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Acetylcholinesterase

II. A Study by Nuclear Magnetic Resonance of the Acceleration of Acetylcholinesterase by Atropine and Inhibition by Eserine

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SUMMARY

A 60-MHz nuclear magnetic resonance method was used to study the binding of acetylcholine to acetylcholinesterase (EC 3.1.1.7). Changes in the linewidth of the *N*-methyl resonance of acetylcholine as a function of time, resulting from association with the enzyme during hydrolysis, were utilized to study the enzyme-substrate interaction. The resonance of the acetate protons of acetylcholine at 132 Hz (downfield from external tetramethylsilane) disappears during hydrolysis, while a new peak, due to newly formed sodium acetate, appears at 118 Hz. The rate of appearance of the free acetate peak was used to measure the initial velocity of the reaction.

In the presence of high concentration of substrate (16.7 mM), eserine and neostigmine decrease substrate hydrolysis by inhibiting its binding to the enzyme. Under the same conditions atropine, (−)-hyoscyamine, and hyoscine accelerate hydrolysis of the substrate without interfering with its binding.

INTRODUCTION

Previous studies (1, 2) have shown that squid acetylcholinesterase (EC 3.1.1.7) contains separate binding sites for atropine and eserine. The present study was undertaken to investigate the effect of atropine on the kinetics of substrate hydrolysis. A high-resolution nuclear magnetic resonance method was used to study the binding of substrate to the enzyme during hydrolysis in the presence and absence of atropine. This method, employed earlier to study the binding of acetylcholine to serum cholinesterase (3), has several advantages over the conventional kinetic methods for analyzing substrate and inhibitor interactions (4).

It is proposed that the enzyme has a second site, distinct from the active center, where the binding of cholinergic ligands can accelerate its catalytic activity. An alternative explanation of the kinetic data is that atropine binds to the second site and prevents substrate inhibition.

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METHODS

Enzyme. Acetylcholinesterase was prepared from the head ganglia of squid (*Loligo opalescens*) as described previously (1). It was dialyzed against double-distilled water and stored frozen as a powder. The enzyme had a specific activity of 1.4 mmoles

of acetylthiocholine hydrolyzed per hour per milligram of protein. The K_m value of acetylthiocholine was 90 μM.

Chemicals. Chemicals were obtained from the following sources: hyoscine (scopolamine) hydrobromide and (−)-hyoscyamine sulfate, Nutritional Biochemicals Corporation; acetylcholine chloride, gift from Hoffmann–La Roche. The sources of the other chemicals used in this study are given in the accompanying paper (1).

Magnetic resonance techniques. For each series a stock acetylcholinesterase solution (specific activity, 1.4 mmoles of acetylthiocholine hydrolyzed per hour per milligram of protein) was prepared in 0.1 M sodium phosphate buffer and brought to pH 7.4 by the addition of small amounts of 0.1 M NaOD. A small quantity of enzyme (0.6 ml) was transferred to standard 5-mm NMR tubes. A small volume of substrate (10 μl of 1.0 M acetylcholine chloride) was injected into the NMR tube, and the reaction was initiated by rapid mixing with the enzyme solution. The tube was placed in the probe, and the acetylcholine spectrum was scanned at various time intervals.

NMR spectra were obtained with a Varian A-60D high-resolution spectrometer. Chemical shifts are expressed in Hertz from tetramethylsilane as external standard. Substrate-binding studies were made at a sweep scan of 10 Hz/cm and a sweep rate of 1 Hz/sec.

All measurements were carried out at a probe temperature of 39°. Every sample was equilibrated at 39° for at least 5 min in a water bath before introduction into the probe. The time between preparation of samples and determination of spectra did not exceed 2 hr, to minimize decomposition of labile compounds.

RESULTS

The binding of acetylcholine to acetylcholinesterase was studied by measuring the change in linewidth of the *N*-methyl group at half-maximal amplitude ($\Delta\nu_{NCH_3}$) 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. This method has been described previously for serum cholinesterase (4).

Substrate binding. The lower trace in Fig. 1 shows a portion of the 60-MHz NMR spectrum of a 16.7 mM solution of acetylcholine chloride (in D_2O phosphate, pH 7.4). The assignment of the acetylcholine spectrum as given in the figure was made previously (4). The peaks are labeled *N*-CH_3

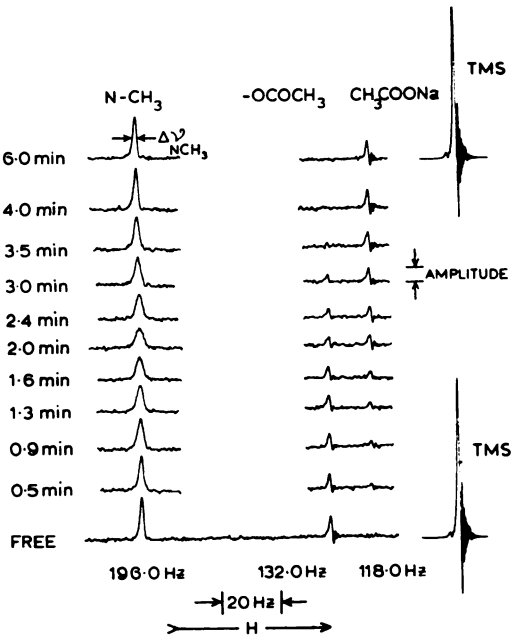

FIG. 1. *Time course of NMR spectrum of acetylcholine chloride (16.7 mM), both free and after addition to acetylcholinesterase (50 mg/ml) in 0.1 M sodium phosphate buffer, pH 7.4, in D_2O*

The temperature was 39°. The lowest trace represents a portion of the NMR spectrum of acetylcholine free in solution. 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 (TMS)] 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.

and —$OCOCH_3$ for the protons of the quaternary ammonium and acetate moieties, respectively. When acetylcholine chloride (16.7 mM) was added to a solution of the enzyme (50 mg/ml) and the spectrum of the solution was scanned repeatedly, the spectrum underwent several changes, as shown in the upper traces of Fig. 1. The *N*-methyl group resonance became broader, reaching a maximum at 2 min, followed by a gradual sharpening. The resonance at 132 Hz due

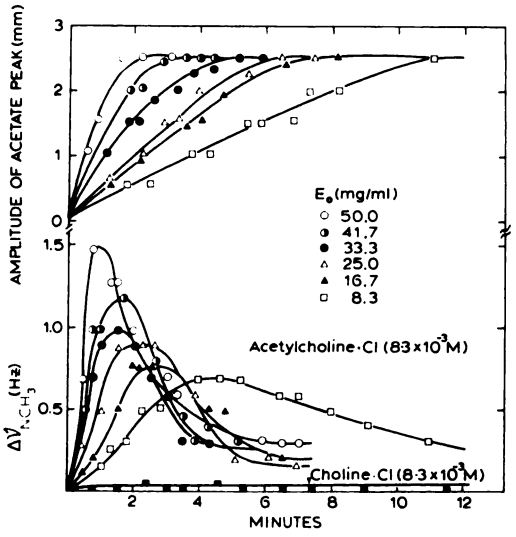

FIG. 2. *Plot of observed linewidth for N-methyl group resonance of acetylcholine (lower graph) and amplitude of sodium acetate group resonance (upper graph) with respect to time at various concentrations of enzyme (E_0) in the presence of the same concentration of acetylcholine chloride (8.3 mM)*

At zero time a solution of acetylcholine chloride (10 μl, 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 at 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 mM) 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; temperature, 39°.

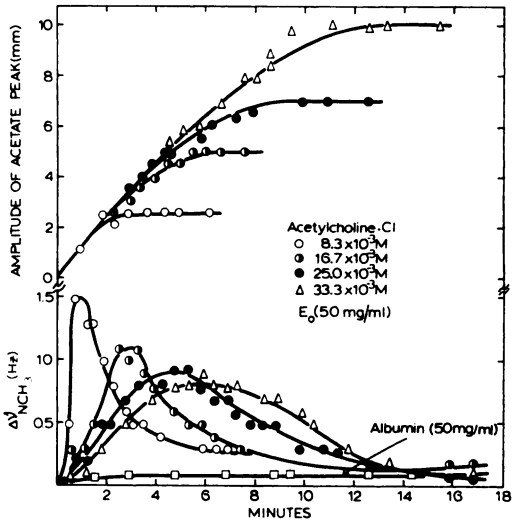

FIG. 3. *Plot of observed linewidth for N-methyl group resonance of acetylcholine (lower graph) and the amplitude of sodium acetate group resonance (upper graph) with respect to time at various concentrations of acetylcholine in the presence of the same concentration of enzyme (E_0 = 50 mg/ml)*

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 at four different concentrations of acetylcholine. Included in the graph is the change in linewidth of the *N*-methyl group of acetylcholine chloride (8.3 mM) when mixed with a solution of albumin (50 mg/ml). The solution contained 0.1 M sodium phosphate buffer, pH 7.4, in D_2O. The total volume was 0.6 ml; temperature, 39°.

to the acetylcholine acetate protons decayed with time, and the resonance at 118 Hz due to the free acetate protons gradually increased, reaching maximal amplitude at 6 min. No further changes occurred after this time.

Although the *N*-methyl protons are markedly broadened, the acetylcholine acetate peaks are broadened only slightly, if at all. Since an acetylated enzyme is formed during hydrolysis (5), the protons of the acetyl-enzyme should give rise to a very broad signal. The predicted linewidth at half-maximal amplitude of this signal is 200–300 Hz (6), assuming a molecular weight of 260,000 for acetylcholinesterase (7). Since the acetyl-enzyme protons and the free acetate protons are not exchangeable, a

single sharp signal is expected from the acetate protons of unhydrolyzed acetylcholine. This signal may be superimposed on a very broad peak arising from the protons of the acetyl-enzyme.

The free acetate resonance was apparently not broadened, since the widths of the absorption peak at half-maximal amplitude (measured at various times) were unchanged (0.4 ± 0.04 Hz). The pH of the solution decreased by 0.1–0.2 unit when 16.7 mM acetylcholine chloride was hydrolyzed by the enzyme.

The progress with time of $\Delta\nu_{NCH_3}$ and the amplitude of the acetate peak are shown in Fig. 2 with six different concentrations of enzyme (E_0) at the same substrate concentration. With each enzyme concentration a characteristic change in line width is observed: a progressive broadening of the N-methyl line, followed by a decrease in linewidth, after which a steady state is reached. At lower enzyme concentrations the maximum line width decreases and the time of maximum broadening is delayed. When

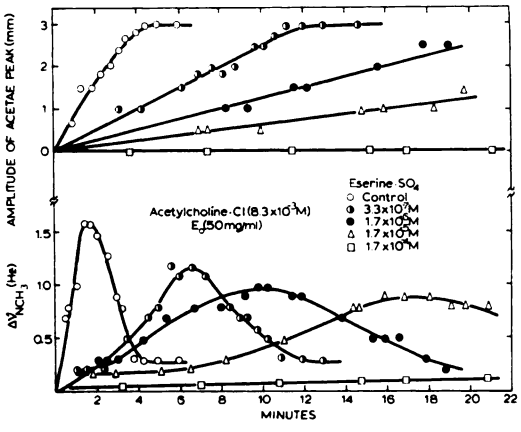

FIG. 4. *Plot of observed linewidth for N-methyl group resonance of acetylcholine (lower graph) and amplitude of sodium acetate group resonance (upper graph) with respect to time at various concentrations of eserine in the presence of the same concentration of enzyme (E_0 = 50 mg/ml) and substrate (acetylcholine chloride = 8.3 mM)*

The experimental procedure was the same as in Figs. 2 and 3, except that the enzyme was incubated with various concentrations of eserine before the addition of substrate. Measurements were made in 0.1 M phosphate buffer, pH 7.4, in D_2O, at 39°.

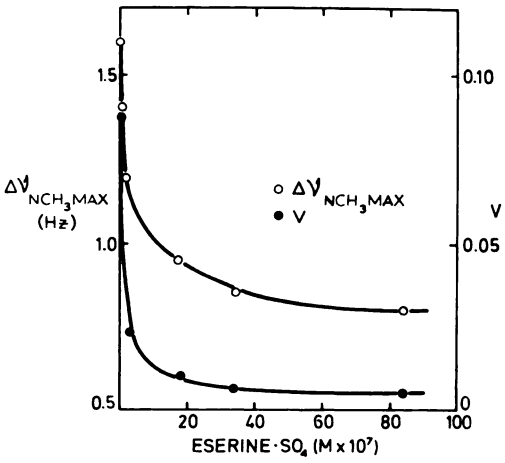

FIG. 5. *Change in maximal linewidth ($\Delta\nu_{NCH_3,max}$) and initial velocity (v) as a function of eserine concentration at constant enzyme (50 mg/ml) and acetylcholine (8.3 mM) concentrations*

Notice that $\Delta\nu_{NCH_3,max}$ and v follow a similar pattern. Measurements were made in 0.1 M phosphate buffer, pH 7.4, in D_2O, at 39°.

choline chloride (8.3 mM) is added to the enzyme (50 mg/ml), its N-methyl resonance is not broadened.

Figure 3 demonstrates that at higher concentrations of substrate the maximum linewidth also decreases and maximum broadening is delayed. When acetylcholine chloride (8.3 mM) is mixed with a solution containing bovine serum albumin (50 mg/ml), its N-methyl resonance line is not broadened. The viscosity of this solution of albumin was approximately the same as the viscosity of a solution of acetylcholinesterase (40 mg/ml).

The amplitude of the free acetate resonance as a function of time is recorded in the upper portions of Figs. 2 and 3. By extrapolating the slopes to zero time, it is possible to calculate the initial velocity, v, of the reaction (8).

It is evident from our present observations that the exchange of acetylcholine between the free and enzyme-bound species is rapid at all times. 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.

The change in linewidth can be interpreted in terms of binding of the substrate to the

active center of the enzyme. The initial step in hydrolysis is the formation of an enzyme-substrate complex, [ES], with a concomitant decrease in the concentration of substrate, [S_0]. Line broadening is due to a decrease in [S_0] during hydrolysis. The subsequent sharpening of the line is due to the appearance of free choline, whose N-methyl resonance and that of acetylcholine are superimposed (196 Hz, downfield from external tetramethylsilane). An increase in the concentration of choline results in a decrease in $\Delta\nu_{NCH_3}$, since choline itself is not bound. As choline formation increases with time, $\Delta\nu_{NCH_3}$ progressively decreases and reaches a plateau.

Effect of anticholinesterases. In the presence of eserine sulfate, the N-methyl group resonance of acetylcholine shows (a) a decrease in the rate of line broadening, indicating a delay in the reaction, and (b) a decrease in maimal broadening ($\Delta\nu_{NCH_3, max}$). These results are shown in Fig. 4 with four different concentrations of eserine. The upper graph shows the effect of increasing concentrations of eserine on the rate of acetate formation. At an eserine sulfate concentration of 0.17 mM, line boradening and hydrolysis were completely abolished.

Figure 5 gives the quantitative data obtained for eserine. A 6 μM concentration of eserine sulfate decreased $\Delta\nu_{NCH_3, max}$ from 1.6 to 0.8 Hz and almost completely inhibited hydrolysis. Similar results were obtained with neostigmine bromide and edrophonium chloride. The experiments with neostigmine are illustrated in Fig. 6.

Effects of other inhibitors. The effects of atropine sulfate, (−)-hyoscyamine sulfate, and hyoscine hydrobromide on acetylcholine binding are uniquely different from those observed with the anticholinesterases. Atropine increased the rate of broadening of the N-methyl resonance without influencing the maximum linewidth (Fig. 7). At 1.7–8.3 mM, atropine accelerates the rate of hydrolysis (upper graph), whereas at higher concentrations (50 mM) hydrolysis and substrate binding are inhibited.

As shown in Fig. 8, increasing the con-

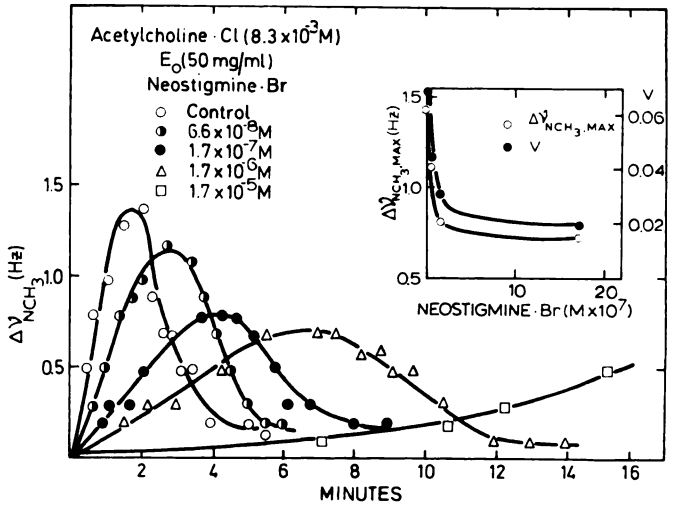

FIG. 6. *Plot of observed linewidth for N-methyl group resonance of acetylcholine with respect to time at various concentrations of neostigmine in the presence of the same concentration of enzyme (E_0 = 50 mg/ml) and substrate (acetylcholine = 8.3 mM)*

The experimental procedure was the same as in Figs. 2 and 3, except that the enzyme was incubated with various concentrations of neostigmine before the addition of substrate. The inset shows the change in maximal linewidth of the N-methyl group of acetylcholine ($\Delta\nu_{NCH_3, max}$) and the initial velocity (v) as a function of the neostigmine concentration at constant enzyme (50 mg/ml) and acetylcholine (8.3 mM) concentrations. Notice that $\Delta\nu_{NCH_3, max}$ and v follow a similar pattern. Measurements were made in 0.1 M phosphate buffer, pH 7.4, in D_2O, at 39°.

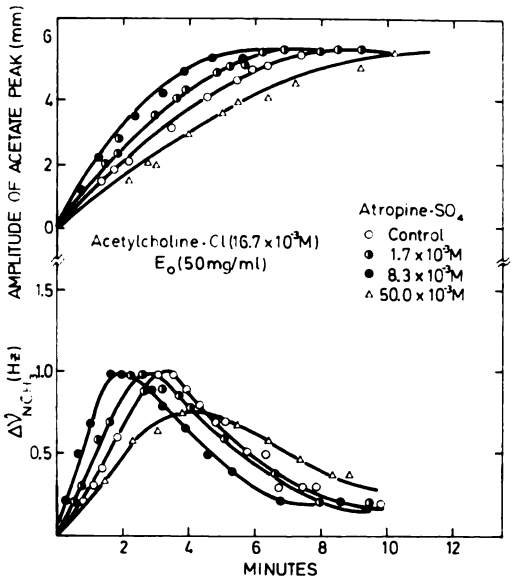

FIG. 7. *Plot of observed linewidth for N-methyl group resonance of acetylcholine (lower graph) and amplitude of sodium acetate group resonance (upper graph) with respect to time at the same concentrations of enzyme (E_0 = 50 mg/ml) and substrate (acetylcholine = 16.7 mM)*

The experimental procedure was the same as in Figs. 2 and 3, except that the enzyme was incubated 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°.

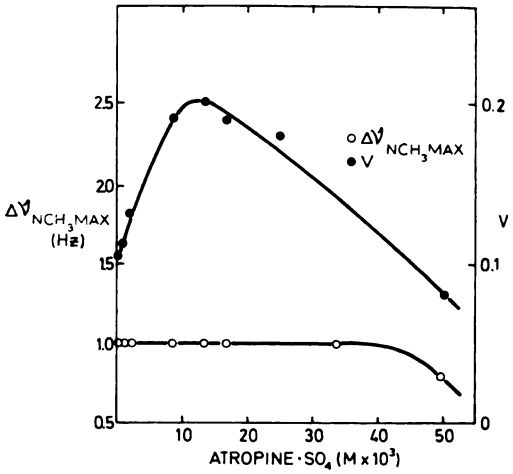

FIG. 8. *Change in maximal linewidth of N-methyl group resonance of acetylcholine ($\Delta\nu_{NCH_3,max}$) and initial velocity (v) as a function of atropine concentration at constant enzyme (50 mg/ml) and acetylcholine (16.7 mM) concentrations*

Notice that a low concentration of atropine (1-15 mM) accelerates hydrolysis without affecting $\Delta\nu_{NCH_3,max}$. All measurements were made in 0.1 M phosphate buffer, pH 7.4, in D_2O, at 39°.

centration of atropine sulfate from 0 to 15 mM results in a 2-fold increase in the rate of hydrolysis, whereas $\Delta\nu_{NCH_3,max}$ does not change.

Similar results were obtained with (−)-hyoscyamine and hyoscine. The results with (−)-hyoscyamine are presented in Fig. 9. Only higher concentrations of (−)-hyoscyamine (45 mM) inhibit hydrolysis and substrate binding.

DISCUSSION

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

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

Roufogalis and Thomas (9) and others (10) have shown 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.* (11) of *exo* and *endo* binding of effectors at the esteratic site level.

Alternatively, atropine may protect the

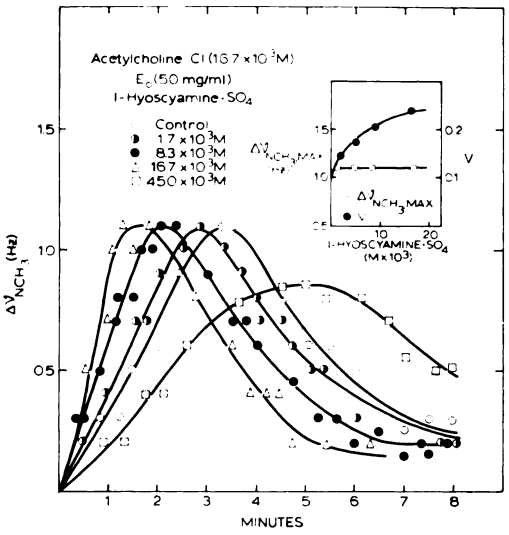

FIG. 9. *Plot of observed linewidth for N-methyl group resonance of acetylcholine with respect to time at various concentrations of* (−)*-hyoscyamine in the presence of the same concentration of enzyme* (E_0 = *50 mg/ml) and substrate (acetylcholine = 16.7 mM)*

The enzyme was incubated with (−)-hyoscyamine before the addition of substrate. Measurements were made in 0.1 M phosphate buffer, pH 7.4, in D_2O, at 39°. The *inset* shows the change in maximal linewidth of the *N*-methyl group of acetylcholine ($\Delta\nu_{NCH_3,max}$) and the initial velocity (v) as a function of the (−)-hyoscyamine concentration at constant enzyme (50 mg/ml) and acetylcholine (16.7 mM) concentrations. Notice that (−)-hyoscyamine (0–20 mM) increases v without affecting $\Delta\nu_{NCH_3,max}$.

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 (12) 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 of the 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.

Because the concentrations of enzyme and atropine used in this study and the ionic strength of the medium were very high, the results may have no physiological significance. Nevertheless the existence of binding sites on acetylcholinesterase, other than the active site, has been confirmed. The physiological significance of these binding sites awaits clarification.

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