for atropine and physostigmine are plotted in this manner in Fig. 2. In these experiments, E_0 was held fixed at 17 μM. Variations of S_0 with $1/\Delta\nu_{obs}$ are linear for both peaks of atropine and physostigmine over the range of conditions used. The following linewidths for the enzyme–inhibitor complexes and dissociation constants are calculated from the least-squares lines. For physostigmine, $\Delta\nu_{ES,NCH_3} = 496 \pm 26$ Hz, $K_D = 5.9 \pm 2.4 \times 10^{-4}$ *M*, $\Delta\nu_{ES,CCH_3} = 488 \pm 16$ Hz, and $K_D = 4.3 \pm 4.0 \times 10^{-4}$ *M*. For atropine, $\Delta\nu_{ES,NCH_3} = 984 \pm 52$ Hz, $K_D = 1.2 \pm 5.8 \times 10^{-4}$ *M*, $\Delta\nu_{ES,phenyl} = 858 \pm 32$ Hz, and $K_D = 3.0 \pm 3.9 \times 10^{-4}$ *M*.

The *N*-methyl and C-methyl groups of physostigmine are immobilized to the same extent when physostigmine binds to the enzyme, as might be expected, since both groups are on the same fused ring system. With atropine the *N*-methyl group is also immobilized to approximately the same extent as the phenyl group.

Since $K_D \ll S_0$, K_D cannot be obtained with great precision. Nevertheless, it is clear that atropine and physostigmine bind with high affinity ($K_D < 1$ m*M*). The K_i values for atropine and physostigmine were then determined from kinetic studies, using acetylthiocholine as the substrate. Whereas physostigmine sulfate was an effective anticholinesterase ($K_i = 1.2$ μM), atropine sulfate inhibited poorly ($K_i = 6.0$ m*M*). If both compounds bind to the active site, both should inhibit substrate hydrolysis. Yet atropine is an ineffective anticholinesterase but binds to the enzyme with high affinity. This indicates that physostigmine may bind to the active site and atropine to another.

That atropine and physostigmine do not bind to the same sites on the enzyme surface was confirmed by adding gallamine triethiodide to a solution of acetylcholinesterase containing either atropine or physostigmine and showing that gallamine reduced the amount of bound atropine but not physostigmine. Table I gives the quantitative data obtained. Gallamine and atropine compete for

Table II—Linewidth Data for *N*-Methyl and Phenyl Protons of Atropine and *N*-Methyl and C-Methyl Protons of Physostigmine Obtained from Association of Atropine and Physostigmine with Acetylcholinesterase, in Absence and Presence of Carbamylcholine Chloride (Carbachol)[a]

Inhibitor (Protons)	Concentration: Inhibitor, *M*	Concentration: Acetylcholinesterase, mg/ml	$\Delta\nu_{obs}$[b], Hz	$\Delta\Delta\nu$, Hz
Atropine (NCH_3)	0.02	0	1.5	—
Atropine (NCH_3)	0.02	4	3.5	2.0
Atropine (NCH_3) Plus carbachol	0.02 0.10	4	2.7	1.2
Atropine (phenyl)	0.02	0	1.0	
Atropine (phenyl)	0.02	4	2.5	1.5
Atropine (phenyl) Plus carbachol	0.02 0.10	4	2.5	1.5
Physostigmine (NCH_3)	0.02	0	1.2	
Physostigmine (NCH_3)	0.02	4	3.2	2.0
Physostigmine (NCH_3) Plus carbachol	0.02 0.10	4	2.3	1.1
Physostigmine (CCH_3)	0.02	0	1.4	
Physostigmine (CCH_3)	0.02	4	3.8	2.4
Physostigmine (CCH_3) Plus carbachol	0.02 0.10	4	3.0	1.6

[a] Measurements were made in 0.1 *M* phosphate buffer, pH 7.4, at 39° in a Varian A-60D spectrometer. [b] See Table I.

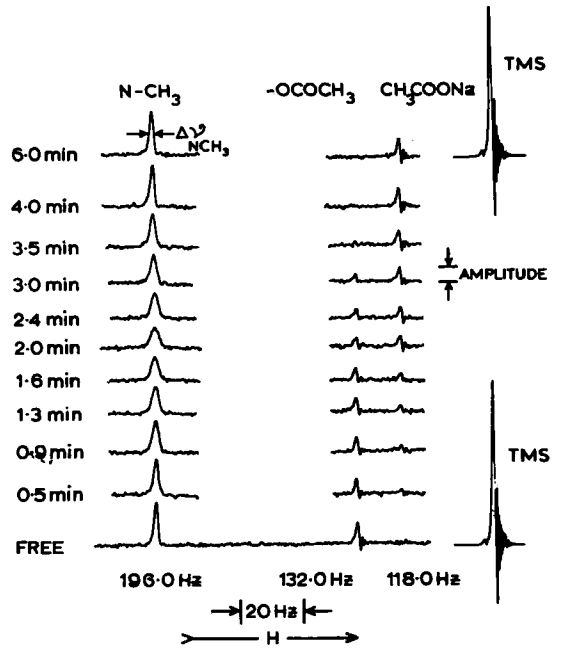

Figure 3—*Time study of the NMR spectrum of acetylcholine chloride (16.7 mM), free and after addition to acetylcholinesterase (50 mg/ml) in 0.1 M sodium phosphate buffer, pH 7.4, in D_2O at 39°. The lower trace represents a portion of the NMR spectrum of acetylcholine free in solution. The upper traces are the scans of the acetylcholine spectrum at various times after addition to acetylcholinesterase. Resonances at 196 and 132 Hz (downfield from external tetramethylsilane) are from protons in the N-methyl and acetate groups of acetylcholine. The resonance at 118 Hz is from sodium acetate protons, a product of hydrolysis of acetylcholine. The arrows on the N-methyl peak indicate the width of the resonance line at half-height ($\Delta\nu_{NCH_3}$). The arrows on the acetate peak indicate its amplitude in millimeters. A solution of tetramethylsilane was used as an external standard, and its resonance appears to the upper field of the acetylcholine resonances. Its spectrum was recorded before and after the experiment. The specific activity of squid acetylcholinesterase was 1.4 mmoles of acetylthiocholine/hr/mg.*

the same sites on the enzyme but gallamine and physostigmine do not.

Carbamylcholine chloride, which resembles the structure of acetylcholine but is not hydrolyzed by acetylcholinesterase, competes with physostigmine binding. The quantitative data are presented in Table II. Carbamylcholine also competes with the binding of atropine but, surprisingly, only competes with the N—CH_3 group of atropine while the binding of the phenyl group remains unchanged.

These results indicate that acetylcholinesterase has groups outside its active site for binding certain inhibitors such as atropine. The most decisive evidence that the atropine binding site is distinct from the active site comes from the following reasoning and observations. If it is assumed that atropine and physostigmine bind at the same site, then any compound able to compete with atropine at its binding site would be expected to compete with physostigmine. If, on the other hand, the atropine and physostigmine sites are distinct, some compounds should be able to displace atropine from its site without necessarily competing with the substrate or physostigmine. That the latter is obtained is demonstrated by the fact that gallamine inhibits atropine binding without inhibiting physostigmine binding.

Gallamine decreased the linewidth of both phenyl and *N*-methyl groups of atropine in the presence of enzyme, as expected under the present conditions, since both gallamine and atropine have a phenyl and a charged quaternary ammonium group. The phenyl group of gallamine competes with the phenyl group of atropine, and the *N*-ethyl group of gallamine displaces the *N*-methyl group of atropine.

The results with carbamylcholine are less clear. One explanation is that there are two different binding sites for atropine. In this case, the atropine molecule binds *via* the *N*-methyl group at one site and *via* the phenyl group at the other. Carbamylcholine may interfere with the binding of atropine at the first site without affecting the second. In the presence of carbamylcholine, therefore, atropine would remain bound at the second site *via* the phenyl group. This is not unlikely, since carbamylcholine does not contain an unsaturated ring.

The second possibility is that atropine binds *via* both the *N*-methyl and phenyl groups and that carbamylcholine interferes with the binding of the *N*-methyl group only, leaving the phenyl group bound. This is consistent with the idea that both groups of atropine are necessary for maximum binding to the enzyme (2, 3).

Binding of Acetylcholine to Acetylcholinesterase—The binding of acetylcholine to acetylcholinesterase was studied by measuring the change in the linewidth at half-maximum amplitude of the *N*-methyl group ($\Delta\nu_{NCH_3}$) as a function of time. The rate of hydrolysis was followed by measuring the amplitude of the acetate peak (118 Hz) as a function of time. The lower trace in Fig. 3 shows a portion of the NMR spectrum of acetylcholine chloride. The peaks are labeled N—CH_3 and —$OCOCH_3$ for the protons of the quaternary ammonium and acetate moieties, respectively.

When acetylcholine chloride was added to a solution of the enzyme and the spectrum of the solution was scanned repeatedly, the spectrum underwent several changes (upper traces of Fig. 3). The N—CH_3 group resonance became broader, reaching a maximum at 2 min, followed by a gradual sharpening of this same resonance. The amplitude of the resonance at 132 Hz (due to the acetylcholine acetate protons) decayed with time, while that at 118 Hz (due to the free acetate protons) gradually increased and reached maxi-

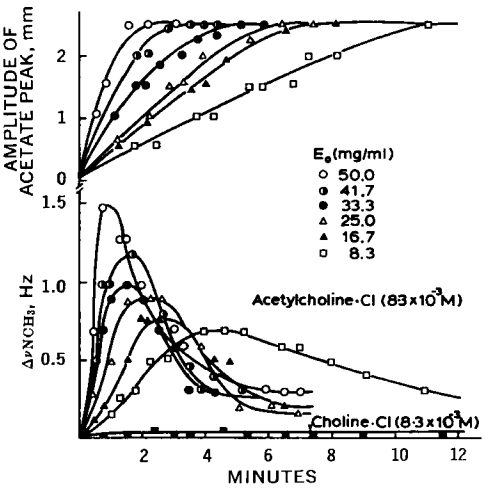

Figure 4—*Plot of the observed linewidth for the* N*-methyl group resonance of acetylcholine (lower graph) and the amplitude of the sodium acetate group resonance (upper graph)* versus *time at varying concentrations of enzyme, E_0, in the presence of the same concentration of acetylcholine chloride (8.3 mM). At zero time, a solution of acetylcholine chloride (10 μl of 1.0* M*) was rapidly mixed with a solution of acetylcholinesterase in the NMR tube. The tube was placed in the probe, and the acetylcholine spectrum was scanned repeatedly. The change in linewidth of the* N*-methyl group and the amplitude of the acetate group were measured from the same experiment. This procedure was repeated using six different concentrations of enzyme. The plot is the difference between the linewidths for acetylcholine chloride in the presence of acetylcholinesterase and in buffer only (0.8 ± 0.05 Hz). Included in the graph is the change in linewidth of the* N*-methyl group of choline chloride (8.3×10^{-3} M) when mixed with a solution of acetylcholinesterase (50 mg/ml). The solution contained 0.1* M *sodium phosphate buffer, pH 7.4, in 98% D_2O. The total volume was 0.6 ml, and the temperature was 39°. The enzyme used was identical to the one used in the experiment shown in Fig. 3.*

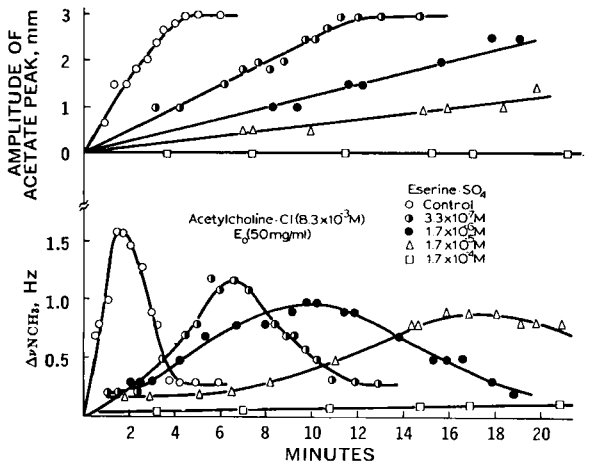

Figure 5—*Plot of the observed linewidth for the* N*-methyl group resonance of acetylcholine (lower graph) and the amplitude of the sodium acetate group resonance (upper graph) versus time at varying concentrations of physostigmine (eserine) in the presence of the same concentration of enzyme (E_0 = 50 mg/ml) and substrate (acetylcholine chloride = 8.3 mM). The experimental procedure was the same as in Figs. 2 and 3, with the exception that the enzyme was preincubated with various concentrations of physostigmine before the addition of substrate. Measurements were made in 0.1* M *phosphate buffer, pH 7.4, in D_2O at 39°.*

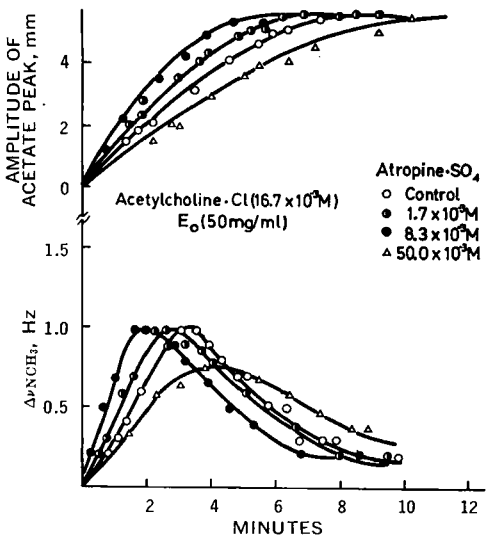

Figure 6—*Plot of the observed linewidth for the* N*-methyl group of resonance of acetylcholine (lower graph) and the amplitude of the sodium acetate group resonance (upper graph) versus time at the same concentrations of enzyme (E_0 = 50 mg/ml) and substrate (acetylcholine chloride = 16.7 mM). The experimental procedure was the same as in Figs. 2 and 3, with the exception that the enzyme was preincubated with various concentrations of atropine before the addition of substrate. Measurements were made in 0.1* M *phosphate buffer, pH 7.4, in D_2O at 39°.*

mum amplitude at 6 min. No further changes occurred after this time. While the acetylcholine acetate peak may be slightly broadened, the free acetate peak was not broadened.

The changes in $\Delta\nu_{NCH_3}$ and the amplitude of the acetate peak are shown in Fig. 4 with six different concentrations of enzyme, E_0, at the same substrate concentration. With each enzyme concentration, a characteristic change in linewidth was observed, *i.e.*, a progressive broadening followed by a decrease in linewidth after which a steady state was reached. At lower enzyme concentrations, the maximum linewidth decreased and the time of maximum broadening was delayed. When choline was added to the enzyme, its *N*-methyl resonance was not broadened. The changes in linewidth are interpreted in terms of binding of the substrate molecule to the active center of the enzyme.

Binding of Acetylcholine to Acetylcholinesterase in Presence of Physostigmine—In the presence of physostigmine sulfate, the *N*-methyl group reasonance of acetylcholine showed a decrease in the rate of line broadening, indicating a delay in the reaction as well as a decrease in maximal line broadening. These results are shown in Fig. 5 with four different concentrations of physostigmine. The upper graph shows the effect of increasing concentrations of physostigmine on the rate of acetate formation. With physostigmine sulfate = 1.7×10^{-4} *M*, line broadening and hydrolysis were completely abolished.

Binding of Acetylcholine to Acetylcholinesterase in Presence of Atropine—The effects of atropine on acetylcholine binding are uniquely different from those observed with physostigmine. In the presence of atropine, the effect on the *N*-methyl resonance was an increase in the rate of line broadening without influencing the maximum linewidth. The upper graph in Fig. 6 shows that atropine ($1.7 - 8.3 \times 10^{-3}$ *M*), under the present conditions, accelerates the rate of substrate hydrolysis. Higher concentrations of atropine (50×10^{-3} *M*) inhibit hydrolysis and substrate binding.

These findings suggest that atropine does not interfere with the binding of the substrate but instead accelerates its hydrolysis. The mechanism by which atropine or its analogs accelerate, if they are not bound at the active site, could be accounted for by allosteric interactions. That is, the binding of atropine transmits a conformational change through the structure of the enzyme which modulates its catalytic activity.

The acceleration by atropine and the inhibition by physostigmine were observed in the presence of high concentrations of substrate. Lower concentrations of substrate could not be used because they caused inaccuracies in linewidth measurements. It is possible, therefore, that these observations depend on the presence of relatively high concentrations of acetylcholine.

Some researchers (17, 18) showed that certain quaternary ammonium compounds potentiate the hydrolytic activity of acetylcholinesterase at high substrate concentrations. They suggested that these compounds accelerate the deacetylation sequence of the enzyme. If atropine accelerates deacetylation by binding to the anionic site of the active center, it must assume an orientation away from the esteratic site; otherwise it will interfere with deacetylation. This may be possible in view of the suggestion by Belleau *et al.* (19) of *exo* and *endo* binding of effectors at the esteratic site level.

Alternatively, atropine may protect the enzyme against substrate inhibition; that is, high concentrations of substrate bind to a regulatory site and exert negative cooperativity toward the active center. Atropine competes with the substrate at the regulatory site and prevents substrate inhibition. This implies that substrate inhibition may be due to an allosteric mechanism. Recent evidence (20) suggests that this may be the correct mechanism for the observed kinetic effects.

It is impossible to conclude, from the data presented here, which of the two mechanisms is correct. Nevertheless a two-site mechanism—the active site and a regulatory site—must be involved in each scheme.

The use of NMR techniques outlined in this paper overcomes some problems of other methods previously used for the study of enzyme–substrate interactions. The experimental procedure described for substrate binding is particularly useful in studying the active site region and the effects of various inhibitors on the enzyme–substrate complex. This method provides the advantage of following the rate of hydrolysis of substrate and its binding at the active site concomitantly.

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ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Research in Anaesthesia and the Department of Pharmacology and Therapeutics, McGill University, Montreal, 101, Canada.*

Presented in part to the Symposium on Drug–Biomolecule Interactions, APhA Academy of Pharmaceutical Sciences, fall meeting, Chicago, Ill., November 1972.

Supported by the Canadian Medical Research Council.

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Drug–Biomolecule Interactions: Fluorescence Studies on Interaction of Aminonaphthalenesulfonic Acid Derivatives with Serum Albumins

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Abstract □ The binding of the three aminonaphthalenesulfonic acid derivatives to human and bovine serum albumins was studied by measuring the fluorescence enhancement of the compounds. The number of binding sites of human and bovine serum albumins for these compounds appears to be one and two, respectively, under the experimental conditions. As the molar ratio of the fluorescent compounds to bovine serum albumin increased, the binding sites appeared to increase for the compounds. The quenching of the native fluorescence of albumin was examined by the successive addition of methanolic solutions of these compounds. 1-Anilinonaphthalene-8-sulfonate quenched the protein fluorescence to a greater extent than the other compounds studied, indicating that 1-anilinonaphthalene-8-sulfonate molecules are bound more closely to the tryptophan residues of albumin. The finding that the three compounds did not quench the fluorescence of tryptophan dissolved in water indicates no direct molecular interaction between tryptophan and the three fluorescent probes. The driving force for binding may be due to the structural characteristics of the amino acid sequence surrounding the tryptophan residues.

Keyphrases □ Aminonaphthalenesulfonic acid derivatives—binding to human and bovine serum albumins, fluorescence enhancement □ Albumin, human and bovine serum—binding of three aminonaphthalenesulfonic acid derivatives, fluorescence enhancement □ Probes—binding of three aminonaphthalenesulfonic acid derivatives to serum albumins, fluorescence enhancement □ Drug–biomolecule interactions—fluorescence studies on interaction of aminonaphthalenesulfonic acid derivatives with serum albumins □ Interactions—drugs with biomolecules, symposium

Interaction of organic molecules with body proteins has resulted in many facets of research interest in biomedical studies. When drugs are bound to plasma proteins, distribution, therapeutic efficacy, and elimination of the drugs may be altered. Biotransformation of organic compounds occurs through interactions of the compounds with metabolizing enzymes. The pharmacological effects of drugs are also thought to be mediated by interactions of the drug molecules with active site proteins.

Because of the clinical implications of such interactions, numerous studies have been carried out concerning these subjects (1, 2). Although aims and experimental techniques vary among investigators, most studies have been directed toward explaining the effects of the interactions.

In this study, fluorescence spectroscopy was employed to study the binding of three fluorescent probes to human and bovine serum albumins. A fluorescent probe is a compound with spectral properties such as optimal excitation and emission wavelengths, fluorescence intensity, and lifetime of the excited state that undergo changes reflecting its molecular environment. When probe molecules bind to protein binding sites, an enhancement of fluorescence emission and wavelength shift occur.