SHORT COMMUNICATION

Studies on Serum Cholinesterase Kinetics by Nuclear Magnetic Resonance Spectroscopy

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SUMMARY

The hydrolysis of acetylcholine by horse serum cholinesterase has been measured by nuclear magnetic resonance spectroscopy. The reaction rate was estimated from the formation of free acetate, whose characteristic frequency of absorption differs from that of the acetate moiety of acetylcholine. From the decay of the acetate signal of acetylcholine and/or the formation of the sodium acetate signal in sodium phosphate buffer, it is possible to follow the rate of the reaction. The calculated K_m for the substrate and the K_i for various inhibitors agree well with values obtained by others using conventional methods.

The technique of nuclear magnetic resonance (NMR) spectroscopy has been little exploited in biological systems, particularly in enzyme kinetics. Jardetzky and his colleagues (1–3) have shown that the formation of intermolecular complexes between small molecules and macromolecules can be detected by observing accompanying changes in the relaxation time. This method should therefore be valuable for the study of enzyme-substrate interactions, as well as enzyme kinetics. A change in chemical shift will occur when either the charge density at a given proton or the shielding of the proton is different in the product from the reactant state. The rate of chemical conversion may then be followed by the decay of the first signal and the formation of the second signal. It should therefore be possible, in certain biochemical reactions, to measure the rate of the reaction by NMR.

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In the experiments described here, the technique of NMR spectroscopy was used to study the hydrolysis of acetylcholine by horse serum cholinesterase, which has received relatively little attention compared to the acetylcholinesterase reaction.

The enzyme used was cholinesterase from horse serum (Sigma type IV, 5.5 μmoles of acetylcholine hydrolyzed per milligram per minute). For the NMR measurements, the samples were prepared by dissolving acetylcholine chloride (British Drug Houses) and horse serum cholinesterase in 99.8% D_2O (Columbia) containing 0.2 M sodium phosphate, pH 7.4. All pH measurements were made on a Radiometer model 22 pH meter. The pH values given correspond to actual meter readings, and were not corrected for the deuterium isotope effect. Drug concentrations are expressed in moles per liter, and enzyme concentrations, in milligrams per milliliter. The inhibitors used were eserine sulfate (Merck), neostigmine bromide (Hoffmann-La Roche), and edrophonium chloride

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(Hoffmann-La Roche) dissolved in 0.2 M sodium phosphate, pH 7.4.

The assay medium contained sodium phosphate buffer (pH 7.4), acetylcholine chloride (0.01–0.2 M), and horse serum cholinesterase (0–100 mg/ml). After the cholinesterase solution had been prepared with phosphate buffer, a small quantity of this was transferred to a standard 5-mm NMR tube. The assay was conducted in a total volume of 1.0 ml. After the addition of substrate (generally 5×10^{-2} M acetylcholine chloride), the reaction was initiated by rapid mixing with the enzyme solution in the NMR tube. The first reading was usually taken 8 sec after mixing of the solutions.

All measurements were made in a Varian A–60 NMR spectrometer operating at 60 MHz. Chemical shifts are expressed in cycles per second from tetramethylsilane as an external standard. The normal temperature of the insert was 39°. Sufficient time was allowed for the tube and its contents to come to thermal balance before recording the spectra. For temperature studies, the tubes containing enzyme and those containing substrate were incubated at the temperature of the insert. The samples were then mixed and readings were immediately taken. All measurements were made at a sweep span of 10 cps/cm and a sweep rate of 2 cps/sec.

The rate of hydrolysis of acetylcholine chloride by cholinesterase can be measured by the liberation of acetate. There is a distinct change in the chemical shift in the NMR spectrum of the acetate protons formed during the hydrolysis reaction from the acetate protons of acetylcholine chloride. The formation of sodium acetate and the disappearance of substrate can be detected by observing the accompanying changes in the two acetate peaks. The decay of the acetate peak of acetylcholine at 132 cps and the formation of the sodium acetate peak at 117 cps is followed by recording the spectra of these two peaks at various times. The first reading was usually taken 8 sec after the solutions had been mixed. This time was found necessary to prevent spinning side bands from interfering with the recording. The rate of the reaction was estimated by measuring the disappearance of the methyl protons of the acetate group of acetylcholine and the appearance of the methyl protons belonging to the newly formed sodium acetate.

The NMR spectra of acetylcholine chloride and a mixture of horse serum cholinesterase in a D_2O–sodium phosphate buffer are shown in Fig. 1. After the addition of cholinesterase (20 mg/ml), the acetylcholine chloride spectrum contains an additional line at 117 cps (Fig. 1b). This is the resonance line of the methyl protons of the sodium acetate newly formed by the hydrolysis of acetylcholine chloride. The spectrum shown in Fig. 1b was taken 8 sec after the enzyme had been mixed with substrate. At the same time, the acetate line of acetylcholine starts to diminish. Four minutes after the addition of acetylcholine chloride to cholinesterase, the acetate line of acetylcholine is one-fourth the height of the original peak height, while the sodium acetate line is three-fourths the height of the original acetate peak of acetylcholine (Fig. 1c).

Figure 2 shows the disappearance of the acetate signal of acetylcholine at 132 cps and the appearance of the sodium acetate signal at 117 cps at various times after mixing 0.1 M acetylcholine chloride and cholinesterase (20 mg/ml). The rate of appearance of the sodium acetate peak and the rate of disappearance of the acetate peak of acetylcholine follow an exponential curve (Fig. 3). The rate of change of the NMR absorption spectrum of the acetate signal of acetylcholine or the sodium acetate signal is thus a good indicator of the rate of the reaction.

The rate of hydrolysis was also measured by plotting the area of the two peaks against time. Both methods gave identical kinetic results. The former method, however, is less accurate at very high protein concentrations (150 mg/ml), where line broadening reduces the peak height.

An increase in enzyme concentration speeds up the initial rate of hydrolysis of acetylcholine. The initial rate was calculated to be 0.11, 0.22, and 0.44 mmole of

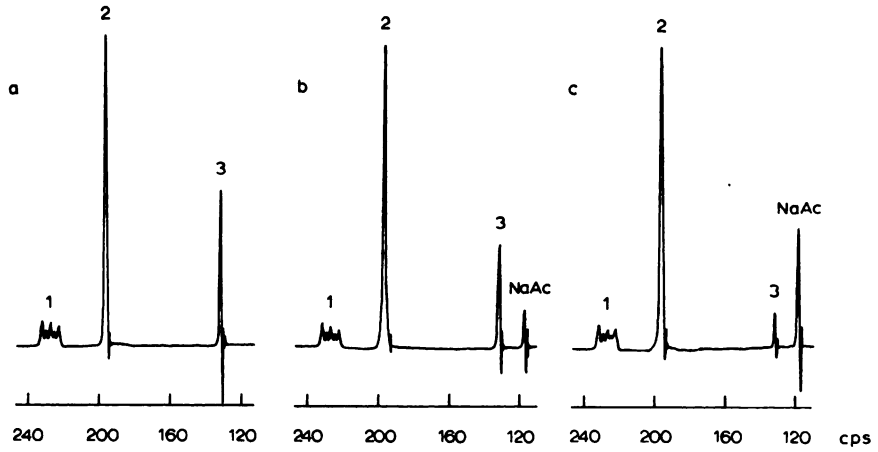

FIG. 1. *NMR spectra of 0.1 M acetylcholine chloride, pH 7.4, at 39°*

a. Acetylcholine chloride (0.1 M) in D_2O–sodium phosphate buffer (0.2 M, pH 7.4). Shifts are from tetramethylsilane as an external standard. The peaks centered at 225 cps are from CH_2CH_2 protons; the large peak at 196 cps is from methyl protons of the quaternary ammonium group; the "singlet" at 132 cps is from the methyl protons of the acetate group. Sample size, 1.0 ml.

b. Acetylcholine chloride in D_2O–phosphate buffer (0.2 M, pH 7.4) containing horse serum cholinesterase (20 mg/ml) 8 sec after mixing of acetylcholine chloride and cholinesterase. The peak at 117 cps is from the methyl protons of sodium acetate (NaAc).

c. Similar spectrum taken 4 min after mixing of acetylcholine chloride and cholinesterase.

acetylcholine per milliliter per minute for cholinesterase concentrations of 20, 40, and 80 mg/ml, respectively. The initial rates were directly proportional to the concentration of enzyme. The specific activity (that is, the number of moles of acetylcholine split per minute per unit weight of protein) was 5.5 μmoles of acetylcholine

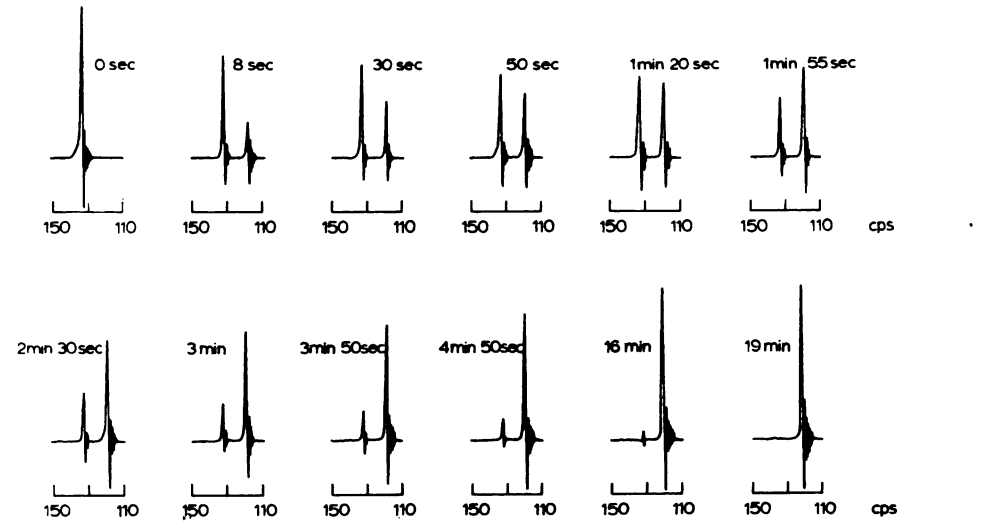

FIG. 2. *Formation and disappearance of acetate protons*

The acetate protons of acetylcholine at 132 cps and the protons of sodium acetate at 117 cps are shown at various time intervals after addition of cholinesterase (20 mg/ml) to 0.1 M acetylcholine chloride in 0.2 M sodium phosphate, pH 7.4, at zero time. Sample size, 1.0 ml; temperature, 39°.

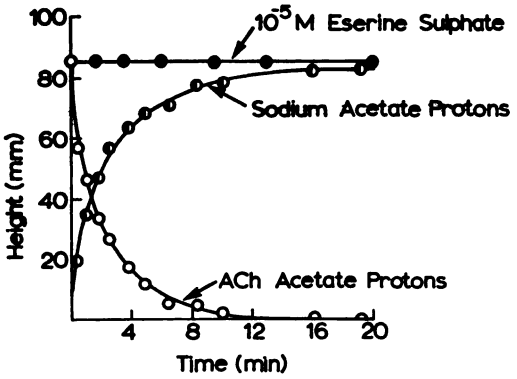

FIG. 3. *Time course of acetylcholine hydrolysis measured by NMR*

The heights of the sodium acetate peak and the acetate peak of acetylcholine (ACh) are plotted as functions of time. The assay mixture contained 0.10 mmole of acetylcholine chloride in 0.2 M sodium phosphate buffer, pH 7.4, in the presence or absence of 10^{-5} M eserine sulfate. The enzyme was 20 μl of a preparation of horse serum cholinesterase (specific activity, 5.5 μmoles of acetylcholine hydrolyzed per milligram per minute; 10 mg/ml) per 1.0 ml of the assay mixture. Temperature, 39°.

per milligram per minute for all concentrations of the enzyme.

The effect of substrate concentration on cholinesterase activity (20 mg/ml) was determined at seven different concentrations of acetylcholine chloride, ranging from 4 to 200 mM, at 38°. The optimum substrate concentration was 10 mM. From a Lineweaver-Burk plot, the K_m was estimated to be 5×10^{-3} M. This is in reasonably good agreement with the value of 3.2×10^{-3} M for horse serum cholinesterase reported by Augustinsson (4).

The influence of pH on the hydrolysis of acetylcholine was studied between pH 6 and 8.6. Optimum activity is obtained at pH 7.5–8.0. The effect of pH changes is generally similar to that described by others (5).

The effect of temperature (T) on the enzymic activity of cholinesterase was evaluated by running the NMR assay at 10°, 15°, 30°, 36°, 40°, 50°, and 54°. The velocity (v) reaches a maximum at 40–50° and falls at higher temperatures, presumably owing to thermal destruction or denaturation of the enzyme protein. The Q_{10} between 10° and 40° was 1.5. From a plot of log v vs. $1/T$, the activation energy was determined graphically to be 7.5 kcal/mole. The Q_{10} and the activation energy for human serum cholinesterase were studied by Shukuya (6) and were calculated to be 1.5 and 7.7 kcal/mole, respectively.

There is a progressive shift of the sodium acetate peak from 117 cps, at the beginning of hydrolysis, to 121 cps, at the end of hydrolysis (Fig. 2). This peak shifts downfield exponentially with time, and the rate of downfield shift depends on the rate of hydrolysis of acetylcholine: the faster the hydrolysis, the more rapid the downfield shift of the acetate peak. Although the shift of the acetate peak is not caused by a change in the pH of the medium, further experiments are necessary to establish the nature of this phenomenon.

All anticholinesterase compounds tested (eserine, edrophonium, neostigmine) acted as competitive inhibitors of cholinesterase. This conclusion is based on the appearance of plots of $1/v$ vs. inhibitor concentration. The corresponding values of K_i for eserine, neostigmine, and edrophonium were 4.5×10^{-8}, 14.0×10^{-8}, and 40.5×10^{-8} M, respectively. These values are in close agreement with those found by other authors, for example, 6.1×10^{-8} M (7), 16×10^{-8} M (7), and 33×10^{-8} M (8), respectively. K_i values were also verified from plots of v/v_i against inhibitor concentration (v_i is the velocity in the presence of inhibitor).

It is clear from the data presented in this paper that the kinetics of hydrolysis of acetylcholine by horse serum cholinesterase can be analyzed with some precision by NMR spectroscopy. The physical parameters evaluated in this study, such as the K_m, K_i, and the activation energy, are in reasonably good agreement with the values found by other authors under similar conditions.

Unlike many direct chemical methods, in which analysis of reactants or products requires discontinuous sampling, the present method makes possible the continuous observation of the reaction.

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