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# Nuclear Magnetic Resonance Studies on Acetylcholinesterase

## The Use of Atropine and Eserine to Probe Binding Sites

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SUMMARY

Nuclear magnetic resonance has been used to study the association of atropine and eserine with acetylcholinesterase extracted from squid head ganglia. Line width changes of the *N*-methyl and phenyl groups of atropine and the *N*-methyl and *C*-methyl groups of eserine resulting from association with the enzyme have been utilized to study the interactions. The observed line widths in the presence of enzyme were similar for atropine and eserine. The addition of tetraethyl pyrophosphate or diisopropyl fluorophosphate diminished the binding of eserine but not of atropine. Kinetic studies, using acetylcholine as the substrate, showed that eserine was a potent cholinesterase inhibitor whereas atropine was a poor anticholinesterase. These results indicate that there are at least two binding sites on the enzyme. One is the active center (where eserine binds), which consists of an anionic and an esteratic site; the other is an anionic site (where atropine binds), which is distinct from the catalytic site.

### INTRODUCTION

Recent studies indicate that acetylcholinesterase contains binding sites which are noncatalytic. Changeux, Leuzinger, and Huchet (1) have shown the reversible binding of acetylcholine to acetylcholinesterase, which was inhibited by eserine, and Changeux (2) has demonstrated the binding of gallamine triethiodide (Flaxedil) and *d*-tubocurarine, two receptor inhibitors which only partially inhibited the enzyme. Kitz *et al.* (3) have shown the presence of an allosteric site where the binding of certain drugs is capable of modulating the activity of the catalytic site through conformational changes in the enzyme. Belleau and his co-workers (4, 5) have shown that at least two anionic sites are present on acetylcholinesterase: site I, which is part of the active center, and site II, located elsewhere on the protein molecule. Their results imply certain similarities between the anionic sites of acetylcholinesterase and the acetylcholine receptors of excitable membranes. The enzyme used in these studies was obtained from the electric organ of *Electrophorus electricus* (1–3) or from bovine erythrocytes (4, 5).

The present paper describes a study of the binding of atropine and eserine to acetylcholinesterase isolated from squid head ganglia, using proton nuclear magnetic resonance ($^1$H NMR) methods (6). These two compounds were used as probes for the two postulated binding sites of acetylcholinesterase. It is known that eserine, which reversibly inhibits acetylcholine hydrolysis, has a high affinity for the catalytic site and

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may be described as an ideal compound to assay hydrolytic sites, whereas atropine may be considered a suitable compound to assay receptor sites since it binds weakly to known macromolecules (7) and has a high affinity for muscarinic receptors (8).

## METHODS

Acetylcholinesterase was extracted from the head ganglia of squid (*Loligo apalescens*, obtained from the U. S. Freezer Company, Monterey, Calif.) with phosphate-buffered NaCl (0.1 M sodium phosphate buffer, pH 7.3, and 0.1 M NaCl) and was purified by ammonium sulfate fractionation. The 20–40% ammonium sulfate fraction was dissolved in phosphate-buffered NaCl, dialyzed against distilled water for 24 hr, and centrifuged, and the supernatant fluid was lyophilized to dryness. The specific activity of the acetylcholinesterase was 20 mmoles of acetylcholine hydrolyzed per milligram per hour.

Other materials were obtained from the following sources: atropine sulfate, Mann Research Laboratories; eserine sulfate, Nutritional Biochemicals Corporation; tetraethyl pyrophosphate, Monsanto Company, Ltd.; diisopropyl fluorophosphate, Mann Research Laboratories; acetylcholine chloride, British Drug Houses; and $D_2O$, Merck Sharp and Dohme, Ltd.

All solutions were dissolved in 99.7% $D_2O$–sodium phosphate buffer (0.1 M or 0.01 M, pH 7.4). The apparent pH of the sample as measured with a glass microelectrode was adjusted with 0.1 N NaOD or 0.1 N DCl in $D_2O$. Solutions were made up to 0.5 ml and placed in standard NMR tubes. The rate of hydrolysis of acetylcholine by acetylcholinesterase with or without inhibitor was measured as described previously (9).

All NMR measurements were made on a Varian A-60D high-resolution spectrometer. Chemical shifts are expressed in Hertz from the external standard, tetramethylsilane. Most line width measurements were made at a sweep width of 5 Hz/cm and a sweep rate of 0.5 Hz/sec. Measured line widths were corrected for instrumental broadening (0.4 Hz), and relaxation rates, $1/T_2$, were calculated from $1/T_2 = \pi\Delta\nu_{1/2}$, where $\Delta\nu_{1/2}$ is the line width of the atropine or eserine protons at one-half maximum peak height and $T_2$ is the transverse relaxation time. For the case of rapid exchange of the small molecule between a site on the protein and the solution, the observed relaxation rate, $(1/T_2)_{obs}$, is a weighted average of the relaxation rates of the bound and free nucleii, each term being weighted by the appropriate mole fraction (10, 11). It was assumed in the present study that the longitudinal relaxation time $T_1$ was equal to $T_2$, and all measurements are reported as $1/T_2$.

## RESULTS

The NMR spectrum at 60 MHz of atropine sulfate exhibits resonances at 443 Hz and 161 Hz (downfield from tetramethylsilane), which can be unambiguously assigned to the phenyl and *N*-methyl group protons, respectively. Eserine sulfate exhibits resonances at 186 Hz and 88 Hz, which can be assigned to the *N*-methyl and *C*-methyl protons, respectively (see Fig. 1). Because these are the most intense resonances in the spectra and are not split by spin-spin coupling, they were best suited for our present study.

*Association of atropine and eserine with acetylcholinesterase.* Typical results are shown in Fig. 1 for the association of atropine and eserine with acetylcholinesterase. At 39° and pH 7.4 in the presence of enzyme (4 mg/ml), the resonance lines of the *N*-methyl and phenyl groups of atropine undergo broadening as a result of association with the enzyme (Fig. 1, left). The resonance lines of the *N*-methyl and *C*-methyl groups of eserine are also markedly broadened in the presence of enzyme (Fig. 1, right). However, within experimental error (estimated to be less than 1%), no significant changes in the positions of these resonance lines could be detected in the presence of acetylcholinesterase.

To confirm that the effects observed in the NMR spectra of atropine and eserine in the presence of acetylcholinesterase were due to association between inhibitor and enzyme rather than to nonspecific viscosity effects in the protein solution, the NMR spectra of atropine and eserine in similarly concentrated solutions of bovine serum albumin were also recorded. It was found that the addition of albumin to either atropine or

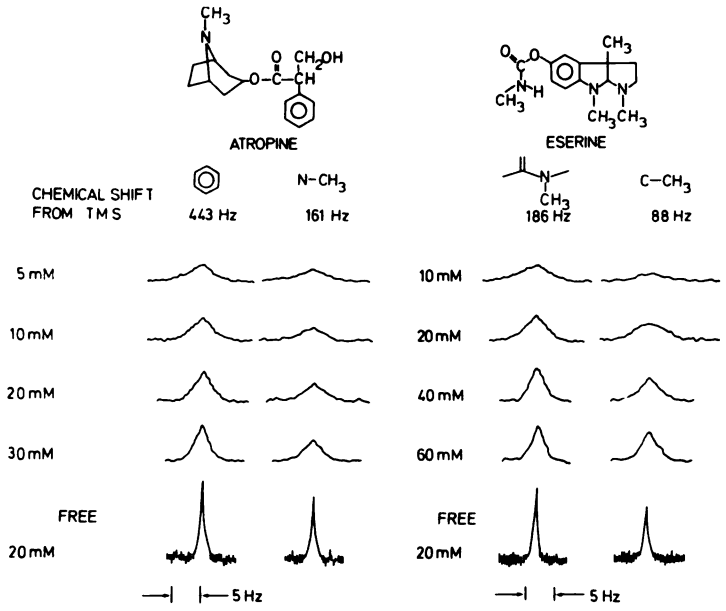


FIG. 1. *Proton NMR spectra of atropine and eserine*

The phenyl and *N*-methyl resonances of atropine and the *N*-methyl and *C*-methyl resonances of eserine in the presence of acetylcholinesterase (4 mg/ml) are shown at various concentrations of atropine and eserine. The solvent was 99.7% $D_2O$ containing 0.01 M sodium phosphate buffer, pH 7.4, at 39°. TMS, tetramethylsilane.

eserine did not result in any line width changes from that of the free inhibitor. Nor did changes in the concentration of the inhibitor affect line widths by more than 10% over the concentration range of atropine or eserine sulfate from $10^{-2}$ M to $10^{-1}$ M.

The effect of acetylcholinesterase concentration on the relaxation rate of the *N*-methyl and phenyl protons of 20 mM atropine sulfate is shown in Fig. 2A; Fig. 2B shows the effect of acetylcholinesterase concentration on the relaxation rate of the *N*-methyl and *C*-methyl protons of 20 mM eserine sulfate. A change in the enzyme concentration from 0 to 5 mg/ml increased the observed relaxation rates of the *N*-methyl and phenyl protons of atropine from 3.2 $sec^{-1}$ to 14.1 $sec^{-1}$ ($\pm 0.7$ $sec^{-1}$) and from 1.5 $sec^{-1}$ to 9.1 $sec^{-1}$ ($\pm 0.6$ $sec^{-1}$), respectively; the relaxation rate of the *N*-methyl group of eserine increased from 0.9 $sec^{-1}$ to 13.4 $sec^{-1}$ ($\pm 0.8$ $sec^{-1}$), whereas that of the *C*-methyl group increased from 1.6 $sec^{-1}$ to 17.9 $sec^{-1}$ ($\pm 1.0$ $sec^{-1}$) for a similar range of enzyme concentration. Thus similar relaxation rates were observed with both inhibitors in the presence of the enzyme.

Under the present conditions of pH 7.4 and 39°, the enzyme-inhibitor complex was short-lived and yielded a spectrum representative of a weighted average of the enzyme-bound and free forms of inhibitor. The effect of inhibitor concentration on line width is shown in Fig. 1. This illustrates that the *N*-methyl and aromatic resonances of atropine and the *N*-methyl and *C*-methyl resonances of eserine become progressively broader as the concentration of inhibitor is decreased. As pointed out earlier (12), this is the opposite to what one would expect if the broadening were due to nonspecific mechanisms. At a given ratio of enzyme to inhibitor, the spectrum of eserine appears to be somewhat broader than that of atropine.

The effect of inhibitor concentration on the relaxation rates at constant enzyme concentration is shown in Fig. 3. The observed relaxation rates of the *N*-methyl and phenyl peaks of atropine increased from 8.8 $sec^{-1}$ ($\pm 1.0$ $sec^{-1}$) to 19.4 $sec^{-1}$ ($\pm 1.2$ $sec^{-1}$) and from 7.5 $sec^{-1}$ ($\pm 0.7$ $sec^{-1}$) to 13.5 $sec^{-1}$ ($\pm 0.9$ $sec^{-1}$), respectively, as the concentration of atropine sulfate was decreased from 40 to 5 mM. The relaxation rates of the

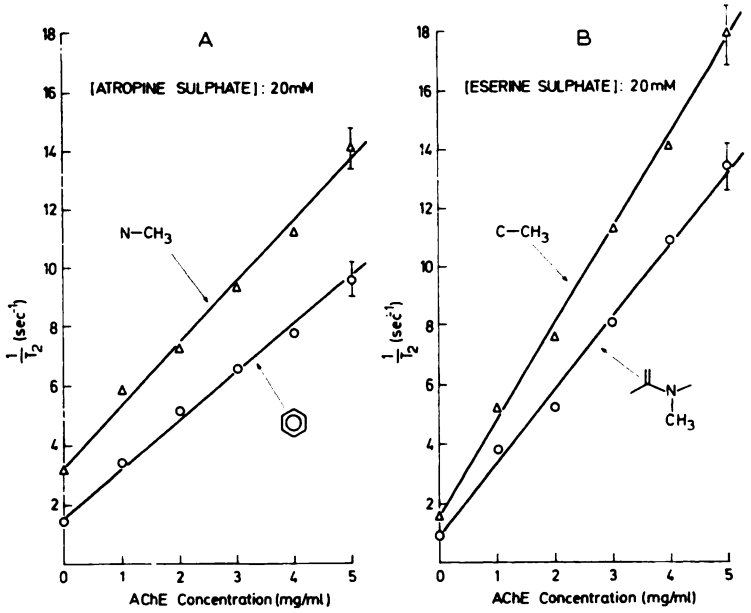


FIG. 2. *Relaxation rates of 20 mM atropine sulfate (A) and 20 mM eserine sulfate (B) as a function of acetylcholinesterase (AChE) concentration*

The solvent was 99.7% $D_2O$ containing 0.01 M sodium phosphate buffer, pH 7.4, at 39°.

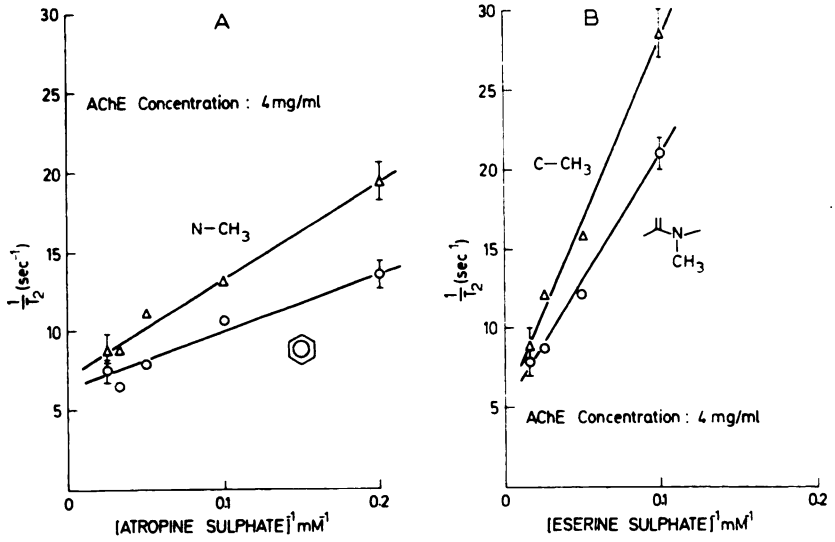


FIG. 3. *Relaxation rates of atropine sulfate (A) and eserine sulfate (B) as a function of the inverse of their respective concentrations at constant acetylcholinesterase (AChE) concentration*

The solvent was 99.7% $D_2O$ containing 0.01 M sodium phosphate buffer, pH 7.4. at 39°.

*C*-methyl and *N*-methyl peaks of eserine increased from 8.8 $sec^{-1}$ ($\pm$1.2 $sec^{-1}$) to 28.5 $sec^{-1}$ ($\pm$1.5 $sec^{-1}$) and from 7.9 $sec^{-1}$ ($\pm$0.9 $sec^{-1}$) to 21.0 $sec^{-1}$ ($\pm$1.0 $sec^{-1}$), respectively, as the concentration of eserine sulfate was decreased from 60 to 10 mM.

Calculation of the relaxation rates of the bound form of the small molecules is possible, as described elsewhere (10). These results are summarized in Table 1. On binding to acetylcholinesterase, the relaxation rate of the *N*-methyl group of atropine increased, from

TABLE 1

*Relaxation rates and $I_{50}$ for atropine and eserine*

$(1/T_2)_{bound}$ and $(1/T_2)_{free}$ are the transverse relaxation rates of the bound and free nuclei, respectively. $(1/T_2)_{bound}$ was obtained from plot of (a) $(1/T_2)_{obs}$ with respect to the total enzyme concentration at constant inhibitor concentration or (b) $(1/T_2)_{obs}$ with respect to the inverse of the inhibitor concentration at constant enzyme concentration. $I_{50}$ is the concentration of inhibitor required to produce 50% inhibition of the enzyme.

| Inhibitor | Group | $(1/T_2)_{free}$ | $(1/T_2)_{bound}$ | $(T_2)_{free}:(T_2)_{bound}$ | $I_{50}$ |
|---|---|---|---|---|---|
| | | $sec^{-1}$ | $sec^{-1}$ | | $M$ |
| Eserine | $CCH_3$ | 1.6 | (a) $16.2 \times 10^3$ | $10.1 \times 10^3$ | $10^{-7}$ |
| | | | (b) $13.0 \times 10^3$ | $8.1 \times 10^3$ | |
| | $NCH_3$ | 0.9 | $12.2 \times 10^3$ | $13.6 \times 10^3$ | |
| | | | $9.1 \times 10^3$ | $10.1 \times 10^3$ | |
| Atropine | $NCH_3$ | 3.2 | $21.2 \times 10^3$ | $6.6 \times 10^3$ | $10^{-1}$ |
| | | | $6.9 \times 10^3$ | $2.2 \times 10^3$ | |
| | Phenyl | 1.5 | $16.4 \times 10^3$ | $10.9 \times 10^3$ | |
| | | | $4.1 \times 10^3$ | $2.7 \times 10^3$ | |

3.2 $sec^{-1}$ to $21.2 \times 10^3$ $sec^{-1}$, by a factor of 6600, and that of the phenyl group, from 1.5 $sec^{-1}$ to $16.4 \times 10^3$ $sec^{-1}$, by a factor of 10,900; the relaxation rate of the *N*-methyl group of eserine increased, from 0.9 $sec^{-1}$ to $12.2 \times 10^3$ $sec^{-1}$, by a factor of 13,600, and that of the *C*-methyl group, from 1.6 $sec^{-1}$ to $16.2 \times 10^3$ $sec^{-1}$, by a factor of 10,100.

*pH dependence of binding.* A study of the effect of pH (5.8–10.1) at constant ionic strength (0.01 M sodium phosphate buffer) on the proton relaxation rates of 20 mM atropine sulfate in the presence of acetylcholinesterase (4 mg/ml) is shown in Fig. 4A. As the pH was increased from 5.8, $(1/T_2)_{obs}$ of both the *N*-methyl and phenyl groups increased, the *N*-methyl curve having a maximum at pH 7.4 and the phenyl curve passing through a plateau in the same pH region. Between pH 8.0 and pH 10.1 there was a sharp increase in both curves. No readings were taken above pH 10.1, as in alkaline solution atropine is hydrolyzed into tropine and tropic acid. There was no further change in $(1/T_2)_{obs}$ below pH 5.8.

The pH dependence of the relaxation rate of the *N*-methyl and *C*-methyl groups of 20 mM eserine sulfate in the presence of acetylcholinesterase (4 mg/ml) is shown in Fig. 4B. As the pH was increased from 6.2, $(1/T_2)_{obs}$ of both groups increased, passing through a plateau at pH 7.3. Above pH 7.5 there was again a sharp increase in both curves until pH 9.4. No further readings were taken above this pH because of the insolubility of eserine sulfate. Below pH 6.2 there was little or no change in $(1/T_2)_{obs}$ of the two groups. Changes in $(1/T_2)_{free}$ for a solution of 20 mM eserine or atropine sulfate as a function of pH were relatively small (less than 3 $sec^{-1}$) when compared to a solution of 20 mM eserine or atropine sulfate and acetylcholinesterase. Chemical shifts of the drugs in the presence of enzyme were not significantly different from the shifts in a solution of the drugs alone throughout the pH range studied.

*Effect of ionic strength on binding.* To study the effect of ionic strength on the atropine-acetylcholinesterase interaction, a 20 mM atropine sulfate–4 mg/ml acetylcholinesterase solution was chosen. This and a control solution of 20 mM atropine sulfate alone were examined in the presence of increasing amounts of NaCl, up to 4 M. The results of relaxation rate measurements are shown in Fig. 5A. An initial increase in the NaCl concentration resulted in a decrease in $(1/T_2)_{obs}$ of both *N*-methyl and phenyl group protons through a minimum at approximately 0.6 M. A further increase in the NaCl concentration resulted in a marked increase in $(1/T_2)_{obs}$, both curves passing through maxima at approximately 3 M NaCl and dropping to 7.0 $sec^{-1}$ and 7.4 $sec^{-1}$ for the *N*-methyl and phenyl peaks, respectively, at 4 M NaCl.

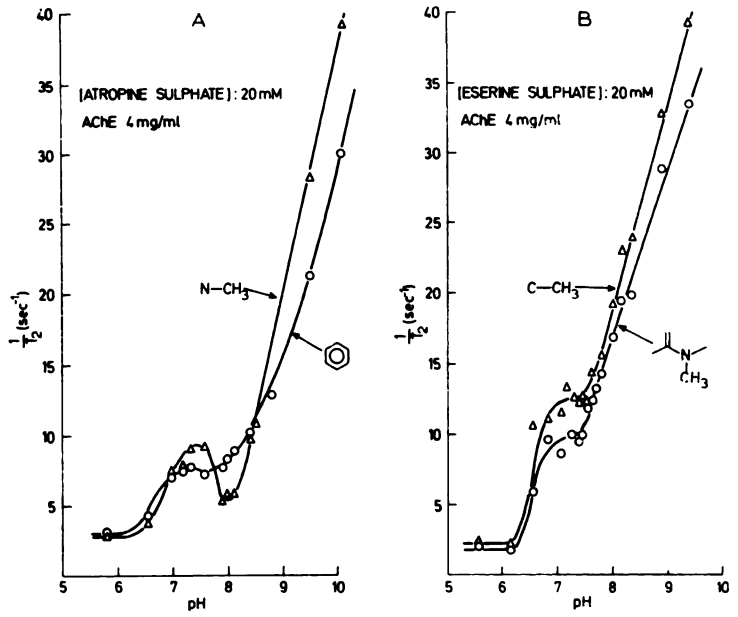


FIG. 4. *pH dependence of relaxation rates of 20 mM atropine sulfate (A) and 20 mM eserine sulfate (B) at a constant concentration of acetylcholinesterase (AChE)*
The solvent was 99.7% $D_2O$ containing 0.01 M sodium phosphate buffer at 39°.

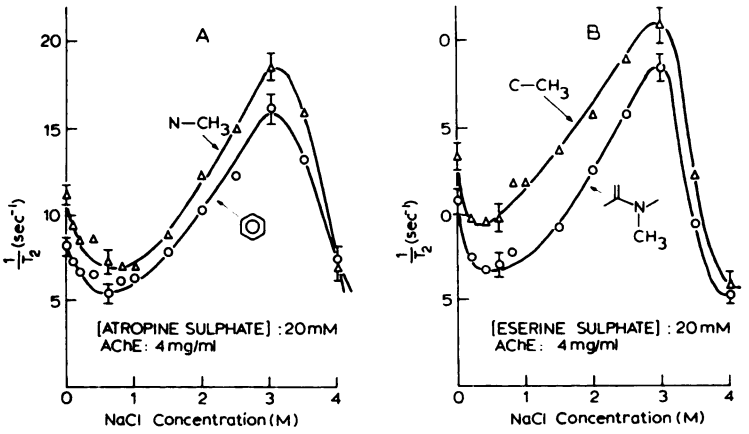


FIG. 5. *Effect of NaCl concentration on relaxation rates of 20 mM atropine sulfate (A) and 20 mM eserine sulfate (B) at a constant concentration of acetylcholinesterase (AChE)*
The solvent was 99.7% $D_2O$ containing 0.01 M sodium phosphate buffer, pH 7.4, at 39°.

Figure 5B shows the effect of NaCl concentration on the relaxation rate of the *N*-methyl and *C*-methyl group protons of 20 mM eserine sulfate with acetylcholinesterase (4 mg/ml). A pattern similar to that of atropine-acetylcholinesterase was established; that is, an initial decrease in $(1/T_2)_{obs}$ to a minimum at approximately 0.6 M NaCl, then a marked increase with increasing NaCl concentration through a maximum at 3 M NaCl, and finally a sharp drop as the NaCl concentration was increased to 4 M. Without acetylcholinesterase, $1/T_2$ of 20 mM eserine sulfate or atropine sulfate changed by approximately 1 $sec^{-1}$ as the NaCl concentration was changed from 0 to 4 M.

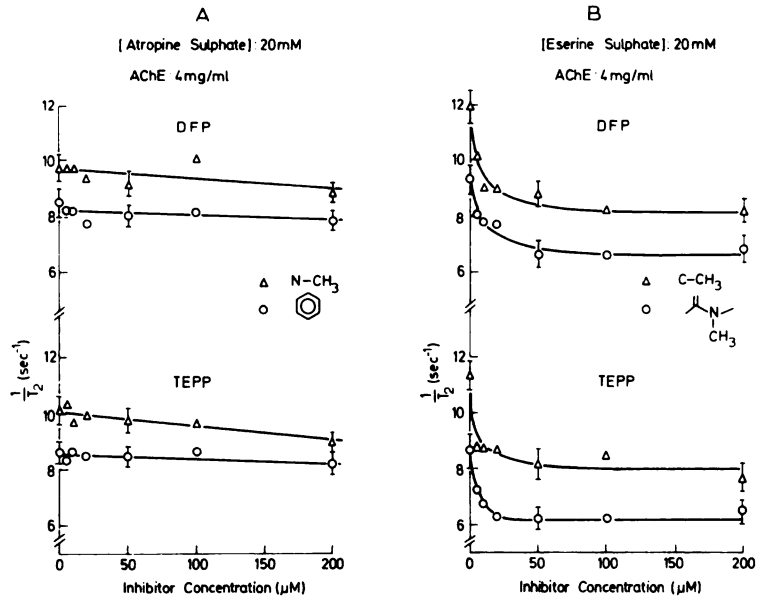


FIG. 6. *Effect of diisopropyl fluorophosphate (DFP) and tetraethyl pyrophosphate (TEPP) on relaxation rates of 20 mM atropine sulfate (A) and 20 mM eserine sulfate (B) at a constant concentration of acetylcholinesterase (AChE)*

The solvent was 99.7% $D_2O$ containing 0.01 M sodium phosphate buffer, pH 7.4, at 39°.

*Effect of diisopropyl fluorophosphate and tetraethyl pyrophosphate on binding.* In these experiments the effects of two irreversible anticholinesterases, tetraethyl pyrophosphate and diisopropyl fluorophosphate, on the binding of atropine and eserine to acetylcholinesterase were studied. The effects of these inhibitors on $(1/T_2)_{obs}$ of atropine and eserine are shown in Fig. 6. The addition of 200 μM diisopropyl fluorophosphate or 200 μM tetraethyl pyrophosphate to acetylcholinesterase (4 mg/ml) plus 20 mM atropine sulfate had little or no effect on $(1/T_2)_{obs}$ of either the *N*-methyl or phenyl group of atropine sulfate. The addition of 100 μM diisopropyl fluorophosphate to acetylcholinesterase (4 mg/ml) plus 20 mM eserine sulfate decreased $(1/T_2)_{obs}$ of the *N*-methyl and *C*-methyl groups from 9.3 $sec^{-1}$ to 6.6 $sec^{-1}$ and from 11.9 $sec^{-1}$ to 8.2 $sec^{-1}$, respectively. Similarly, the addition of 100 μM tetraethyl pyrophosphate to acetylcholinesterase (4 mg/ml) plus 20 mM eserine sulfate decreased $(1/T_2)_{obs}$ of the *N*-methyl and *C*-methyl groups from 8.6 $sec^{-1}$ to 6.2 $sec^{-1}$ and from 11.3 $sec^{-1}$ to 8.0 $sec^{-1}$, respectively. A further increase in the concentration of either inhibitory agent had little or no effect on the relaxation of either the atropine or eserine peaks in the presence of the enzyme. The selectivity in inhibiting the binding of eserine without preventing the binding of atropine suggests the existence of two distinguishable binding sites for inhibitors on acetylcholinesterase.

*Effect of atropine and eserine on the catalytic activity of acetylcholinesterase.* A direct test of the above postulate was made by studying the effect of atropine and eserine on the catalytic activity of the enzyme. Acetylcholinesterase was incubated with atropine sulfate or eserine sulfate in various concentrations ($10^{-8}$–$10^{-1}$ M) for 20 min at pH 7.4 and 39°. Acetylcholine chloride (10 mM) was then added, and the rate of hydrolysis was followed by NMR methods as described earlier for serum cholinesterase (9). The results are summarized in Table 1. While eserine is a potent anticholinesterase ($I_{50} = 10^{-7}$ M), atropine is a poor cholinesterase inhibitor ($I_{50} = 10^{-1}$ M).

## DISCUSSION

The broadening of the spectra of atropine and eserine observed in the presence of acetylcholinesterase is due to a restricted motion of the small molecule when bound to the enzyme. The similarity of the ratio $(T_2)_{free}:(T_2)_{bound}$ for the phenyl group ($10.9 \times 10^3$) and *N*-methyl group ($6.6 \times 10^3$) of atropine suggests that both ends of the molecule are immobilized to the same extent by the interaction. On binding, the relaxation rates of the *C*-methyl group and *N*-methyl group of eserine are enhanced by factors of $10.1 \times 10^3$ and $13.6 \times 10^3$, respectively. These groups are also immobilized to approximately the same extent by the interaction. Such behavior might have been expected for eserine, since both methyl groups are on the rigid ring system and would experience a similar restriction of motion when eserine binds to a macromolecule.

To explain the observed variation in $1/T_2$ with pH and ionic strength, we may initially consider the following possibilities: (a) alteration of the state of aggregation of acetylcholinesterase, which is sensitive to both pH and ionic strength, (b) change in the rate of exchange of the inhibitor between the enzyme-inhibitor complex and the solution, (c) change in the affinity of the inhibitor for the enzyme, i.e., change in binding constant, and (d) increase in the number of negative charges on the enzyme as the pH is raised.

As plots of $1/T_2$ with respect to the inverse of the inhibitor concentration at constant enzyme concentration, or $1/T_2$ with respect to enzyme concentration at constant inhibitor concentration, are linear at pH 7.4, 0.01 M sodium phosphate, and 39°, and as one of the underlying requirements for linearity is rapid exchange on the NMR time scale, we may assume such rapid exchange to occur. Although such plots were not constructed for other conditions of pH and ionic strength, it is suspected that for most or all of the conditions in the present experiments, the residence lifetime of the inhibitors on the enzyme molecule is sufficiently short and constant and need not be considered further.

Acetylcholinesterase is known to aggregate at low ionic strength (0–0.2 M NaCl) (13). The somewhat larger relaxation rate at very low ionic strength and the minimum at 0.6 M NaCl could be explained by increased aggregation of acetylcholinesterase at low ionic strength. The binding constant need not change to give the observed effect. The increased relaxation rate can be attributed to the higher molecular weight of the aggregated enzyme entity, which confers upon the bound inhibitor an increased correlation time. The decrease in $1/T_2$ through a minimum at 0.6 M NaCl can be explained by disaggregation of the enzyme at the higher ionic strength. Although the observations can be explained by the aggregation phenomenon, the binding constant could conceivably be influenced by aggregation, and could contribute to the $1/T_2$ curve. The increase in $1/T_2$ at an NaCl concentration above 0.6 M appears to be due to an increase in the binding constant caused by enhanced hydrophobic interaction between the inhibitor ring system and the enzyme, favored by high ionic strength. A larger decrease in $1/T_2$ between 0 and 0.6 M NaCl, caused by disaggregation, may be masked by the increase in binding due to the increasing dominance of hydrophobic forces at higher salt concentrations. At very high concentrations of NaCl (4 M), the decrease in $1/T_2$ could result from a conformational change at the binding site of the enzyme, giving rise to a decreased binding affinity for the inhibitor. Precipitation of the enzyme was evident, and the consequent decrease in enzyme concentration could further cause a drop in the plot of $1/T_2$ with respect to NaCl concentration.

The pH dependence of the observed relaxation rates of both atropine and eserine in the presence of acetylcholinesterase exhibits a sharp increase at alkaline pH. An increase in the pH results in an increase in the net number of negative charges on the enzyme. Formation of more negative charges with increasing pH would cause more atropine or eserine to bind. If the binding were electrostatic, $1/T_2$ would be expected to increase with an increase in the pH, but should decrease above the pK values of the inhibitors (9.8 for atropine, 8.5 for eserine) as the inhibitors become neutral species. It is likely that the increase in $1/T_2$ is due to an increase in electrostatic interactions between the posi-

tively charged inhibitors and the negatively charged protein. A decrease in $1/T_2$ above the pK values of the inhibitors did not occur, however.

It is difficult to say what the primary binding forces are in the inhibitor-acetylcholinesterase complex, although it is more than likely that both electrostatic and hydrophobic forces play a role. It would be interesting to examine the binding of tropine and tropic acid, the former containing only the aliphatic ring and charged *N*-methyl group, and the latter containing the phenyl ring. In this way some separation of the two effects may be achieved.

If both compounds bind at the catalytic site, both should inhibit acetylcholine hydrolysis. Yet atropine, which binds strongly to the enzyme, is ineffective as an inhibitor of cholinesterase. Consequently, it must bind to a site other than the catalytic site. The two binding sites can be clearly distinguished, since diisopropyl fluorophosphate or tetraethyl pyrophosphate interferes with the binding of eserine but not of atropine.

According to Leuzinger, Goldberg, and Cauvin (14), the acetylcholinesterase molecule consists of two different polypeptide chains of the same molecular weight and contains two sets of each kind. Changeux *et al.* (15) have suggested that one subunit functions as the catalytic site and that the subunit of the other class functions as the acetylcholine receptor. Much recent evidence indicates that acetylcholinesterase might play the role of receptor as well as enzyme (1–5). The physiological significance of this property of acetylcholinesterase, however, is not yet clear. Whether or not acetylcholinesterase functions as the postsynaptic receptor for acetylcholine remains to be shown.

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