Nuclear Magnetic Resonance Study of the Interaction between Acetylcholine and Horse Serum Cholinesterase

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SUMMARY

The mechanism of interaction of acetylcholine (ACh) and horse serum cholinesterase was investigated by relaxation time measurements on the high resolution proton magnetic resonance spectrum of ACh. Upon addition of cholinesterase, there is a transient increase in the relaxation rate of the methyl protons of the quaternary ammonium group and of the acetate group of ACh, lasting only a few minutes. After an initial sharp rise, the line width reaches a maximum and then decays exponentially. The time-dependent broadening of the ACh peaks in the presence of horse serum cholinesterase is correlated with the binding to the enzyme. Both the maximal line width and the time of maximum broadening depend on the ACh to enzyme ratio and on the temperature. The higher the enzyme concentration, the faster the reaction and the shorter the time to maximum line broadening. The higher the substrate concentration, the longer the time to maximum line broadening. A rise in temperature from 6° to 45° increases the rate of hydrolysis of ACh as well as the initial rate of interaction between ACh and cholinesterase. No such transient maximum line broadening is seen in the presence of cholinesterase inhibitors, such as eserine, neostigmine, and edrophonium.

INTRODUCTION

It has long been recognized that the molecular morphology of the active surface of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) consists of two subsites, the "anionic site" and the "esteratic site." The anionic site is concerned with binding positively charged heteroatoms carrying alkyl substituents and orienting the substrate molecule, as well as promoting activity at the esteratic site (1). That portion of the catalytic surface which is responsible for the hydrolysis is termed the esteratic site (2). In contrast, most other preparations of cholinesterases (acylcholine acyl-hydrolase, EC 3.1.1.8) have not been pure enough to allow detailed studies of the active surface. The great affinity of cholinesterase for cationic substrates and inhibitors, however, suggests that the active center is also composed of a negative group, the anionic site, in addition to the ester-binding groups, the esteratic site (3). Eley and Stone (4) concluded that electrostatic forces are unimportant for plasma cholinesterase and that the main point of attack of this enzyme is on the ester group. However, the problem must be regarded as unsolved until more direct evidence is presented.

Such direct evidence can be obtained by the technique of nuclear magnetic resonance, which has recently been used to study the hydrolysis of ACh[1] by horse serum cholinesterase (5, 6). If a substrate

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[1] The abbreviation used is: ACh, acetylcholine.

molecule interacts with the enzyme during the course of hydrolysis, it would find itself in a highly constrained environment and hence its molecular motion would be much reduced compared to molecules in solution. Because the molecules occupy fixed positions, the environment of the substrate would tend to resemble that found in the solid state. Measurement of the molecular motion of ACh at the active site of the cholinesterase should provide a sensitive measure of the degree of binding to the enzyme.

Using the technique of nuclear magnetic resonance (NMR) spectroscopy, it is possible to measure the rate of rotation of molecules containing atoms with nuclear magnetic moments. The restriction of rotational motion of the groups stabilized by interaction leads to a shortening of the relaxation times and a corresponding broadening of the resonance lines (7). Selective broadening of one or more peaks can thus be interpreted in terms of specific interactions involving the corresponding chemical groups. The present paper gives the results of an investigation of the specific complex formed between horse serum cholinesterase and ACh in the presence and absence of cholinesterase inhibitors.

METHODS

The enzyme used throughout was cholinesterase from horse serum (type IV, 5.5 μmoles of acetylcholine hydrolyzed per milligram per minute, Sigma Chemical Company). For the NMR measurements, the samples were prepared by dissolving acetylcholine chloride (British Drug Houses) and horse serum cholinesterase in a 99.8% D_2O (Columbia)–sodium phosphate buffer (0.1 M, pH 7.0). Drug concentrations are all expressed in moles per liter, and enzyme concentrations, in milligrams per milliliter. The inhibitors used were neostigmine bromide (Hoffmann-La Roche), eserine sulfate (Merck), edrophonium chloride (Hoffmann-La Roche), and tetraethyl pyrophosphate (Monsanto) of 40% purity. Crystalline bovine serum albumin, lecithin, and chondroitin sulfate were obtained from Sigma Chemical Company. These were dissolved in the same buffer. Various concentrations of cholinesterase, with or without inhibitor, were then pipetted into standard 5-mm NMR tubes. The acetylcholine solution was injected into the bottom of the tubes (total volume, 1.0 ml), the tubes were thoroughly shaken, and the NMR measurements were made 8 sec after mixing. All measurements were made in a Varian A-60 NMR spectrometer at 60 Mc/sec. Chemical shifts are expressed in cycles per second from tetramethylsilane as an external standard. The temperature of the insert was 40°. Sufficient time was allowed for the tube and its contents to come to thermal balance before measurements were taken. For temperature studies, the tubes containing the cholinesterase as well as the acetylcholine chloride were incubated at the temperature of the instrument insert. The samples were then mixed and readings immediately started.

All relaxation measurements were made at a sweep span of 10 cps/cm and a sweep rate of 2 cps/sec. Measured line widths were corrected for instrumental line width (0.4 cps) and values of the relaxation rate, $1/T_2$, were calculated from the spectral line width using the formula

$$\frac{1}{T_2} = \pi\Delta\nu_{1/2}$$

where $\Delta\nu_{1/2}$ is the line width at one-half maximum peak height.

RESULTS

Horse serum cholinesterase alone, in concentrations up to 300 mg/ml, did not give rise to any peaks in the NMR spectrum under the present experimental conditions.

The NMR spectrum of 0.1 M ACh·Cl is shown in Fig. 1a. The spectrum consists of low-field protons (peaks 1) centered at 225 cps downfield from tetramethylsilane, which were assigned to the 4 hydrogens on the choline skeleton of ACh. The methyl protons of the quaternary ammonium group (peak 2) are identified by a singlet at 196 cps downfield from tetramethylsilane. The peak at 132 cps (peak 3) is due to methyl protons of the acetate group

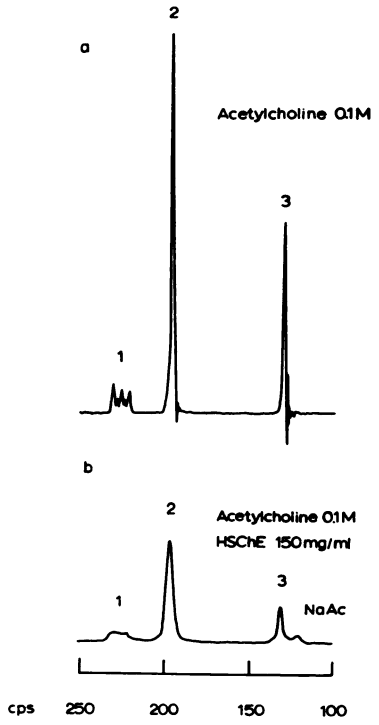

FIG. 1. *NMR spectra of 0.1 M acetylcholine chloride, pH 7.0 at 40°*

a. Acetylcholine chloride in D_2O–sodium phosphate buffer (0.1 M, pH 7.0). Shifts are from tetramethylsilane as an external standard. The multiple peak (*1*) centered at 225 cps is attributed to CH_2CH_2 protons; the large peak (*2*) at 196 cps is from methyl protons of the quaternary ammonium group; the "singlet" peak (*3*) at 132 cps is from the methyl protons of the acetate group. Sample size, 1.0 ml.

b. Acetylcholine chloride, in the same buffer solution, 60 sec after addition of horse serum cholinesterase (HSChE) (150 mg/ml, final concentration). The peak at 120 cps is from the methyl protons of sodium acetate (NaAc).

in ACh. Measurements of line width will be confined to the two single peaks, 2 and 3. Figure 1b shows the spectrum of ACh in the presence of horse serum cholinesterase at a concentration of 150 mg/ml. The symmetrical resonance signals of the quaternary ammonium and acetate peaks were considerably broadened and reduced in amplitude by the presence of enzyme.

After the addition of cholinesterase (150 mg/ml), the ACh·Cl spectrum also contained an additional line at 120 cps (Fig. 1b). This was the resonance line of the methyl protons of the sodium acetate newly formed by the hydrolysis of ACh·Cl.

The selective broadening of the peaks is interpreted as a specific interaction between ACh and enzyme. This interaction between enzyme and substrate takes place in D_2O buffer solution and depends on factors such as time, concentration of enzyme, concentration of substrate, temperature, and the presence or absence of inhibitors.

Time-dependent broadening of acetylcholine peaks in the presence of cholinesterase. When ACh·Cl (0.1 M) was added to horse serum cholinesterase (150 mg/ml), there was a time-dependent broadening of the ACh spectral peaks (Fig. 2). First, there was a sharp increase in the line widths of both peaks 2 and 3; these lines then reached a maximum broadening, usually after 1–10 min, depending on the enzyme concentration. The line width then began to decrease at an approximately exponential rate. The broadening of the quaternary ammonium peak reached a maximum 2 min after mixing (Fig. 2a). Changes in the line width of the acetate peak followed a comparable time course, reaching a maximum at 3 min after mixing and then steadily decreasing. Such transient line broadening was also observed with the sodium acetate formed during hydrolysis. The line width of the sodium acetate peak followed a slower time course, reaching a maximum at 10 min and then decaying more or less exponentially. Although the rate of attaining maximum line width of the sodium acetate protons is slower when compared to the rate of change of the widths of the ACh spectral peaks, these peaks became much broader than the ACh peaks. Increasing the sodium acetate concentration without added enzyme did not alter the width of the acetate peak. Nor did sodium acetate (0.02–0.1 M) alone, in the presence of various concentrations of enzyme (50–200 mg/ml), show such an increase in line width. The plateau where these line widths of both quaternary ammonium and acetate peaks eventually leveled off was always higher than that observed for ACh in free solution.

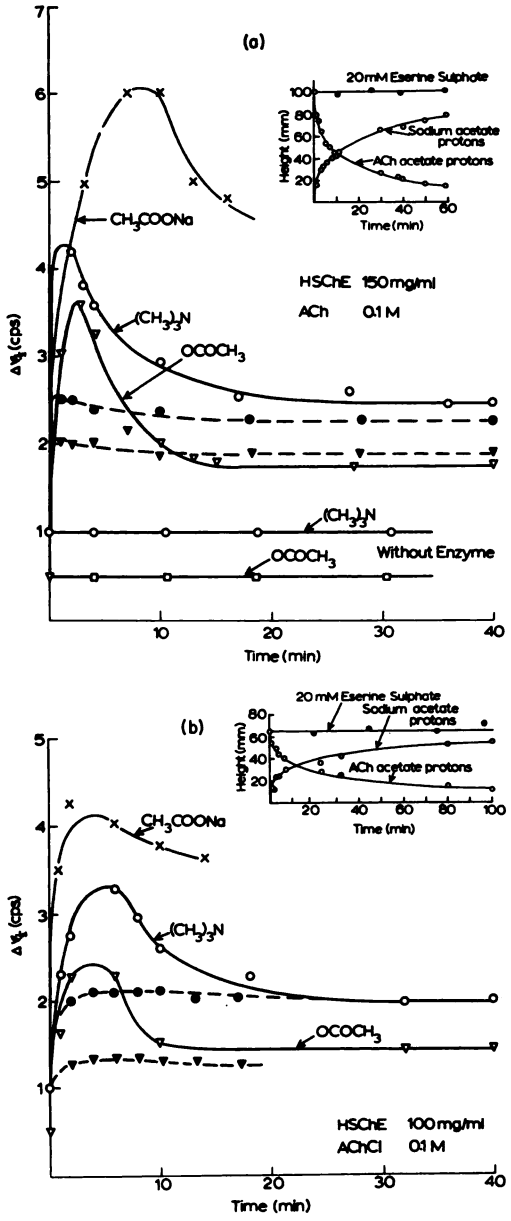

FIG. 2. *Change in line width* ($\Delta\nu_{1/2}$, *cycles per second*) *of quaternary ammonium and acetate peaks of ACh·Cl and the peaks of newly formed sodium acetate, in the presence of cholinesterase in* D_2O*–sodium phosphate buffer (0.1* M, *pH 7.0, 40°*)

a. At zero time, ACh·Cl (0.1 M) was mixed with a solution of horse serum cholinesterase (HSChE) (150 mg/ml) in the presence or absence of eserine sulfate (20 mM). ○ and ▽, Methyl protons of the quaternary ammonium and acetate groups of ACh·Cl, respectively; ● and ▼, the same chemical groups in the presence of eserine sulfate (20 mM). In the inset, the height of the sodium acetate and ACh acetate peaks is plotted as a function of time. The sample size was 1.0 ml.

b. Similar to Fig. 2a, but with a different concentration of cholinesterase (100 mg/ml).

Also shown is the line width of the quaternary ammonium and acetate groups of ACh·Cl (1.0 M) mixed with a sodium phosphate buffer at zero time in the absence of enzyme. The final concentration of ACh·Cl was 0.1 M.

The rate of hydrolysis of ACh can be followed from the formation of the free acetate, which has a different chemical shift from ACh acetate (5). The insets in Fig. 2 show the time course of ACh hydrolysis.

Effect of cholinesterase concentration on binding. An increase of cholinesterase concentration raised the maximum degree of line broadening of the quaternary ammonium line (peak 2). Figure 3 shows the results of a series of measurements of peak 2, made at a fixed ACh concentration (0.1 M) and varied enzyme concentrations. Increasing the cholinesterase concentration from 60 to 300 mg/ml increased the maximum line width of peak 2 from 2.2 to 6.5 cps. The inset shows that an increase in the enzyme concentration shortened the time at which maximum line broadening occurred.

It can be seen from Fig. 4 that raising the enzyme concentrations from 0 to 300 mg/ml caused an increase in the relaxation rate of the methyl protons of the quaternary ammonium group of 0.1 M ACh·Cl from 3 sec^{-1} to 21 sec^{-1}, i.e., by a factor of 7, whereas that of the methyl protons of the acetate group increased from 1.5 sec^{-1} to 23 sec^{-1}, i.e., by a factor of 15. The values are derived from measurements of the line width at maximum broadening. There was a larger increase in the relaxation rate for the acetate moiety than for the quaternary ammonium group. The acetyl moiety is therefore preferentially stabilized by the interaction, compared with the methylammonium derivative.

Effect of acetylcholine concentration on binding. The effect of changing the ACh concentration on the maximal line width and the time of maximum broadening of the

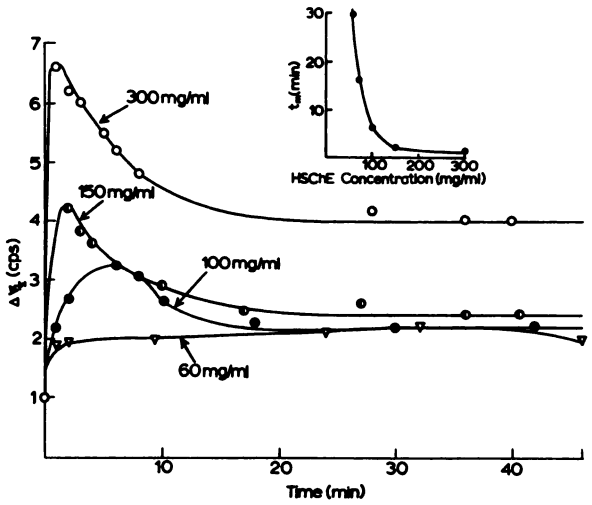

FIG. 3. *Change in line width* ($\Delta\nu_{1/2}$, *cycles per second*) *of the quaternary ammonium peak of ACh·Cl* (*0.1* M) *in the presence of four different concentrations of cholinesterase*

The inset shows the times at which maximum broadening occurred (t_m), as a function of horse serum cholinesterase (HSChE) concentration.

quaternary ammonium line in the presence of horse serum cholinesterase is illustrated in Fig. 5. The maximum rate of initial line broadening was observed with 0.03 M ACh·Cl, and the minimum, with 0.2 M ACh·Cl. Maximum line broadening for 0.03, 0.07, 0.1, and 0.2 M ACh·Cl was observed at 20 sec, 4 min, 7 min, and 20 min, respectively.

The maximum broadening of peak 2 was also greatest at low ACh concentrations. Figure 5 shows that the maximum line widths of peak 2 for 0.03, 0.07, 0.1, and 0.2 M ACh·Cl in the presence of cholinesterase (100 mg/ml) were 3.8, 3.4, 3.2, and 2.6 cps, respectively.

Since the line width is related to the relaxation rate, the relaxation rate depends on the ACh to cholinesterase ratio rather than on the enzyme concentration alone, and for a given enzyme concentration the maximum relaxation rate decreases with increasing ACh concentration. Increasing the ACh concentration from 0.03 to 0.2 M decreased the relaxation rate of the methylammonium protons from 11.9 to 8.3 sec^{-1}.

In the present system, the major part of the ACh present remains free in solution and a minor portion is bound to the enzyme. Provided that the rate of exchange of ACh molecules between the free and bound state is rapid, the observed relaxation rate is given by (8)

$$\left(\frac{1}{T_2}\right)_{\text{observed}} = \alpha\left(\frac{1}{T_2}\right)_{\text{bound}} + (1-\alpha)\left(\frac{1}{T_2}\right)_{\text{free}}$$

If $(1/T_2)_{\text{free}} = 3.2$ sec^{-1} for the methyl protons of the quaternary ammonium group and α, the ratio of bound ACh to free ACh in the solution, is taken as $1/175$, the relaxation rate of the bound species is approximately 1200 sec^{-1}. The ratio $T_{2(\text{free})}/T_{2(\text{bound})}$ indicates that the rotation of the quaternary ammonium group is reduced 380-fold when it is maximally bound to the enzyme.

Effect of cholinesterase inhibitors on the enzyme-substrate complex. A narrowing of the spectral lines in the presence of the inhibitor can be interpreted as inhibition of the active sites, and it should be possible to inhibit selectively the binding to the anionic or the esteratic site (or both). The initial line broadening of the quaternary ammonium and acetate groups of ACh by cholinesterase, as well as the enzymatic hydrolysis, was prevented by 20 mM

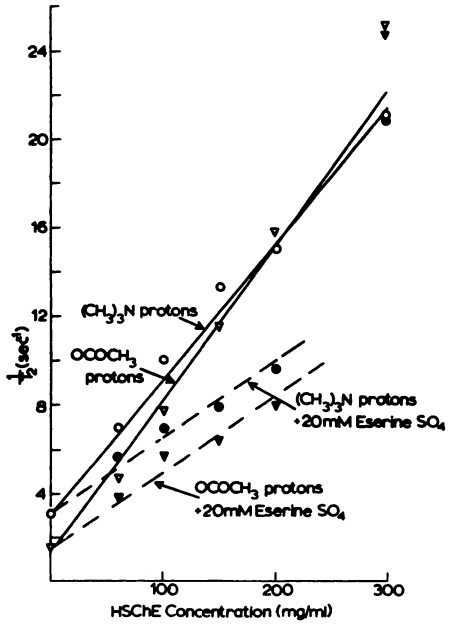

FIG. 4. *Relaxation rates ($1/T_2$) of 0.1 M ACh·Cl peaks (pH 7.0, 40°) as a function of cholinesterase concentration in the presence or absence of eserine*

Relaxation rates were calculated from Fig. 2, at the time of maximum line broadening. HSChE, horse serum cholinesterase.

eserine, as shown in Fig. 2a and b. However, this was not the case with very high concentrations of enzyme (i.e., 300 mg/ml); the presence of 20 mM eserine sulfate enhanced the line broadening of the ACh peaks. There was a similar narrowing of

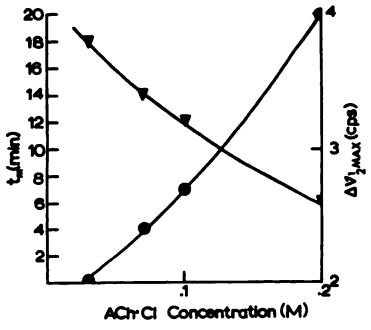

FIG. 5. *Effect of acetylcholine concentration on the maximal line width ($\Delta\nu_{1/2\,max}$, ▼) and the time of maximum broadening (t_m, ●) of the methylammonium peak at 40°*

The horse serum cholinesterase concentration was 100 mg/ml.

both peaks 2 and 3 after addition of 20 mM neostigmine bromide or edrophonium chloride to a solution of ACh·Cl (0.1 M) and cholinesterase (100 mg/ml). Tetraethyl pyrophosphate (20 mM) prevented the broadening of the acetate peak only, whereas the peak for the quaternary ammonium group remained unchanged but did not show the usual subsequent exponential decay.

Several other compounds, among them glutamate, glucose, and ether, were tested at reasonable concentrations (40 mM) and found not to interfere with the broadening of either the quaternary ammonium or acetate peaks of ACh.

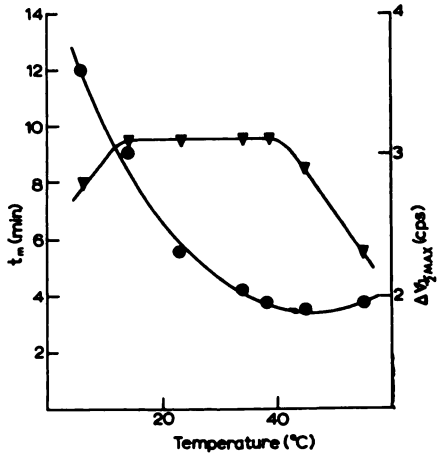

FIG. 6. *Effect of temperature on the maximal line width ($\Delta\nu_{1/2\,max}$, ▼) and the time of maximum broadening (t_m, ●) of the methylammonium peak*

The concentrations of horse serum cholinesterase and acetylcholine chloride were 100 mg/ml and 0.1 M, respectively.

Effect of temperature on binding. The effect of temperature on the maximum line width and the time of maximum line broadening of the quaternary ammonium group of ACh when added to horse serum cholinesterase is shown in Fig. 6. The times at which maximum line broadening was reached (t_m) were 12, 9, 5.5, 4.2, 3.8, and 3.5 min for 6°, 14°, 23°, 34°, 38°, and 45°, respectively. Increasing the temperature above 45° produced no further change in the rate of initial line broadening.

There was also a slight but significant

change in the amount of interaction at various temperatures. The maximum line widths of the quaternary ammonium line at 6°, 14°, 23°, 34°, 38°, 45°, and 55° were 2.8, 3.1, 3.1, 3.1, 3.1, 2.9, and 2.3 cps, respectively. This shows that interaction was maximal between 14° and 38°.

Controls. For comparison, the ACh spectrum was examined in the presence of three viscous substances (chondroitin sulfate, bovine serum albumin, and lecithin) dissolved at a final concentration of 100 mg/ml in 0.1 M sodium phosphate buffer and mixed with ACh·Cl (0.1 M) in a 1.0-ml volume. Spectra were recorded immediately after mixing. The respective line widths of the ACh spectrum in the presence of chondroitin sulfate, bovine serum albumin, and lecithin were 2.2, 2.0, and 1.9 cps for the methyl protons of the quaternary ammonium group, and 1.5, 1.4, and 1.3 cps for the methyl protons of the acetate group of ACh. These values did not change significantly up to 30 min after mixing ACh with these macromolecules. When a phosphate buffer was added to an ACh solution, there was no change in the ACh line width (Fig. 2a). Changes in the concentration of ACh in the absence of macromolecules did not produce a significant change in line width of the characteristic peaks of the ACh spectrum. Increasing the ACh·Cl concentration from 0.01 to 1.0 M increased $\Delta\nu_{1/2}$ from 0.93 to 1.01 cps and from 0.48 to 0.55 cps for the methylammonium and acetate protons, respectively.

Chemical shift of the sodium acetate peak. There was a progressive shift of the sodium acetate peak from 117 cps at the beginning of hydrolysis to 121 cps at the end of hydrolysis. This peak shifted downfield exponentially with time and at a rate dependent on the rate of hydrolysis of ACh: the faster the hydrolysis, the more rapid the downfield shift of the acetate peak.

DISCUSSION

The selective time-dependent broadening of the acetylcholine spectral peaks in the presence of cholinesterase indicates specific short-lived interactions involving the quaternary nitrogen and acetate groups of ACh and the receptive site of the enzyme. The rate of initial line broadening was greater for the quaternary ammonium group than for the acetate group. A possible interpretation is that the quaternary ammonium group acts as an initial anchor of the molecule on the anionic surface of the enzyme and that this is followed by a more pronounced interaction between the acetate group of ACh and the esteratic site of the enzyme, leading to hydrolysis. When the interaction is complete, the width of peaks 2 and 3 decay to a steady level which is higher than that for these two peaks in free solution. This may be due to an interaction between choline and the horse serum cholinesterase or to a nonspecific interaction between choline or unreacted ACh and the protein, which has now undergone structural alterations. The quaternary ammonium peaks of choline and ACh are superimposable.

The change in line width of the sodium acetate protons probably reflects an interaction between acetate and the enzyme. As the reaction approaches completion, the line width of the sodium acetate protons also progressively declines. The sodium acetate, which at first is presumably bound to the enzyme, is then released into the environment along with choline. As the chemical groups of these molecules are released by the enzyme, they attain a greater freedom of rotation, which decreases the relaxation rates, and therefore a decrease in line width is observed. Control experiments showed that it is unlikely that the observed changes are due to an increase in sodium acetate concentration.

Both the maximal interaction and the time at which maximal interaction takes place depend on the ACh to enzyme ratio and on temperature. The fact that the relaxation rate increases as the ACh to enzyme ratio is decreased indicates that there is rapid exchange between the free and bound species. In the case of slow exchange, one would expect the sharp peaks of the free species to be superimposed on the broad peaks of the bound species. For a given ratio of acetylcholine to enzyme,

therefore, it is possible to assume that the observed relaxation rate is an average of the relaxation rate of the free and bound species. It should therefore be possible to calculate the fraction of ACh participating in the complex for any given ACh to enzyme ratio. The present data, however, do not provide adequate information about the stoichiometry of the complex, and it is therefore impossible to calculate precisely the equilibrium constant involved.

Although peak 2 is the broadest, as indicated from measurements shown in Fig. 2a and b, it is readily seen that the relaxation rate of peak 3 is changed by a larger factor than the relaxation rate of peak 2. It is this relative increment, rather than the absolute amount of broadening, which suggests that the methyl protons of the acetate group are preferentially stabilized by acetylcholine-cholinesterase interaction. This is not surprising, since the function of the enzyme is to hydrolyze the acetate group; for optimal enzyme-substrate interaction, strong binding is likely to be essential.

That these observations with horse serum cholinesterase were not caused by artifacts of mixing (inhomogeneity) is shown by the facts that (a) line broadening was blocked by cholinesterase inhibitors, and (b) when ACh was mixed with highly viscous materials, only a small permanent line broadening was observed.

Eserine, neostigmine, and edrophonium (20 mM) partially inhibited the interaction of both the quaternary ammonium and acetate groups with the active surface of the cholinesterase, whereas tetraethyl pyrophosphate inhibited only the esteratic site of the enzyme. The hydrolysis of ACh was completely inhibited by 20 mM eserine, neostigmine, edrophonium, and tetraethyl pyrophosphate. These foreign molecules are presumably bound to the active sites, thus preventing access of the substrate molecule to the active surface.

A definitive analysis of the downfield shift of the sodium acetate peak is difficult on the basis of the present data. Further experiments are necessary to clarify this phenomenon.

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REFERENCES

1. R. Kitz and I. B. Wilson, *J. Biol. Chem.* 238, 745 (1963).
2. B. Holmstedt, *in* "Cholinesterases and Anticholinesterase Agents," Handbuch der experimentellen Pharmakologie (G. B. Koelle, ed.), Vol. 15, p. 428. Springer, Berlin, 1963.
3. K.-B. Augustinsson, *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Vol. 4, p. 521. Academic Press, New York, 1960.
4. D. D. Eley and G. S. Stone, *Biochem. J.* 49, 30P (1951).
5. G. Kato, *Mol. Pharmacol.* 4, 640 (1968).
6. G. Kato, *Proc. Can. Fed. Biol. Soc.*, 11, 87 (1968).
7. O. Jardetzky, *Advan. Chem. Phys.* 7, 499 (1964).
8. O. Jardetzky and N. G. Wade-Jardetzky, *Mol. Pharmacol.* 1, 214 (1965).