Studies of Catalysis by Acetylcholinesterase. I. Fluorescent Titration with a Carbamoylating Agent*

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ABSTRACT: The reaction of acetylcholinesterase with the *N*-methyl-7-hydroxyquinolinium iodide ester of dimethylcarbamic acid has been followed spectrofluorometrically. In an excess of substrate the observed rate of carbamoylation of the enzyme is strictly first order and is much greater than the concentration-independent rate of decarbamoylation, allowing a titration of enzyme active sites. The method is sensitive to enzyme normalities in the nanomolar region, and from the turnover number and the enzyme normality, the number of active sites per molecule can be calculated. The effects of inhibitors on both the carbamoylation and decarbamoylation rates have been analyzed. Competitive inhibition constants for the inhibition of carbamoylation are the same as those obtained for the inhibition of enzyme-catalyzed acetic acid ester hydrolysis and support the idea that the same enzyme active site is involved in both cases. Some competitive inhibitors of acetylcholine hydrolysis and of carbamoylation are bound to the carbamoyl-enzyme as well as to the free native enzyme. These "inhibitors" are positive effectors of the rate of carbamoyl-enzyme hydrolysis and thereby support previous proposals of a second *allosteric* binding site.

Acetylcholinesterase is a membrane-bound esterase present in cholinergic nerves. It catalyzes the hydrolysis of acetylcholine, a chemical transmitter of the nerve impulse. Catalysis apparently proceeds by formation of an intermediate acyl-enzyme which is then hydrolyzed (Wilson *et al.*, 1950). In contrast to serum cholinesterases, acetylcholinesterase maintains a high specificity for the acyl portion of carboxylic esters and a relatively low specificity for the alcohol-leaving group. Acyl groups containing more than about four carbon atoms are hydrolyzed very inefficiently if at all by this enzyme (Nachmansohn and Wilson, 1951; T. L. Rosenberry, unpublished observations).

A second class of substrates also reacts with acetylcholinesterase. This class consists of esters or acyl halides of phosphoric, carbamic, and methanesulfonic acids (Wilson, 1966). These compounds are potent inhibitors of the enzyme. They apparently form a covalent enzyme intermediate which, unlike acetyl-enzyme, is hydrolyzed to regenerate active enzyme very slowly, if at all, in aqueous solutions. Half-lives for the hydrolysis of carbamoyl-enzymes are of the order of minutes; those of phosphoryl-enzymes, of the order of hours; and those of methanesulfonyl-enzyme are too small to be measured in the absence of certain nucleophilic reactivators (Kitz and Wilson, 1962).

The study of the catalytic mechanism of acetylcholinesterase has usually been restricted to inferences derived from an analysis of the steady-state kinetics of hydrolysis of various substrates (sometimes in the presence of inhibitors). Transient kinetic studies involving the natural substrate acetylcholine and other esters of acetic acid are difficult, because the sequence of steps in a single catalytic turnover is completed in less than 100 μsec (Lawler, 1961). However, transient kinetic studies with the second class of slowly reacting substrates are feasible, and one such study involving the ester *N*-methyl-(7-dimethylcarbamoxy)quinolinium iodide, M7C[1] (I), is reported here.

Various esters of dimethylcarbamic acid have been shown to react with acetylcholinesterase to form a common intermediate whose breakdown is the rate-controlling step in hydrolysis (Wilson *et al.*, 1961). This intermediate is presumably the dimethylcarbamoyl-enzyme and is analogous to the acetyl-enzyme assumed to be formed during the hydrolysis of esters of acetic acid (Wilson and Bergmann, 1950; Krupka, 1964). The rate of formation of the dimethylcarbamoyl-enzyme is specified by the dissociation constant for the ester–enzyme complex and the specific rate constant for the formation of dimethylcarbamoyl-enzyme from the complex. These two kinetic parameters are often difficult to separate, but this study reports both parameters for the dimethylcarbamic acid ester M7C and analyzes the effect of inhibitors on the relevant rates and equilibria. This ester has been selected because the zwitterionic form of the hydroxyquinolinium leaving group, M7H (II), is uniquely fluorescent allowing for the direct observation of acylation of enzyme catalytic sites. Due to the low concentrations of product required for analysis, such structurally identifiable fluorescent changes are particularly valuable when enzyme samples are precious.

The hydrolyses of both acetyl and dimethylcarbamoyl ester substrates are assumed to proceed through an acyl-enzyme intermediate. One may inquire as to whether the same enzyme functional group, presumably a reactive serine hydroxyl (Wilson, 1966), is acylated in both cases. While this question must be resolved by protein degradation studies, less stringent criteria for the identity of binding sites for the two substrate classes can be investigated kinetically. Two necessary conditions for substantiating the identity of binding sites are the

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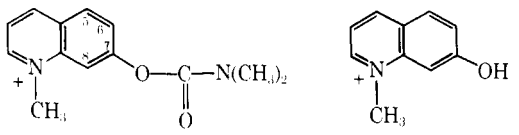
[1] The following abbreviations are used: M7H, *N*-methyl-7-hydroxyquinolinium iodide (M5H is the corresponding 5 isomer); and M7C, *N*-methyl-(7-dimethylcarbamoxy)quinolinium iodide.

following. (1) Reversible inhibitors which compete with either type of substrate must show the same *competitive* inhibition constant against both. (2) During the simultaneous enzymatic hydrolysis of both types of substrate, the substrates must show reciprocal competitive inhibition. That is, the kinetically determined apparent substrate binding constant and its competitive inhibition constant against second substrate must be the same. The first condition is clearly demonstrable as is indicated below utilizing the dimethylcarbamic acid ester substrate M7C and a series of acetic acid ester substrates.

In a subsequent paper (Rosenberry and Bernhard, 1971)[2] the second condition is examined. Unlike the former condition, the latter is demonstrable only under specific conditions of substrate concentration. This observation indicates that the detailed reaction mechanism contains certain complexities, and a discussion of several alternate mechanistic schemes will be presented.

Materials and Methods

Substrate and Inhibitors. N-Methylhydroxyquinolinium Iodides (II) were prepared by dissolving the corresponding hydroxyquinolines (K & K Chemical Co.) in a mixture of tetrahydrofuran and methyl iodide. After allowing the mixtures to stand at room temperature for a few days the crude precipitates were removed by filtration and recrystallized from methanol–anhydrous ethyl ether. M7H gave mp 240–241° (lit. Prince (1966a) mp 249–250°) and λ_{max} 406 nm (0.01 N potassium hydroxide) (ϵ 10,000) (lit. Prince (1966a) λ_{max} 406 nm (ϵ 8500)). M5H gave mp 239.5–240.5° (lit. Prince (1966a) mp 237–238°) and λ_{max} at 460 nm (0.01 N potassium hydroxide) (ϵ 3800) (lit. Prince (1966a) λ_{max} 460 nm (ϵ 3900)).



I M7C

II M7H

N-Methyl-(7-dimethylcarbamoxy)quinolinium iodide (I) was prepared by the following procedure. A solution of 0.50 g (3.4 mmoles) of 7-hydroxyquinoline (K & K Chemical Co.) and 2.5 g (23 mmoles) of dimethylcarbamoyl chloride (Baker) in 50 ml of reagent pyridine was refluxed overnight at 70°. Solvent was removed under reduced pressure; the remaining gum was dissolved in 80 ml of ethyl acetate and washed twice with 20 ml of water, twice with 20 ml of 0.1 N potassium hydroxide, and twice with 20 ml of water. The organic portion was dried over sodium sulfate and the solvent was removed, leaving a light brown oil. Methyl iodide (30 ml) was added and 0.60 g of yellow crystals (1.1 mmoles, 50% yield) formed on standing overnight. The crude material was dissolved in hot methanol, filtered through charcoal, and recrystallized in a mixture of methanol–anhydrous ether to give 0.12 g (10% yield) of yellow needles. The low yield is at least partially due to the fact that the product is strongly absorbed to charcoal. M7C gave mp 198.5–199.5° (lit. Kitz *et al.* (1967) mp 197°) and λ_{max} 320 nm (0.05 M phosphate, pH 6.85) (ϵ 8000).

Anal. Calcd for $C_{13}H_{15}IN_2O_2$: C, 43.57; H, 4.23; I, 35.44; N, 7.82. Found: C, 43.59; H, 4.56; I, 35.52; N, 7.72.

Choline chloride (Matheson, Coleman & Bell Chemical Co.) was recrystallized from anhydrous ethanol–anhydrous ether. 1-Naphthol (Matheson, Coleman & Bell Chemical Co.) was recrystallized from cyclohexane and sublimed; mp 96.5–97.0° (lit. 96°) and λ_{max} 293 nm (0.05 M phosphate, pH 6.85) (ϵ 4900).

Enzyme. Acetylcholinesterase from the electric eel was obtained from Sigma Chemical Co. The enzyme arrived in a frozen solution of 0.02 M ammonium sulfate at a concentration of 500 μM units/ml,[3] with about 0.4 mg/ml of protein. This enzyme preparation had a specific activity of about 70 mmoles/(mg of protein)(hr) for the hydrolysis of acetylcholine, a specific activity about 10% that reported for purified crystalline enzyme (Leuzinger *et al.*, 1969). The frozen solution was thawed, divided into 50-μl portions, and immediately refrozen and stored until needed.

Rate Measurements. Reaction rates were measured at pH 6.85 ± 0.05 in an aqueous buffered solvent (0.05 M phosphate) which contained 0.1 M NaCl and 0.01 M $MgCl_2$. Methanol was added to a final concentration of 1.6% for reasons of solubility when 1-naphthol was present as an inhibitor. The presence of methanol had no noticeable effect on the rate of reaction of enzyme with M7C.

The reaction of enzyme with M7C was followed by the formation of the fluorescent product, the zwitterionic form of M7H (Prince, 1966a,b). The pK_a for M7H is 5.85; and when the hydroxyl group is protonated as in II, no fluorescence is observed. The reaction was monitored on a Hitachi recording fluorescence spectrophotometer, MPF-2A. The excitation wavelength was 400 nm and the emission wavelength was 500 nm. Slit widths on both monochrometers were adjusted manually. The observed rates were the same under all conditions of slit adjustment, although the signal was more stable with small excitation slit widths. The fluorescence emission intensity was calibrated by comparison with a standard M7H solution.

All rates were measured at 25 ● 1°. The nonenzymatic hydrolysis of M7C was negligible, even at the extremely low enzyme concentrations to be described (see also O'Brien *et al.*, 1966).

Analysis of Fluorometric Data from the Reaction of M7C with Acetylcholinesterase. The enzyme-catalyzed hydrolysis of M7C has been monitored by the appearance of the fluorescent product M7H as described above. The experimental trace obtained for this reaction is shown in Figure 1. As reported previously for other esters (Wilson, 1966), the carbamoylation[4] rate appears to be much greater than the decarbamoylation rate. An initial burst is observed, which corresponds closely to a stoichiometric titration of enzyme active sites, followed by a slow steady-state hydrolysis. This reaction ourse is analyzable in terms of the acyl-enzyme model (eq 1).

$$E + CX \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} E\cdot CX \xrightarrow{k_2} E\text{–}C + X^- \xrightarrow[H_2O]{k_3} E + C\text{–}OH + H^+ \quad (1)$$

The dimethylcarbamoyl group is denoted by C; X is the fluo-

[2] Submitted for publication.

[3] One micromolar unit of acetylcholinesterase is defined by the supplier as that quantity which will hydrolyze 1 μmole of acetylcholine/min at pH 8.0 and at 37°. Several different lots of the enzyme were used in this study, and the quantity defined by the supplier as 1 μM unit was observed to hydrolyze from 0.8 to 1.6 μmoles of acetylcholine per minute at pH 6.85 and 25°.

[4] The term carbamoylation is used in this paper to refer to dimethylcarbamoylation of the enzyme. The term decarbamoylation refers to hydrolysis of the dimethylcarbamoyl-enzyme.

rescent zwitterionic leaving group (M7H); and EC is the dimethylcarbamoylated enzyme. The acylation and deacylation rates are labeled k_2 and k_3 and are considered irreversible.

This model includes both an enzyme–substrate complex and an acyl-enzyme as intermediates in the catalytic pathway, as both have been implicated in the catalytic mechanism of esterases (Bender and Kezdy, 1964; Wilson, 1966). Experimental conditions can be set such that the enzyme concentration is several orders of magnitude smaller than the concentrations of reacting species. Kinetic analytical techniques are such that the concentrations of substrates and inhibitors can be maintained effectively constant during the measurement of minute extents of transformation to products.

Equations which express the rates of formation and disappearance of each enzyme species arising from eq 1 may be formulated. The general solutions to these rate equations have been discussed elsewhere (Rosenberry, 1969). A reasonable simplifying assumption is to consider reversibly linked enzyme species to be in virtual equilibrium. This leads to

$$K_c = \frac{k_{-c}}{k_c} = \frac{[E][CX]}{[E \cdot CX]} \tag{2}$$

We shall in this study have occasion to consider the simultaneous interaction of a variety of reversibly bound ligands, L_i, with free enzyme. For convenience, the sum of [E] and $[E \cdot L_i]$ is defined as $[E] \cdot L$, where the parameter L is a partition given by

$$L = 1 + \sum_i \left(\frac{[L_i]}{K_{L_i}} \right) \tag{3}$$

The rate equations for all enzyme species containing reversibly linked ligand can be summed in terms of such a partition; this procedure simplifies the kinetic treatment of reaction at an enzyme site which, in this study, we assume may bind only one of a variety of reversibly bound ligands at a time (Rosenberry, 1969). Given the generality of the equilibrium assumption in eq 2, in the presence of reversibly bound ligands the acyl-enzyme model can be summarized according to eq 4.

$$E \times L \rightleftharpoons E\text{—}C \times N \qquad \begin{matrix} X^- \\ \uparrow \\ C\text{—}OH \\ + \\ H^+ \end{matrix} \tag{4}$$

The parameter N is a partition analogous to L (eq 3) for species reversibly bound to EC (such species are considered further in the Results and Discussion section). The rate equations and the two partitions define the observable rates k_{12} and k_{21} in terms of k_2 and k_3, the two specific rates of chemical transformation in the acyl-enzyme model, as shown in eq 5a and b.

$$k_{12} = \frac{k_2[CX]}{K_c L} \tag{5a}$$

$$k_{21} = \frac{k_3}{N} \tag{5b}$$

The solutions to the differential equations generated from eq 4 are well known (Frost and Pearson, 1953). In terms of the measurable concentration variable [X], a solution is given in

$$[X]_t = \frac{k_{12}E_0}{\alpha}\left[k_{21}t + \frac{k_{12}}{\alpha}(1 - e^{-\alpha t}) \right] \tag{6}$$

where $\alpha = k_{12} + k_{21}$, $[X] = 0$ at $t = 0$, and E_0 is the total concentration of enzyme active sites or the enzyme *normality*.

The experimental trace in Figure 1 also corresponds to a graphical representation of eq 6. The rate constants k_{12} and k_{21} have been determined in the following manner. The time constant α is given by the quantity (-2.3)(slope) derived from a plot of $\log \Delta_t$ *vs.* t; the antilog of the intercept of this plot at $t = 0$ is given by

$$\Delta_{(t=0)} = E_0(k_{12}/\alpha)^2 \tag{7}$$

At late times the time-dependent variation in [X] reaches a constant steady-state rate (eq 8). Hence, defining Q as the

$$\left(\frac{d[X]}{dt} \right)_{\text{steady state}} = \frac{k_{12}k_{21}E_0}{\alpha} \tag{8}$$

quotient of eq 8 divided by eq 7, the rate constants k_{12} and k_{21} defined in eq 5 and 6 can be evaluated directly from the data (eq 9).

$$k_{21} = \frac{Q\alpha}{Q + \alpha} = \alpha - k_{12} \tag{9}$$

Note the functional dependence of specific rate on substrate and other ligand concentrations in eq 5a. This equation is analogous to that utilized in steady-state enzyme kinetic analysis with the exception that k_{12} is independent of E_0. Thus if $1/k_{12}$ is plotted against $1/[CX]$ for systems containing only enzyme and M7C, the intercept of the plot is $1/k_{12}$ and the ratio of the slope to the intercept is K_c, the dissociation constant for the $E \cdot CX$ species.

In the presence of an inhibitor, the enzyme species $E \cdot I$ adds the term $[I]/K_I^I$ to the partition L. If, in the presence of a fixed concentration of inhibitor, reciprocal plots of $1/k_{12}$ *vs.* $1/[CX]$ are first carried out, the slopes of a series of such plots may be graphed *vs.* [I]. If such a replot is linear, the ratio of the intercept to the slope is defined as K_{comp}, the *competitive* inhibition constant for the inhibitor (Dixon and Webb, 1958). For the model described by eq 1 and 3, K_{comp} is K_I, the dissociation constant for the $E \cdot I$ species.

The model in eq 1 and 3 predicts no variation in the intercepts of the reciprocal plots with variation in [I]. However, the occurrence of a significant variation, if linear in [I], may be expressed in terms of a finite *uncompetitive* inhibition constant, K_{uncomp}. This constant is obtained similarly to K_{comp} and is defined as the ratio of the intercept to the slope obtained from a plot of the above-mentioned reciprocal plot intercepts *vs.* [I].

Statistical Analysis. Several dissociation constants in this paper and in subsequent papers could be estimated by more than one experimental procedure. A statistical analysis of the data was carried out so that quantitative comparisons of two estimates could be made.

The substrate concentrations and k_{12} values for each set of data points at a fixed concentration of inhibitor were treated by a weighted least-squares procedure on the IBM 360 computer. Experimental points were analyzed in $1/k_{12}$ *vs.* $1/$

TABLE I: Effect of Various Compounds on Carbamoylation and Decarbamoylation Rates.[a]

Compound	K_{comp} (μM)	K^{I}_{uncomp} (μM)	c	K_I^C (μM)
M7C	*b*		3.4 ± 1.1	83.5 ± 55.0
M5H	2.22 ± 0.70	*c*	*d*	*d*
Choline chloride	819 ± 63	*c*	7.36 ± 0.50	1600 ± 200
1-Naphthol	1630 ± 780[e]	571 ± 148	*d*	*d*

[a] Rate measurements were made on the fluorometer. K_{comp}, K^{I}_{uncomp}, and the error factors were calculated as outlined in the Materials and Methods section; c and K_I^C were determined using eq 12. [b] The apparent substrate dissociation constant, K_C = 10.2 ± 0.61 μM. [c] No significant uncompetitive inhibition could be observed up to the following maximal inhibitor concentrations: [M5H] = 38 μM; [choline chloride] = 6.3 mM. [d] No significant change in the decarbamoylation rate was observed up to the following maximal inhibitor concentrations: [M5H] = 38 μM; [1-naphthol] = 0.76 mM. [e] The experimental slope from which K_{comp} was determined was significantly greater than zero only at the 0.90 confidence level.

[M7C] plots to determine the slope and intercept of each data set. Estimated variances of the slope and intercept were calculated according to the equations of Wilkinson (1961). Each $1/k_{12}$ value was weighted by $(k_{12})^2$ which is equivalent to considering the variance of k_{12} to be a constant percentage of k_{12} for the entire data set. The estimated variance of the ratio of the slope to the intercept was calculated by assuming the values to be related by the linear form $y = mx + b$. Several values of k_2 and K_0 were obtained in the course of various experiments. The weighted means of these values were obtained using the reciprocal of the variance of each individual value as the weight for that value. The estimated variance of the weighted mean was obtained as outlined by Margenau and Murphy (1943).

The slopes and intercepts were plotted *vs.* [I] as outlined above. Each slope and intercept value was weighted by the reciprocal of its variance. The variances of these replot slopes and intercepts could be estimated in two ways, from the least-squares deviations of the replots themselves or from the known variances of the initial slopes and intercepts. The two estimates were usually close and the larger was assumed. The apparent inhibition constant is defined above as the ratio of the replot intercept to the replot slope. The approximation that the variance of this ratio is normally distributed is valid only when the variance is small. Some of the estimates of the inhibition constants with relatively large errors violate this approximation, and the estimate of the variance by the above method in these cases is a very crude one.

The values listed in Table I are given with their standard deviations, defined as the square root of the estimated variance. In some cases no significant uncompetitive inhibition was observed, since the replot slope was not significantly greater than zero at the 95% confidence level (Freund *et al.*, 1960).

The number of points used in constructing the initial reciprocal plots varied from 3 to 15 but was usually 5. The number of lines constructed in the family of lines for a given inhibitor varied from 3 to 5 but was usually 3.

Results and Discussion

The Fluorometric Detection of the Reaction of M7C with Acetylcholinesterase. Figure 1 is the first reported example in which the rates of both acylation and deacylation of acetylcholinesterase are monitored by direct observation of the loss of the leaving group from an ester. In the technique generally used, the acylation rates for slowly reacting esters of carbamic or phosphoric acid are measured by the time-dependent inhibition of enzyme activity toward acetic acid esters (Iverson and Main, 1969; O'Brien, 1968; Kitz *et al.*, 1967). Parallel experiments are required to demonstrate that the leaving group X is released in the reaction, and this has been demonstrated both by spectrophotometric determination of X (Bender and Stoops, 1965) and by the isolation of radioactively labeled HX in the assay mixture (O'Brien *et al.*, 1966). In Figure 1 and in fact in *every* carbamoylation reaction discussed in this paper, a strict adherence to a single exponential is observed; log Δ_t *vs.* t plots are linear, within experimental error, over the entire presteady-state region. This result contrasts with the report of Iverson and Main (1969), who ascribed minor deviations from linearity in plots of log (activity) *vs.* t to the presence of small amounts of molecular forms of acetylcholinesterase different from the predominant forms.

Because enzyme decarbamolyation is much slower than carbamoylation and enzyme dephosphorylation is negligible in the absence of nucleophilic reactivators, esters of carbamic and phosphoric acid may be used to titrate the enzyme sites active in the acylation reactions. Either the initial "burst" of a leaving group like fluorescent M7H ($\Delta_{t=0}$ in eq 7) or the number of moles of acyl group remaining attached to the en-

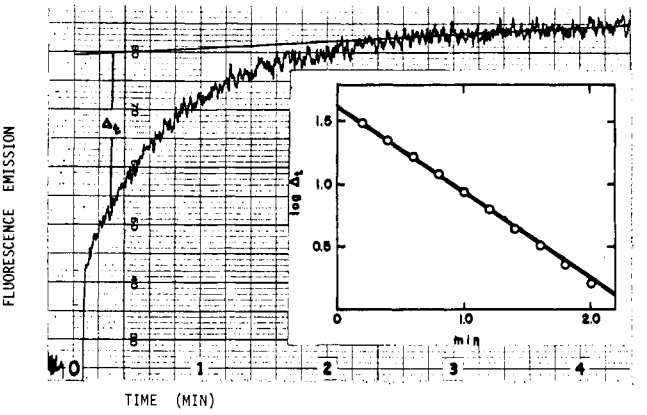


FIGURE 1: The reaction of M7C with acetylcholinesterase as monitored on the fluorometer. Enzyme was 1.27 × 10^{-9} N; [M6C] = 4.6 μM. Reaction conditions are described in the Methods section. The insert shows the agreement of the experimental to a first-order plot. The line in the insert plot is a best fit estimated by eye.

TABLE II: Turnover Numbers for Eel Acetylcholinesterase.[a]

Sp Act. of Enzyme Prepn[b]	Titrant	Turnover No. (min^{-1})	Exptl Condns	Source
1 132	Dimethylcarbamoyl fluoride	8.3×10^5	pH 7.0, 25°, ionic strength $\Gamma = 0.16$	Wilson and Harrison (1961)
2a 660	Tetram, a phosphate thioester	6.8×10^5	pH 7.0, 25°, $\Gamma = 0.2$	Kremzner and Wilson (1964)
2b 12	Tetram	7.8×10^5	pH 7.0, 25°, $\Gamma = 0.2$	Kremzner and Wilson (1964)
3 18	Diisopropyl fluorophosphate	5.4×10^5 [c]	pH 7.4, 38°, $\Gamma = 0.14$	Michel and Krop (1951)
4 Not reported	*o*-Nitrophenyl dimethylcarbamate	10.0×10^5 [d]	pH 7.8, 25°, $\Gamma = 0.2$	Bender and Stoops (1965)
5 70	M7C	8.0×10^5	pH 6.85, 25°, $\Gamma = 0.2$	This paper
6 730			pH 7.0, 25°, $\Gamma = 0.2$	Leuzinger *et al.* (1969)

[a] The turnover number is defined as the ratio of the maximum hydrolysis rate of acetylcholine extrapolated from reciprocal plots (V_{max}) to the enzyme normality as determined by the titrant. [b] The units here are (mmoles of acetylcholine hydrolyzed)/(mg of protein) (hr). The acetylcholine concentration was usually the "optimum" concentration, or that which gives the maximum observed rate. [c] The velocity at 0.015 M acetylcholine rather than V_{max} was used in the calculations. [d] The substrate was phenyl acetate rather than acetylcholine. This ester has about the same k_{cat} as acetylcholine (Krupka, 1966).

zyme can be utilized for determining the acitive-site normality. It has usually been assumed, because of the qualitative similarities in the effects of reversibly bound organic cations on catalytic reactivity and on the rate of modification of active-site serine by acylating and/or phosphorylating reagents, that the enzyme normality measured with the titrant acylating agent is the same as the enzyme normality of the sites active in the hydrolysis of esters of acetic acid (Lawler, 1961; Kremzner and Wilson, 1964). While this conclusion does not necessarily follow, its use allows the comparison of enzyme turnover numbers for acetylcholine reported by several workers. These turnover numbers are listed in Table II and are surprisingly consistent.

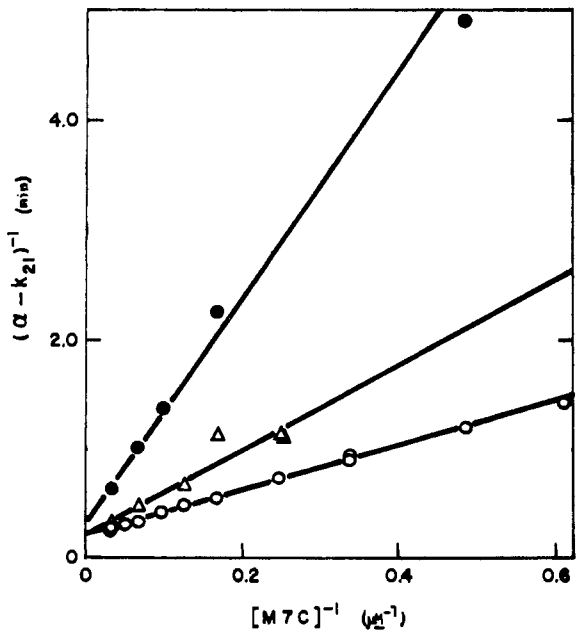


FIGURE 2: Carbamoylation of enzyme by M7C and inhibition of this reaction by choline. The enzyme concentration is 8.1×10^{-9} N. The quantity $(\alpha - k_{21})^{-1}$ is obtained as described in the Methods section. (○) No inhibitor, (△) 0.65 mM choline chloride, and (●) 3.25 mM choline chloride. The lines were calculated from a least-squares analysis of the data shown.

A molecular weight of 260,000 ± 10,000 has been reported for a preparation of crystalline and electrophoretically pure eel acetylcholinesterase (Leuzinger *et al.*, 1969). The molecule was found to consist of four subunits. The specific activity of this preparation (730 mmoles of acetylcholine/mg of protein per hr) may be used to calculate a turnover based on the *molarity* of the enzyme (mol wt 2.6×10^5) of 3.1×10^6 min^{-1}. Comparing this turnover number with the one based on *normality* determined in this paper (Table I), one obtains a value of 3.9 carbamoylation sites/molecule or one site per subunit. This value agrees with the estimate of four active sites per molecule obtained by Kremzner and Wilson (1964) and fails to confirm a preliminary report of two active sites per molecule (Leuzinger *et al.*, 1969). Recent studies also disagree with both the number of subunits per molecule (Froede and Wilson, 1970; Millar and Grafius, 1970) and the specific activity of the pure enzyme preparation (Chen *et al.*, 1971)[5] which are given above. These studies bear directly upon the assumptions used in calculating the number of four active sites per molecule and make this value much more tenuous (for further discussion of this point, see Chen *et al.*, 1971).[5]

The observed dependence of the carbamoylation rate on the concentration of M7C is plotted according to eq 9 (with $L = 1 + [CX]/K_c$) in the lower reciprocal plot of Figure 2. The reciprocal plot is linear within experimental error, with $k_2 = 5.06 \pm 0.25$ min^{-1} and $K_c = 10.2 \pm 0.6$ μM. These values are in excellent agreement with a previous report that $k_2/K_c = 42$ μM^{-1} min^{-1} for M7C in a similar assay medium at pH 7.0 (Kitz *et al.*, 1967).

The decarbamoylation rate (k_{21}) was calculated from eq 9, at low concentrations of M7C where $N = 1$ (eq 5b). The observed value of k_3 is 0.0254 ± 0.0021 min^{-1} in excellent agreement with a value of 0.026 min^{-1} obtained by a different

[5] Y. T. Chen, H. W. Chang, and T. L. Rosenbery, manuscript in preparation.

method but in a similar assay medium at pH 7.0 (Wilson *et al.*, 1961).

Inhibition of Carbamoylation. The effect of the inhibitor choline chloride on the carbamoylation rate is also shown in Figure 2. Table I lists K_{comp} for three inhibitors of carbamoylation. The values of K_{comp} here are identical, within experimental error, to those obtained for the same three inhibitors, M5H and 1-naphthol (Rosenberry and Bernhard, 1971)[2] and choline chloride (Krupka, 1965), during the hydrolysis of esters of acetic acid. (A rather large error is observed for the inhibition constants for 1-naphthol because the inhibitor has a limited solubility.) This result supports the premise that the substrate binding site is the same for ester substrates of both acetic acid and dimethylcarbamic acid.

Despite the lower precision of measurements of inhibition by 1-naphthol, an *uncompetitive* inhibition term as well as a competitive form of inhibition is apparent from the data. The molel of eq 1 and 3 does not allow for uncompetitive inhibition (see Methods); modifications of this model which account for the observation will be considered in a subsequent paper (Rosenberry and Bernhard, 1971).[2]

Acceleration of Decarbamoylation. Certain organic and inorganic cations which competitively inhibit both the carbamoylation of the enzyme and the overall hydrolysis of acetic acid esters are known to accelerate decarbamoylation, presumably by binding at the active site of the carbamoylated enzyme (Wilson, 1967; Kitz *et al.*, 1970). The effect is sometimes enhanced by the inclusion of a nucleophilic group in the cation, indicating that the enzyme may be catalyzing a transcarbamoylation reaction (Roufogalis and Thomas, 1969). A simple extension of the acyl-enzyme model (eq 10) allows a quantitative treatment of data on the acceleration of decarbamoylation.

$$\begin{array}{c} \mathrm{I} \\ \updownarrow \\ \mathrm{H_2O} + \mathrm{E\text{-}C} \xrightarrow{k_3} \mathrm{E} + \mathrm{C\text{-}OH} + \mathrm{H^+} \\ K_I^C \updownarrow \\ \mathrm{H_2O} + \mathrm{E\text{-}C\cdot I} \xrightarrow{ck_3} \mathrm{EI} + \mathrm{C\text{-}OH} + \mathrm{H^+} \end{array} \qquad (10)$$

The species EC and EC·I are related by the partition N (eq 4), and the constant c expresses the decarbamoylation rate of the EC·I complex relative to that of EC. The extended model can still be summarized by eq 4 if it is realized that k_{21} is redefined by the new rate equations so as to take account of the "partitioning" of decarbamoylation *via* two parallel pathways, from EC and from EC·I. This is conveniently notated by a rate partition N_0 (eq 11).

$$k_{21} = k_3 \frac{N_0}{N} \qquad (11)$$

where

$$N_0 = 1 + \frac{c[\mathrm{I}]}{K_I^C}$$

The k_{21} defined in eq 11 is also determined experimentally as outlined in eq 9.

The decarbamoylation rate was studied in the presence of "inhibitors" at low concentrations of M7C where it was assumed that no M7C was bound to the carbamoylated enzyme. In this case the N and N_0 partitions contain only [I] as a variable and eq 11 can be rewritten in the form of eq 12 (Rosenberry, 1969).

$$\frac{k_3}{k_{21} - k_3} = \frac{1}{c - 1} + \frac{K_I^C}{(c - 1)[\mathrm{I}]} \qquad (12)$$

A plot of $k_3/(k_{21} - k_3)$ *vs.* 1/[I] was made to obtain c and K_I^C. The results for various compounds are included in Table II. The dissociation constant for the desorption of the compound to the carbamoyl enzyme, K_I^C, and the relative acceleration of the decarbamoylation rate, c, are determined according to eq 12. 1-Naphthol and M5H have little effect on the decarbamoylation rate; M7C stimulates the rate significantly; and choline chloride increases the rate some 7-fold at (choline) concentrations which are saturating for the carbamoylated enzyme. The values of 0.026 min^{-1} for k_3 and 0.191 min^{-1} for ck_3 in the presence of saturating amounts of choline are in excellent agreement with the rates at which acetylcholinesterase activity returns to enzyme preincubated with M7C (T. L. Rosenberry, unpublished observations). These rates are presumably limited by decarbamoylation of the enzyme.

Decarbamoylation rates measured by this technique have been reported by Kitz *et al.* (1970), who found a K_I^C of 4 mM for choline under somewhat different assay conditions using an equation identical with eq 12. This result shows satisfactory agreement with the K_I^C of 1.6 mM for choline chloride in Table II. The extensive data collected by Kitz *et al.* (1970) on the acceleration of decarbamoylation by various compounds were interpreted in terms of possible allosteric interactions. Our results support such an interpretation. Data from this laboratory concerning further evidence for such interactions will be presented in subsequent papers.

Conclusions

The carbamoylating agent, M7C, is a useful titrant for determining the normality of enzyme sites in solution due to the intensity and convenient wavelength of fluorescence emission in aqueous solution for the product, M7H. The fact that the rate of the stoichiometric reaction can be measured also is a powerful aid in determining the homogeneity of the active sites and in comparing the specific reaction rate constant k_2 in various enzyme preparations.

The direct observation of the reaction of a carbamoylating agent with acetylcholinesterase has allowed for separate analysis of the effects of modifiers on the rates of carbamoylation and of decarbamoylation. Competitive inhibitors of carbamoylation inhibit this process to the same extent that they inhibit the acetylcholinesterase-catalyzed hydrolysis of acetic acid esters; thus they support the premise that both acetyl and carbamoyl esters are hydrolyzed at the same site, albeit at very different velocities. The technique for direct observation of decarbamoylation reveals that a variety of modifiers accelerate this hydrolytic process. A model for this acceleration identical with that of Kitz *et al.* (1970) involving the formation of a ternary enzyme–carbamoyl–modifier complex has been proposed. These results demonstrate that there exist specific site–modifier interactions which positively effect the rate of deacylation whereas they competitively inhibit acylation. Hence, the extent of acylation under steady-state conditions is regulated by the modifier concentration. The possibility that such regulation occurs *in vivo* is increased by the observation that the specific rates of acetylation and deacetylation with acetylcholine are comparable (Wilson and Cabib, 1956). If the

acetyl-enzyme plays an important role in the cholinergic membrane distinct from the catalytic function of acetylcholinesterase, regulated acyl-enzyme levels are of obvious physiological significance.

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