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The Use of Nuclear Magnetic Resonance to Describe the Binding of Atropine Analogues to Acetylcholinesterase

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SUMMARY

Proton magnetic resonance has been used to study the association of atropine and several of its analogues with acetylcholinesterase as indicated by changes in line width of the *N*-methyl and phenyl group resonances of the smaller molecule. Atropine and its analogues are bound to a site distinct from the active center of the enzyme. A direct involvement of the positively charged nitrogen and the phenyl group in the complex formation of atropine was established. Tropine and tropic acid bind very weakly, and a small alteration in the tropine moiety of atropine decreases its affinity for this site on the enzyme when compared with atropine. The *l*-form of atropine is more tightly bound than the *d*-isomer. The conclusions reached suggest a model for the site on the acetylcholinesterase molecule in which atropine bridges between a negative and a hydrophobic subsite.

INTRODUCTION

We have recently demonstrated by the use of nuclear magnetic resonance (NMR) techniques the interaction of atropine and eserine with acetylcholinesterase extracted from head ganglia of squid (1). It was possible to show that eserine is bound to the active center while atropine, a muscarinic inhibitor (2), is bound to a separate site. Thus it was evident that there are at least two distinct binding sites on the surface of the enzyme.

The pH and ionic strength of the solution had a marked effect on the line width of the inhibitors in the presence of enzyme. It was not possible, however, to determine whether the changes were due to the degree of aggregation or the degree of ionization of the enzyme, or both.

The present communication presents an NMR study of the binding of tropine and tropic acid to acetylcholinesterase and provides an interpretation of the line width changes observed in the resonances of atropine. Tropine and tropic acid were used to study the mechanism of binding of atropine, because the first contains the aliphatic and charged *N*-methyl groups while the second contains the phenyl ring. It is shown that both hydrophobic and electrostatic forces play a part in stabilizing the atropine molecule on the surface of the enzyme.

In these studies the five analogues of atropine shown in Table 2 were also examined. The results suggest that these inhibitors could be utilized as an effective probe for investigating the environment of the atropine-binding site on acetylcholinesterase.

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[1] Scholar of the Canadian Medical Research Council.

METHODS

All NMR measurements were made on a Varian A-60D high-resolution spectrometer. Samples were dissolved in 99.7% D_2O (Merck) in sodium phosphate buffer (0.1 and 0.01 M, pH 7.4), and equilibrated at the temperature of the insert (39°) before the spectra were recorded. When the concentration of enzyme was varied, the pH and ionic strength of the solution were constant. When the ionic strength of the solution was varied by adding NaCl, the pH of each sample was maintained constant at 7.4 by the addition of a small volume of 0.1 M DCl or NaOD.

The rate of hydrolysis of acetylcholine chloride in the presence or absence of inhibitors is described elsewhere (3).

Measured line widths were corrected for instrumental broadening (0.4 Hz), and relaxation rates were calculated from $1/T_2 = \pi\Delta\nu_{1/2}$, where $\Delta\nu_{1/2}$ is the line width at half-maximal peak height and $1/T_2$ is the transverse relaxation rate. It was assumed that the longitudinal relaxation time T_1 was equal to T_2, and all results are reported as $1/T_2$. A more detailed description of the theory and methods is given elsewhere (4, 5).

Acetylcholinesterase was extracted from the head ganglia of frozen squid (*Loligo apalescens*, U. S. Freezer Company, Monterey, Calif.) as described in a previous communication (1). Its specific activity was 20 mmoles of acetylcholine hydrolyzed per milligram per hour. Other materials were obtained from the following sources: atropine sulfate, Mann Research Laboratories; hyoscine (scopolamine) hydrobromide, tropine, *dl*-tropic acid, homatropine hydrobromide, and *l*-hyoscyamine sulfate, Nutritional Biochemicals Corporation; scopolamine aminoxide, Hoffmann-Toff, Inc.; and apoatropine, K and K laboratories.

RESULTS AND DISCUSSION

Table 1 presents the chemical shift of the *N*-methyl and phenyl protons of the atropine analogues tested. These two peaks were selected to study the association of the inhibitors with the enzyme, since they had the most intense resonances in the spectra. For each inhibitor the change in the observed relaxation rate, $(1/T_2)_{obs}$, of the resonance peak was plotted against (a) the acetylcholinesterase concentration at constant inhibitor concentration and (b) the inverse of the inhibitor concentration at constant enzyme concentration.

TABLE 1

Chemical shifts and I_{50} values of various atropine analogues

Inhibitor	Resonance[a]		I_{50}[b]
	Phenyl	*N*-Methyl	
	Hz	*Hz*	*mM*
Atropine	444	161.0	50
l-Hyoscyamine	445	162.0	50
Homatropine	448.8	160.3	10
Apoatropine	447.3	165.0	4
Scopolamine	444.7	157.5	100
Scopolamine aminoxide	448.8	188.2	50
Tropine		165.5	20
Tropic acid	442		>100

[a] With reference to tetramethylsilane (external).

[b] I_{50} is the concentration of inhibitor required to produce 50% inhibition of the enzyme, and was determined as described elsewhere (3).

A plot of $(1/T_2)_{obs}$ of the *N*-methyl peak of tropine and the phenyl peak of tropic acid against the acetylcholinesterase concentration is shown in Fig. 1. Figure 2 shows the effect of the inverse inhibitor concentration on $(1/T_2)_{obs}$ for the resonances of tropine and tropic acid at constant enzyme concentration (40 mg/ml). An increase in the enzyme concentration at constant inhibitor concentration or a decrease in the inhibitor concentration at constant enzyme concentration resulted in a linear increase in $(1/T_2)_{obs}$ for each inhibitor tested in the concentration range of 5–50 mM.

Controls. The possibility existed that a change in pH or ionic strength of the solution could affect the line widths of the inhibitors or that the observed line width changes in the presence of enzyme were produced by changes in the viscosity of the solution (6). The line width of each inhibitor was therefore measured as a function of pH (pH 5–11), ionic strength (0–4 M NaCl), and inhibitor

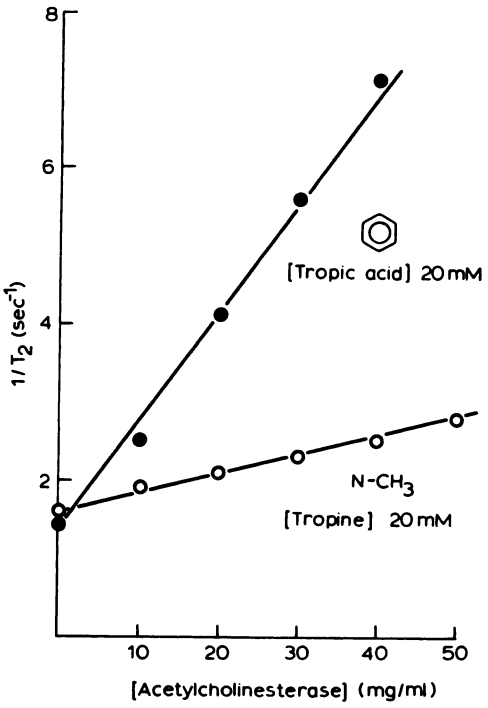


FIG. 1. *Relaxation rates of phenyl protons of tropic acid and N-methyl protons of tropine as a function of concentration of acetylcholinesterase (squid head ganglia)*

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

concentration (5–500 mM). The effects of these three variables on the line widths of the inhibitors alone were small (less than 1 Hz) compared to changes in the presence of acetylcholinesterase, and therefore did not contribute to the observed changes.

The addition of a concentrated solution of bovine serum albumin (Sigma Chemical Company) (20 mg/ml) resulted in only a small change in the line width of each inhibitor (less than 0.5 Hz). An increase in the inhibitor concentration (at constant enzyme concentration) resulted in a decrease in the relaxation rates of each peak. As described elsewhere (6), it is unlikely that the observed results in the presence of acetylcholinesterase were due to viscosity, inhibitor-inhibitor interaction, or direct intermolecular relaxation by the protein in the absence of complex formation.

Under the present conditions, the enzyme-inhibitor complex was probably short-lived and therefore yielded a spectrum representative of a weighted average of the enzyme-bound and free states of the inhibitors. This assumption is not unreasonable, since plots of $(1/T_2)_{obs}$ with respect to the inverse of inhibitor concentration were linear for each inhibitor studied.

Effect of ionic strength on binding of tropine, tropic acid, and atropine. It was hoped that additional information on the nature of the binding forces could be obtained from a study of the dependence of the binding of tropine and tropic acid on ionic strength. Figure 3 shows the relation between NaCl concentration and $1/T_2$ for the resonances of tropine, tropic acid, and atropine in the presence of acetylcholinesterase.

An increase in the NaCl concentration from 0 to 1 M results in a decrease in $1/T_2$ of the *N*-methyl peak of tropine, with no further change at higher salt concentrations. With tropic acid, an increase in the NaCl concentration from 0 to 3 M results in an

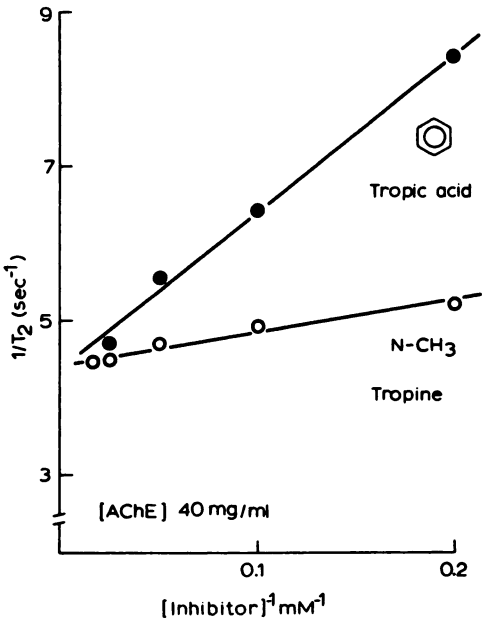


FIG. 2. *Relaxation rates of phenyl protons of tropic acid and N-methyl protons of tropine as a function of inverse concentration of tropic acid and tropine at constant enzyme concentration*

The buffer was 0.01 M sodium phosphate, pH 8.0, at 39°.

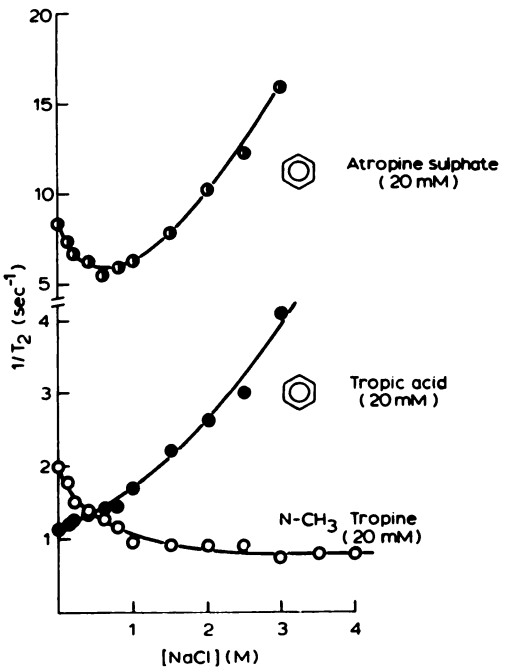


FIG. 3. *Effect of NaCl concentration on relaxation rates of phenyl protons of atropine and tropic acid and N-methyl protons of tropine in the presence of acetylcholinesterase (4 mg/ml with atropine sulfate and 10 mg/ml with tropic acid and tropine)*

The buffer was 0.01 M sodium phosphate, pH 7.4, at 39°. After the addition of NaCl to the inhibitor-enzyme solution, the pH of each sample was adjusted to 7.4 with 0.1 M NaOD or 0.1 M DCl. Notice change in the ordinate scale for atropine sulfate.

increase in $1/T_2$ of the phenyl peak from 1.2 to 4.1 sec^{-1}. With atropine a decrease in $1/T_2$ of the phenyl peak is observed between 0 and 0.6 M NaCl, followed by an increase with a maximum in $1/T_2$ at 3 M NaCl. The protein precipitates at higher salt concentrations (4 M NaCl). A similar pattern was established for the *N*-methyl peak of atropine (1).

Tropine contains the positively charged *N*-methyl portion, while tropic acid contains the phenyl portion of atropine. The decrease in $1/T_2$ in the tropine curve is presumably due to a dissociation of the tropine-acetylcholinesterase electrostatic complex by an increase in ionic strength. The increase in $1/T_2$ for the phenyl group of tropic acid with increase in ionic strength of the solvent is consistent with a "hydrophobic" nature of the complex (7). The initial decrease in $(1/T_2)_{obs}$ in the atropine curve may therefore have been caused by a dissociation of electrostatic bonds between the positively charged *N*-methyl group and the protein, and the increase in $1/T_2$ at higher salt concentration may have been due to hydrophobic bonding between the phenyl group and the protein. A larger decrease in $1/T_2$ between 0 and 0.6 M NaCl may be prevented by an increase in binding as a result of increasing dominance of hydropobic forces at higher salt concentrations. This does not exclude the possibility that aggregation of the enzyme causes the initial changes in $1/T_2$ between 0 and 0.6 M NaCl (8).

Effect of pH on binding of tropine and tropic acid. The pH dependence of $1/T_2$ for the

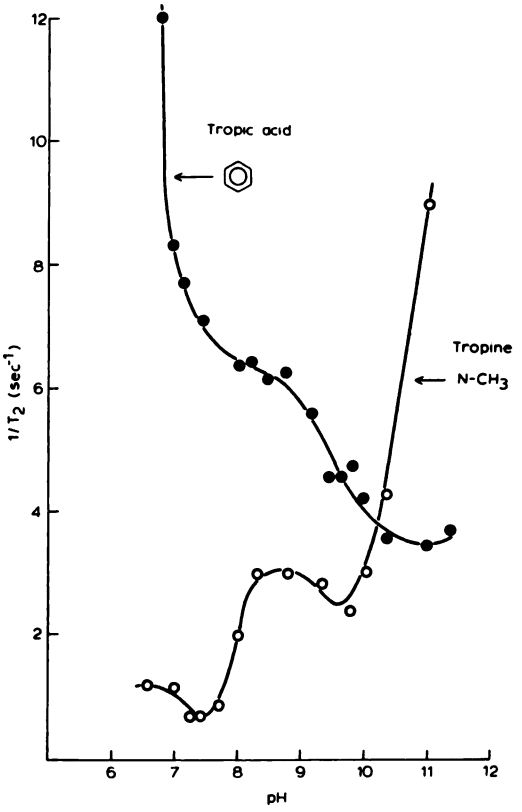


FIG. 4. *pH dependence of relaxation rates of N-methyl protons of tropine (20 mM) and phenyl protons of tropic acid (20 mM) in the presence of acetylcholinesterase (40 mg/ml)*

The buffer was 0.01 M sodium phosphate at 39°.

resonances of tropine and tropic acid in the presence of acetylcholinesterase (40 mg/ml) is shown in Fig. 4. Raising the pH from 7.2 to 11 resulted in an increase in $1/T_2$ of the tropine peak and a decrease in $1/T_2$ of the tropic acid peak. Both curves had a plateau at pH 8.7. A rise in the pH from 6 to 10 resulted in an increase in $1/T_2$ of both the *N*-methyl and phenyl peaks of atropine in the presence of acetylcholinesterase (4 mg/ml) (1).

The most probable causes of pH-dependent changes in $1/T_2$ are due to changes in the degree of ionization of a group on the enzyme. Increasing the pH results in an increase in the number of negative charges on the protein. One would therefore expect more tropine and atropine molecules to bind as the pH is raised, since in solution both are positively charged. An increase in the fraction of molecules stabilized results in an increase in $1/T_2$ of the interacting species. This was observed for tropine (Fig. 4) and atropine (1). If this hypothesis is correct, an increase in the pH should result in a decrease in the number of negatively charged molecules being bound, and hence a decrease in $1/T_2$ of the interacting species. Such a decrease in $1/T_2$ (with an increase in the pH) was observed with tropic acid (Fig. 4), which in solution has a negatively charged carboxyl group (pK 3.5). The binding of tropic acid is therefore attributed to electrostatic and hydrophobic interactions.

These studies may explain the observed effects of pH on $1/T_2$ of the atropine peaks, and reinforce the argument that electrostatic forces play a role in stabilizing the atropine molecule.

Binding of atropine analogues. It is assumed that all the atropine analogues tested bind to the same site as atropine. The I_{50} values shown in Table 1 indicate that these compounds are rather ineffective inhibitors of acetylcholinesterase, which suggests that they are bound only weakly, if at all, to the active center.

Since the alteration between the free and enzyme-bound states of the inhibitors is rapid, the relaxation rate of the bound nuclei, $(1/T_2)_{\text{bound}}$, can be calculated from the slope of a plot of $(1/T_2)_{\text{obs}}$ with respect to the inverse of the inhibitor concentration (5). The stabilization factor is given by the ratio $(T_2)_{\text{free}}/(T_2)_{\text{bound}}$, where $(T_2)_{\text{free}}$ is the relaxation time of the free nuclei (7). This term reflects the change of correlation times for each group (7). A comparison of $(1/T_2)_{\text{bound}}$ and the stabilization factors is given in Table 2.

On binding to acetylcholinesterase, the relaxation rate of the *N*-methyl protons of atropine increased from 3.2 to 5600 sec^{-1}; that of the phenyl protons increased from 1.5 to 5000 sec^{-1}. The stabilization factor indicates that the rotational rates of the *N*-methyl and phenyl groups were reduced by factors of 1750 and 3330, respectively. In contrast, the rotational rates of the corresponding groups of *l*-hyoscyamine were reduced by factors of 3470 and 6850, respectively. Therefore the *l*-isomer of atropine is stabilized to a greater extent than the *d*-isomer. This suggests that the atropine-binding site may be stereospecific.

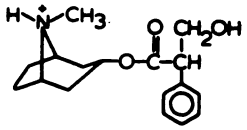
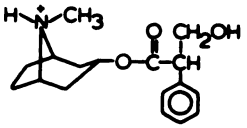
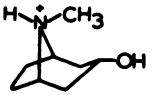
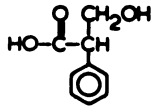
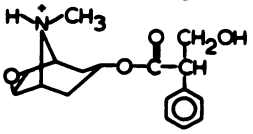
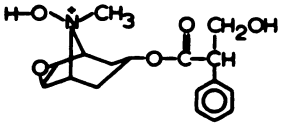
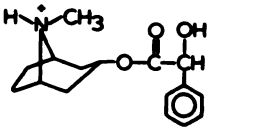
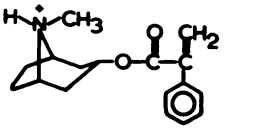
The stabilization factor of the phenyl protons of atropine in the bound state was somewhat greater than that of the *N*-methyl protons, perhaps because atropine may interact through the phenyl or the *N*-methyl group or both. Our studies on the effects of pH and ionic strength on binding show that both groups are involved in complex formation with acetylcholinesterase. Although the aromatic group is more stabilized than the methyl, it is not apparent which of the two groups is the primary binding site.

In contrast to atropine, its hydrolysis products, tropine and tropic acid, are only weakly bound. This is apparent from the relatively small changes in the relaxation rates on binding. It seems, therefore, that the entire atropine molecule in a special configuration is essential for maximum binding.

Scopolamine differs from atropine in that it contains an additional oxygen atom in the aliphatic ring. Its phenyl and particularly its *N*-methyl groups are immobilized to a lesser extent than in atropine. The additional oxygen atom proximal to the *N*-methyl group therefore interferes with binding. It is possible that this bulky oxygen atom partially

TABLE 2

Relaxation rates of N-methyl and phenyl protons of atropine analogues both free and bound to acetylcholinesterase

INHIBITOR	Group	$(1/T_2)_{free}$*	$(1/T_2)_{bound}$*	$\frac{(T_2)_{free}}{(T_2)_{bound}}$
 ATROPINE	N-CH_3	3.2	5,600	1,750
(dl-HYOSCYAMINE)	phenyl	1.5	5,000	3,330
 l-HYOSCYAMINE	N-CH_3	3.0	10,400	3,470
	phenyl	1.4	9,600	6,850
 TROPINE	N-CH_3	1.6	28	17.5
 TROPIC ACID	phenyl	1.4	125	89.3
 SCOPOLAMINE	N-CH_3	2.6	1,940	746
(HYOSCINE)	phenyl	1.9	2,200	1,160
 SCOPOLAMINE	N-CH_3	2.8	103	36.8
AMINOXIDE	phenyl	2.1	167	79.6
 HOMATROPINE	N-CH_3	1.8	3,900	2,160
	phenyl	1.2	4,500	3,750
 APOATROPINE	N-CH_3	2.0	3,770	1,880
	phenyl	0.7	1,200	1,720

*in sec^{-1}

prevents the access of the *N*-methyl group to a cleft at the anionic binding site.

Scopolamine aminoxide is bound even less strongly than scopolamine. The interaction reduced the mobility of the *N*-methyl and phenyl group protons by factors of only 36.8 and 79.6, respectively. This molecule contains an oxygen atom bonded to the nitrogen. The addition of a bulky substituent directly to the *N*-methyl group is therefore detrimental to binding.

In changing from scopolamine to scopolamine aminoxide, only the structure of the *N*-methyl portion of the molecule is altered,

but the relaxation rates of both *N*-methyl and phenyl peaks change concomitantly. This indicates that the *N*-methyl group may be the primary binding site and that the change in the relaxation rate of the phenyl group results from an increase in the rotational correlation time of the entire molecule. This does not exclude the possibility that the phenyl group is also stabilized by hydrophobic interaction.

A slight alteration in the structure of atropine at the tropic acid moiety (as in homatropine) does not significantly affect the stabilization factor of the phenyl or the *N*-methyl group. Removal of the alcohol group (as in apoatropine) results in a significant decrease in the stabilization factor of the phenyl group compared to that of atropine or homatropine. It therefore appears that the hydroxyl group of tropic acid is involved in the binding of atropine, probably through the formation of hydrogen bonds.

Since the structural details of the flexible atropine analogues are not known (9), a detailed interpretation of the observed effects cannot be made from the present data. However, our results suggest that there are three subsites for the binding of atropine. Examination of a molecular model of atropine (built from Corey-Pauling-Koltun models) provides some clues to the possible structure of the binding site for atropine. Assuming that atropine exists in the "boat form," it can be predicted that the electrostatic binding site is 8 A away from the hydrophobic subsite and 6 A away from the hydrogen-bonding site. The two latter sites are separated by about 3 A.

The findings are consistent with a pocket-shaped anionic binding site which allows penetration of the *N*-methyl group into the cleft but prevents the entry of more bulky substituents.

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