Studies of Catalysis by Acetylcholinesterase. Synergistic Effects of Inhibitors during the Hydrolysis of Acetic Acid Esters†

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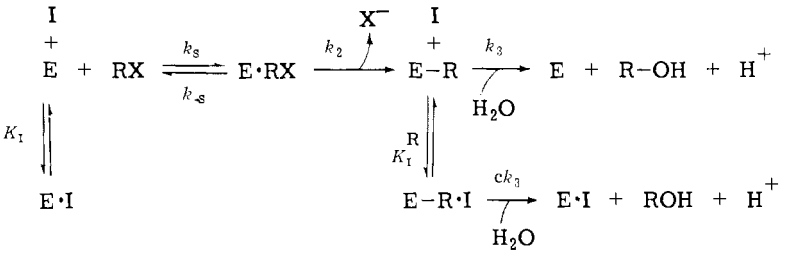
ABSTRACT: The inhibition patterns of several inhibitors of acetylcholinesterase were studied during the steady-state hydrolysis of three acetic acid esters. The *competitive* and *uncompetitive* inhibition constants were tabulated. Data on competitive inhibition are consistent with the assumption that all three acetic acid ester substrates and a dimethylcarbamic acid ester substrate are hydrolyzed at the same enzyme active site. Certain pairs of compounds appeared to bind simultaneously to the enzyme in a ternary complex, and such pairs acting in concert inhibited acetylcholine hydrolysis to an extent many times greater than that observed for either compound alone. A formal model of enzyme catalysis is proposed to account for these *synergistic* effects, and detailed examination of the inhibition data test the predictions of specific molecular interactions within the model. Compounds which act synergistically show evidence of simultaneously interacting with two regions of the active site; these regions are proposed to become separated as a result of a conformational change of the enzyme. A potential role for this conformational change in the cholinergic receptor mechanism of excitable membranes is discussed.

In a previous paper (Rosenberry and Bernhard, 1971), the reaction of acetylcholinesterase with an ester of dimethylcarbamic acid, MeQn1-7-CxNMe$_2$,[1] was investigated. This slowly reacting substrate can be used to titrate the enzyme, and the enzyme active sites may be compared to those for the rapidly hydrolyzed acetic acid ester substrates. Two criteria were proposed to establish that the binding sites for both substrate classes are identical: the observance both of identical inhibition constants for a given competitive inhibitor against all substrates and of reciprocal competitive inhibition during the simultaneous enzymatic hydrolysis of a substrate from each class. Results presented in this paper further substantiate that the former criterion for the identity of both classes is satisfied. The latter criterion is demonstrated here within the group of acetic esters, although certain deviations are seen at higher acetylcholine concentrations. The extension of the latter criterion to both substrate classes is examined in a subsequent paper (Rosenberry and Bernhard, 1972).[2] A simple model (eq 1) for acetylcholinesterase hydrolysis of an ester RX



(1)

(Krupka and Laidler, 1961a; Rosenberry and Bernhard 1971), although generally consistent with the available data, failed to account for the fact that 1-naphthol inhibits the acylation step (k_2) of the reaction with MeQn1-7-CxNMe$_2$ both *competitively* and *uncompetitively*.[3]

An extensive analysis of the uncompetitive components for

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[1] Abbreviations used are: MeQn1-7-CxNMe$_2$, *N*-methyl(7-dimethylcarbamoxy)quinolinium iodide; MeQn1-7-OH, *N*-methyl-7-hydroxyquinolinium iodide (MeQn1-5-OH is the corresponding 5 isomer); MeQn1-7-OAc, *N*-methyl-7-acetoxyquinolinium iodide; PyrCHNOH, 2-pyridinecarbaldoxime methiodide. These abbreviations differ from those used in a previous paper (Rosenberry and Bernhard, 1971) and were recommended by Dr. Waldo E. Cohn, NAS–NRC Office of Biochemical Nomenclature, Oak Ridge National Laboratory.

[2] Manuscript in preparation.

[3] The term *uncompetitive inhibition* as used in this paper refers to the experimental observation that the extrapolated maximum catalytic rate at infinite substrate concentration as determined from *linear* plots of $1/v_0$ *vs.* 1/[S] (Lineweaver and Burk, 1934) is smaller in the presence of inhibitor than in its absence. No change in this maximum rate is predicted for competitive inhibition. Uncompetitive inhibition is usually pictured as a result of complex formation between inhibitor and some enzyme–substrate intermediate formed during catalysis (Mahler and Cordes, 1966). The same inhibitor can bind both to free enzyme and to some enzyme intermediate. In this case the conventional plots of $1/v_0$ *vs.* 1/[S] show increases both in slope and in intercept when inhibitor is added. The term *competitive inhibition component* will be used to describe increases in slope caused by the inhibitor; and the term *uncompetitive inhibition component* will be used in describing inhibitor-dependent increases in intercept. The term noncompetitive inhibition is often applied to the special case which obtains when the competitive and the uncompetitive inhibition constants are found to be equal. Since our treatment of the data includes but is not restricted to this case, no further reference to noncompetitive inhibition will be made.

a number of inhibitors of the acetylcholinesterase-catalyzed hydrolysis of acetylcholine has been carried out by Krupka (1964). His results suggest that all uncompetitive inhibition components arise due to the formation of an inhibitor–acetyl-enzyme complex in which deacylation is at least partially blocked, and therefore his data are accounted for by the model in eq 1. In this paper we investigate the inhibition patterns observed with a variety of acetic acid ester substrates. Although particular inhibitors follow a pattern predicted by eq 1 quite nicely, other inhibitors show large uncompetitive components with some substrates which are not predictable according to this model. The interactions among enzyme, substrate, and such inhibitors are analyzed; and an expanded form of the model in eq 1 is shown to adequately describe the results.

Materials and Methods

Substrates and Inhibitors. *N*-Methylhydroxyquinolinium iodides and 1-naphthol were prepared as described previously (Rosenberry and Bernhard, 1971). *N*-Methylacetoxyquinolinium iodides were synthesized by a procedure similar to the following typical preparation of MeQn1-7-OAc. A solution of 0.20 g (1.4 mmoles) of 7-hydroxyquinoline (K & K) in 10 ml of acetic anhydride was allowed to stand at 37° for 40 hr. Methyl iodide (5 ml) was added and the mixture was stirred in a reflux system at 50° for 24 hr. Reagent ethyl ether was added to ensure complete precipitation, and the solution was filtered. The crude solid was recrystallized three times from methanol–ethyl ether and gave 0.27 g of yellow needles (0.81 mmole, 59% yield), mp 224.0–224.5°, lit. (Prince, 1966) mp 220–221°. MeQn1-5-OAc gave mp 191.2–191.7°, lit. (Prince, 1966) mp 186.5–187.5°.

The spectrophotometric extinction coefficients for the various quinolinium compounds are listed in Table I. Both the methods of preparation and the extinction coefficients differ slightly from an earlier report (Prince, 1966).

The synthesis of 1-naphthyl acetate was from 1-naphthol and acetic anhydride in triethylamine. It was twice recrystallized from Skelly solv B, mp 46.0–46.3°, lit. mp 46–49°; the extinction coefficient is given in Table I. 1- and 2-naphthylamines (Aldrich) were twice recrystallized from cyclohexane. 1-Naphthylamine gave mp 50.3–50.8° (lit. mp 50°) and λ_{max} at 304 nm (0.05 M phosphate, pH 6.85), ϵ_{304} 5200. 2-Naphthylamine gave mp 110.5–111.5° (lit. mp 111–112°) and λ_{max} at 278 nm (0.05 M phosphate, pH 6.85), ϵ_{278} 5600. The corresponding acetamides were prepared by dissolving the naphthylamine in acetic anhydride, filtering the precipitate, and recrystallizing from cyclohexane–dichloromethane. *N*-Acetyl-1-naphthylamine gave mp 162.5–163.0° (lit. mp 160°) and λ_{max} at 280 nm (0.05 M phosphate, pH 6.85), ϵ_{280} 6800. *N*-Acetyl-2-naphthylamine gave mp 135.0–136.0° (lit. mp 134°) and λ_{max} at 282 nm (0.05 M phosphate, pH 6.85), ϵ_{282} 7000. Acetylcholine chloride (Sigma) was either used directly or recrystallized from anhydrous ethanol–anhydrous ether. In either case an acid titration equivalent of 98–101% of the calculated value was observed after hydrolysis by acetylcholinesterase. Decamethonium bromide (K & K) and 2-PyrCHNOH[1] (Aldrich) were used without further purification. Flaxedil (gallamine triethiodide) was a gift of Dr. J.-P. Changeux, Institute Pasteur.

Enzyme. Acetylcholinesterase from the electric eel, solubilized and partially purified from its native membrane-bound state to a specific activity of 70 mmoles of acetylcholine hydrolyzed per mg of protein per hr, was obtained from the Sigma Chemical Co. as described previously (Rosenberry and Bernhard, 1971). During the kinetic studies the enzyme stock was either buffered solvent or buffered solvent containing 1% gelatin (Wilson and Bergmann, 1950). If no gelatin was added to stabilize the solution, it was necessary to monitor the time-dependent decay of enzyme activity and to correct the observed rates for this decay. No time-dependent loss of enzyme activity was observed in the presence of gelatin over a period of a few hours. On addition of enzyme to the assay mixture the dilution was always sufficient to maintain a final gelatin concentration of less than 0.007%, a concentration which has no demonstrable effects on the enzyme activity.

In agreement with a previous report (Kitz *et al.*, 1970), the use of enzyme purified to a greater extent than above has little effect on the kinetic results. When enzyme purified by the procedure of Leuzinger and Baker (1967) to a specific activity of 500 mmoles/mg of protein per hr was used in the system shown in Figure 1, no quantitative difference in the results was obtained (T. L. Rosenberry and I. P. Yang, unpublished observations).

Solvent. The aqueous solvent for these studies contained 0.1 M NaCl and 0.01 M $MgCl_2$. For rates observed on the spectrophotometer, 0.05 M phosphate (as a mixture of Na_2HPO_4 and KH_2PO_4) was added as a buffer. The pH was adjusted to 6.85 ± 0.05 unless otherwise noted. Both ionic strength (Changeux, 1966) and specific ions (Roufogalis and Thomas, 1968) have been reported to affect the apparent values of the kinetic parameters. A solution of 1.6% methanol was used to prevent the precipitation of naphthalene derivatives; this amount of methanol had no effect on the catalyzed hydrolysis of either acetylcholine or MeQn1-7-OAc.

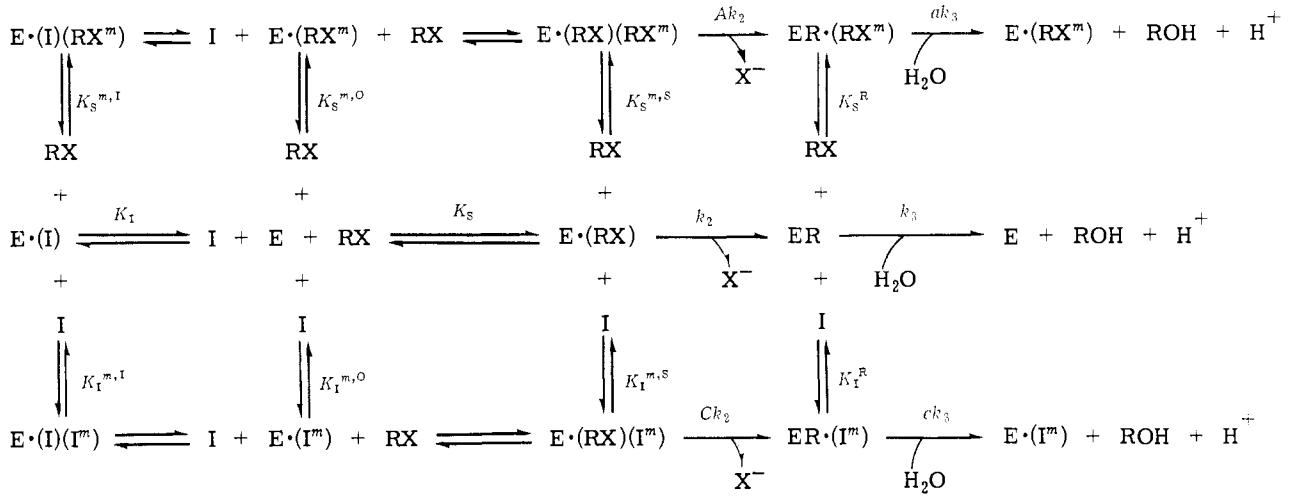
Rate Measurements. Reaction rate measurements were carried out in one of three ways. (1) Acetylcholine hydrolysis was measured on a Radiometer-Copenhagen, Ole Dick pH-Stat using 0.100 N KOH to titrate the protons released during hydrolysis. The pH-Stat assay volume was 90 ml. (2) MeQn1-7-OAc hydrolysis was measured on a Hitachi MPF-2A recording fluorescence spectrophotometer. The appearance of the fluorescent product MeQn1-7-OH was monitored as described previously (Rosenberry and Bernhard, 1971). (3) All

TABLE I: Spectrophotometric Extinction Coefficients.[a]

Compound	$\lambda_{max}^{H_2O}$	ϵ	$\lambda_{max}^{0.10\ N\ KOH}$	ϵ	Rate Studies[b] λ	Rate Studies[b] $\Delta\epsilon$
MeQn1-5-OH			460	3,800		
			272	33,000		
MeQn1-5-OAc	316	6800	460	3,900[c]	320	−5,000
			272	32,600	270	25,200
MeQn1-7-OH			406	10,100		
MeQn1-7-OAc	317	7900	406	10,000[c]	406	4,200
1-Naphthol			333	7,400		
1-Naphthyl acetate	279	6100	333	7,400[c]	235	24,000

[a] Units for λ are nm and for ϵ are M^{-1} cm^{-1}. [b] Conditions for the enzymatic hydrolysis of acetic acid esters are described in the Methods section. The difference extinction $\Delta\epsilon$ (ϵ product minus ϵ reactant) is observed at the given wavelength. [c] The ester is presumed to be totally converted to its hydrolysis product.

SCHEME I: Model Including an Acyl-enzyme and a Modifier Site Distinct from the Active Site.[a]



[a] A substrate RX or an inhibitor I may bind either to the active site or to the modifier site. At the active site, RX may react to form the acyl-enzyme ER with loss of the leaving group X; ER may then be hydrolyzed to regenerate free enzyme. The binding of either RX or I to the modifier site may perturb the acylation (k_2) and/or deacylation (k_3) rate constants by an amount indicated by a proportionality factor. The dissociation constants for ligand binding at the modifier site are denoted by the superscript "m" in the free enzyme and by the superscript "R" in the acyl-enzyme. The superscript "m" is followed by "O" if the active site is free of ligand; by "I" if the active site has bound inhibitor; and by "S" if the active site has bound substrate. The dissociation constants for ligand binding at the active site have no superscript.

other substrate hydrolyses were monitored on the Cary Model 14 spectrophotometer at the wavelength indicated in Table I. The rates of 1-naphthyl acetate hydrolysis were measured both on the Cary 14 and on the pH-Stat.

All rates were measured at 25 ± 1° and were corrected for nonenzymic hydrolysis and for any dilution factors. Nonenzymic hydrolysis was significant only for the *N*-methylacetoxyquinolinium iodides. MeQn1-7-OAc rates were also adjusted to compensate for product present during the initial rate measurement (see below). A more comprehensive tabulation of reaction rates and their dependence on particular ligand concentrations is available elsewhere (Rosenberry, 1969). Further kinetic details are available from the authors.

Analysis of Reaction Rate Data. The simple model in eq 1 fails to account for all inhibitor ligand interactions with acetylcholinesterase as noted earlier. Consequently, interaction of the inhibitor with both the enzyme–substrate complex and the acyl-enzyme as well as with the free enzyme must be considered. Scheme I outlines an allosteric or "modifier" site for such interaction (in addition to the substrate binding site or the "active site") and contains all the enzyme species consequent to an acyl-enzyme model of catalysis. Ligands may bind to the modifier site, but undergo no further reaction; however, ligand binding at the modifier site may perturb reactions at the active site and hence give rise to uncompetitive inhibition components. The concept of a modifier site allows a general treatment of several models for uncompetitive inhibition considered by Krupka and Laidler (1961a). Such a site model is in contrast to *allosteric site cooperativity*, where ligand-dependent *intersubunit* interactions can lead to rates of reaction which vary with ligand concentration in nonhyperbolic fashion (Monod *et al.*, 1965). The solutions to the appropriate rate equations arising from this modifier-site model are indicated in the Appendix.

Experimentally, inhibition measurements could be fitted, within limits of error, to eq 2 (a single exception is noted in the Results section), where $k_{cat}[E_0]$ and K_{app} are the constants determined from reciprocal plots of $1/v_0$ *vs.* 1/[RX] in the absence of inhibitor; K_{comp} and $K_{uncomp}{}^{I}$ are obtained from

$$\frac{1}{v_0} = \frac{1}{k_{cat}(E_0)}\left[1 + \frac{[I]}{K_{uncomp}{}^{I}} + \frac{[RX]}{K_{uncomp}{}^{S}} + \frac{K_{app}}{[RX]}\left(1 + \frac{[I]}{K_{comp}}\right)\right] \quad (2)$$

replots of the slopes and intercepts of the reciprocal plots *vs.* [I] as described previously (Rosenberry and Bernhard, 1971; additional data may be found in Rosenberry (1969) or be obtained by writing to the authors); and $K_{uncomp}{}^{S}$ is estimated by a procedure described below. The agreement of the data with eq 2, which was not *a priori* predictable from Scheme I, was utilized to simplify the formal kinetic model as shown in the following paragraph.

In establishing a correspondence between the theoretical velocity expression arising from Scheme I (eq 9A in the Appendix) and the experimental in eq 2 we utilize the apparent experimental fact that only inhibition patterns in which $1/v_0$ is linear in [I] occur. In other words the apparent K_{comp} and $K_{uncomp}{}^{I}$ of eq 2 are independent of concentration variables. According to a literal interpretation of Scheme I, *uncompetitive* inhibition having these properties can occur only if occupancy at the modifier site blocks bond-breaking reactions completely at the active site.[4] In this case the $K_{uncomp}{}^{I}$ in eq 2 is formulated in the kinetic parameters of Scheme I as shown in eq 3.

$$\frac{1}{K_{uncomp}{}^{I}} = \frac{k_{cat}}{k_2 K_I{}^{m,S}} + \frac{K_{app}}{K_I K_S{}^{m,I}} + \frac{k_{cat}}{k_3 K_I{}^{R}} \quad (3)$$

[4] For Scheme I to conform to eq 2 and to give rise to uncompetitive inhibition components either the acylation step k_2 or the deacylation step k_3 must be blocked completely (either M_0 or $N_0 = 1$; see eq 7A–8A in the Appendix). If $M_0 = 1$, the conformity further requires that $1/K_I{}^{m,I} = 0$ (no E·(I)(I^m) species may be formed). Trivial binding at the modifier site (*i.e.*, that which has no effect on kinetic constants at the active site and $M_0 = M^i$ or $N_0 = N$) may occur but would not be kinetically detectable. The formulation of the inhibition constants in eq 3 and 4 assumes the general case that $M_0 = N_0 = 1$.

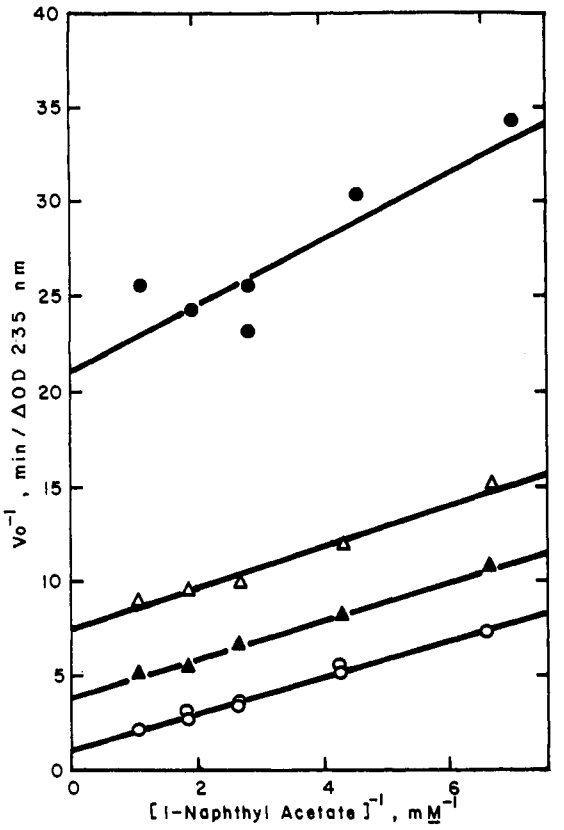


FIGURE 1: Inhibition of 1-naphthyl acetate hydrolysis by 2-PyrCHNOH. (○) No inhibitors; (▲) plus 10.4 μM 2-PyrCHNOH; (△) plus 25.6 μM 2-PyrCHNOH; (●) plus 98.0 μM 2-PyrCHNOH. The lines were calculated from a least-squares analysis of the data shown (see Methods section).

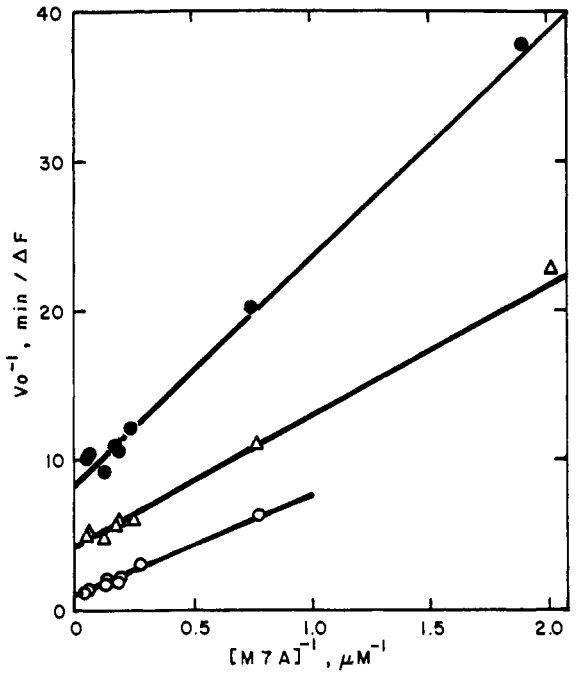


FIGURE 2: Inhibition of MeQnl-7-OAc hydrolysis by 1-naphthol. The change in sample fluorescence (ΔF) was an arbitrary value which was calibrated by a standard MeQnl-7-OH solution. (○) No inhibitor; (△) plus 0.84 mM 1-naphthol; (●) plus 2.09 mM 1-naphthol. The lines were calculated from a least-squares analysis of the data shown.

where k_{cat} and K_{app} are mechanistically defined in the Appendix. Expressions for K_{comp} and K_{uncomp}^{S} also are given in the Appendix (see eq 10A–13A).

Substrate Inhibition as a Special Case of Uncompetitive Inhibition. At high [S] for some acetic acid esters, substrate inhibition occurred and the reciprocal plots deviated from linearity. When such a deviation was obtained, the difference between [$1/v_0$ observed] and [$1/v_0$ extrapolated from assumed linear double-reciprocal plots at low [S]] was calculated and defined as Δ. A plot of Δ *vs.* [S] was then made and found to be linear in the cases observed. The slope of this line was used to define uncompetitive inhibition by substrate (eq 4).

$$K_{uncomp}^{S} = \frac{1}{(\text{slope})(k_{cat}[E_0])} \quad (4)$$

Correction of MeQnl-7-OAc Reaction Rates for the Presence of Product. Since the reaction product MeQnl-7-OH is a potent competitive inhibitor of MeQnl-7-OAc hydrolysis, reaction velocities had to be corrected for small amounts of MeQnl-7-OH formed before the initial rate measurement. Uncorrected values were substituted into eq 2, and the $1/v_0$ component estimated to arise from the ratio, [[MeQnl-7-OH]/K_{comp}], was subtracted. The correction amounted to between 5 and 10% of the $1/v_0$ value.

Statistical Analysis. The weighted least-squares analysis used to analyze the data has been described previously (Rosenberry and Bernhard, 1971). The tabulated inhibition constants are obtained as ratios, and the error term listed is defined as the square root of the estimated variance of the value. 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.

Results

Measurement of Inhibition Constants. Examples of the reciprocal plots used to display the initial reaction rate data are shown in Figures 1 and 2. For certain substrate and inhibitor combinations as in Figure 1, the uncompetitive inhibition component is relatively large, and the system displays the rare case of nearly parallel lines in reciprocal plots for inhibition in a single substrate system (Mahler and Cordes, 1966).

Values for K_{comp} and K_{uncomp}^{I} for inhibitors and three different acetic acid esters are listed in Tables II and III. Table IV lists K_{app}, the apparent Michaelis constants, and K_{uncomp}^{S}, the substrate inhibition constants, for the three acetic acid esters. According to the model of Scheme I, these parameters are related to the dissociation constants for complexes of inhibitors with either free enzyme or some enzyme intermediate in the manner described in eq 3 and in the Appendix (eq 10A–13A).

Two factors led to relatively low precision of some of the measurements. (1) Inhibitors in group D (Tables II and III) are naphthalene derivatives and the solubilities of these compounds in 1.6% methanol are of the same magnitude as the observed dissociation constants. (2) When the observed K_{comp} and K_{uncomp}^{I} differ widely, two different ranges of substrate concentrations are required for the measurement of the

TABLE II: K_{comp} Values for Various Acetic Acid Esters and Inhibitors.[a]

Inhibitor	Substrate		
	Acetylcholine	1-Naphthyl Acetate	MeQnl-7-OAc
Group A			
Flaxedil	107 ± 12	344 ± 64 98 ± 32[b]	173 ± 15
Group B			
MeQnl-5-OH	2.20 ± 0.17	3.48 ± 0.17	2.74 ± 0.32
MeQnl-7-OH	3.41 ± 0.48	5.58 ± 0.27	3.50 ± 0.35
Decamethonium bromide	5.83 ± 0.46	9.55 ± 0.70	7.90 ± 0.37
Group C			
2-PyrCHNOH	69 ± 13	109 ± 32	76.1 ± 8.2
MeQnl-7-OAc	3.04 ± 0.78 3.69 ± 0.51[c]	*d*	
MeQnl-5-OAc	*d*	*d*	6.4 ± 1.4[e]
Group D			
1-Naphthyl acetate	535 ± 114		765 ± 124
1-Naphthol	2340 ± 600	2210 ± 890	1730 ± 530
Group E			
Acetylcholine		227 ± 21	173 ± 14[e]

[a] K_{comp} values were calculated as outlined in the Methods section. Units are μM. [b] Measured on the pH-Stat (see text). [c] Measurements were made at pH 6.25 ± 0.10. [d] No measurements were made. [e] Replot lines were curved and K_{comp} was estimated at very low acetylcholine concentrations; see Results section.

TABLE III: $K_{uncomp}{}^{I}$ Values for Various Acetic Acid Esters and Inhibitors.[a]

Inhibitor	Substrates		
	Acetylcholine	1-Naphthyl Acetate	MeQnl-7-OAc
Group A			
Flaxedil	*b*	*b*	*b*
Group B			
MeQnl-5-OH	6.71 ± 0.72	6.4 ± 1.1	58 ± 23[c]
MeQnl-7-OH	16.1 ± 1.8	8.1 ± 1.4	104 ± 18
Decamethonium bromide	13.1 ± 1.0	19.9 ± 2.9	*b*
Group C			
2-PyrCHNOH	*b*	4.61 ± 0.63	*b*
MeQnl-7-OAc	26.6 ± 7.4[d] 19.1 ± 7.9[c-e]	*f*	
MeQnl-5-OAc	*f*	*f*	*b*
Group D			
1-Naphthyl acetate	*b*		39.4 ± 2.1[d]
1-Naphthol	4100 ± 730[e]	*b*	312 ± 22
Group E			
Acetylcholine		1180 ± 440[d]	*g*

[a] $K_{uncomp}{}^{I}$ values were calculated as outlined in the Methods section. Units are μM. [b] No significant uncompetitive inhibition was observed at inhibitor concentrations which gave the significant competitive inhibition constants in Table II, so $K_{uncomp}{}^{I} \gg K_{comp}$. [c] Uncompetitive inhibition was significant only at the 90% confidence level. [d] In these two-substrate systems $K_{uncomp}{}^{I} = K_G$ (see Appendix). [e] Measurements were made at pH 6.25 ± 0.10. [f] No measurements were made. [g] Replot lines were curved; see Results section.

two parameters. Insufficient data at higher substrate concentrations often limited the precision of the larger uncompetitive inhibition constants. The values listed are sufficiently precise for the purpose of this paper.

Comparison of K_{comp} Values. The K_{comp} values in Table II are independent of ligand concentration variables with the single exception of the inhibition of MeQnl-7-OAc by acetylcholine. Thus the data generally conform to the simplified

form of the model introduced in the Methods section, where K_{comp} reflects inhibitor binding both to the active site and to the modifier site in the free enzyme (eq 12A in the Appendix). It is also noteworthy that according to Scheme I, when a second substrate is added to the assay as an inhibitor, the $K_{comp} = K_{app}$ for that substrate (eq 15A in the Appendix).

The competitive inhibition constants themselves are quite consistent from one acetic acid ester substrate to another throughout the varied list of inhibitors. Furthermore, reciprocal competitive inhibition is observed during the simultaneous hydrolysis of two substrates; *i.e.*, each substrate competitively inhibits the other with a competitive inhibition constant equal to its K_{app}. When acetylcholine inhibits the hydrolysis of a substrate this relationship holds only at lower acetylcholine concentrations; at higher concentrations the K_{comp} for acetylcholine decreases (see further description below). A somewhat surprising results does occur in that the constants for inhibitors measured with acetylcholine as substrate are about 1.5 to 2 times smaller than those determined with either 1-naphthyl acetate or MeQn1-7-OAc as substrate. While this difference is not always significant for a single inhibitor, the trend is unmistakable and significant. The only differences in experimental conditions between inhibition of acetylcholine hydrolysis and inhibition of 1-naphthyl acetate or MeQn1-7-OAc hydrolysis are the use of the pH-Stat method and the absence of 0.05 M phosphate buffer for measuring hydrolysis rates with acetylcholine.

The effect of phosphate buffer on the enzymatic hydrolysis of 1-naphthyl acetate was determined. Buffer concentrations of 0.02, 0.05, and 0.20 M in the standard 0.1 M NaCl and 0.01 M $MgCl_2$ solutions gave the same reaction velocities. The precision of the data was such that any buffer inhibition constant would have to be much greater than 0.20 M. Smith and Alberty (1956) have reported that complexing of magnesium cations and orthophosphate anions is significant under these conditions. However, deletion of 0.01 M $MgCl_2$ from the solvent during either acetylcholine hydrolysis in the pH-Stat or 1-naphthyl acetate hydrolysis in the spectrophotometer gave results consistent with those of Roufogalis and Thomas (1968) and indicated that $MgCl_2$ acts as a weak competitive inhibitor. A magnesium phosphate complex therefore does not account for the observed discrepancies in K_{comp}.

The apparent Michaelis constant (K_{app}) for 1-naphthyl acetate could be measured on both the pH-Stat and the spectrophotometer, and both values are shown in Table IV. There does appear to be a significant difference in the two values, and the value obtained on the pH-Stat is the same as the competitive inhibition constant for 1-naphthyl acetate measured during the hydrolysis of acetylcholine. Inhibition of 1-naphthyl acetate by flaxedil was also measured on both the spectrophotometer and the pH-Stat. The K_{comp} values obtained are shown in Table II, and they differ in the same manner as the K_{app} values for 1-naphthyl acetate. The difference between the two methods does not have a satisfactory explanation unless some change in substrate affinity for enzyme occurs between 0 and 0.02 M phosphate buffer. Such a change would have to be independent of buffer concentration above 0.02 M from the results described in the previous paragraph. The data do indicate, however, that the variance in the measured affinities arises from the methodology and not from a difference in the enzyme active site for various substrates.

Comparison of $K_{uncomp}{}^I$ Values. In Table III the compounds have been divided into five groups to facilitate discussion. Flaxedil in group A was the only inhibitor tested which gave no significant uncompetitive components against all three acetic acid ester substrates. In agreement with the results for competitive inhibition, all observed uncompetitive inhibition constants are concentration independent except for acetylcholine inhibition of MeQn1-7-OAc hydrolysis. Unlike the results for competitive inhibition, however, wide variations in the value of the uncompetitive inhibition constant for a given inhibitor are observed from one acetic acid ester substrate to another.

TABLE IV: Apparent Substrate Michaelis Constants and Substrate Inhibition Constants.[a]

Acetate	K_{app}[b] (μM)	$K_{uncomp}{}^I$ (mM)
Acetylcholine	111.5 ± 7.4 (7)	44 ± 19
	94.2 ± 8.5 (3)[f]	
1-Naphthyl acetate	879 ± 30 (8)[d]	[c]
	522 ± 48 (2)[e]	
MeQn1-7-OAc	6.05 ± 0.16 (8)	0.53 ± 0.15
	6.54 ± 0.70 (2)[f]	
MeQn1-5-OAc	5.74 ± 0.88 (2)	0.435 ± 0.055

[a] Parameters were calculated as outlined in the Methods section. [b] These values are averages of the number of experiments given in parentheses. The standard deviation listed is the standard deviation of the mean. [c] No substrate inhibition was observed with 1-naphthyl acetate. [d] Measured on Cary 14 at 235 mμ. [e] Measured on pH-Stat. [f] Measured at pH 6.25; binding constants measured at pH 6.25 were not significantly different from those measured at pH 6.85.

In certain cases one observes a much larger uncompetitive than competitive inhibition component, as shown in Figures 1 and 2. Such cases are extremely sensitive to variations in the structure of the inhibitor. This may be seen in Table V where the inhibition of MeQn1-7-OAc hydrolysis by a series of naphthalene derivatives is shown. The K_{comp} values are roughly constant, the $K_{uncomp}{}^I$ values range from some 20 times smaller than K_{comp} in the case of 1-naphthyl acetate to much larger than K_{comp} in the case of *N*-acetyl-1-naphthylamine. This difference between corresponding acetoxy and acetamido derivatives of naphthalene is striking.

Synergistic Inhibition. While inhibitors for which $K_{uncomp}{}^I < K_{comp}$ are found when either 1-naphthyl acetate or MeQn1-7-OAc is the substrate, no such inhibitor was obtained when the substrate was acetylcholine. The effects of a substrate–inhibitor system for which $K_{uncomp}{}^I < K_{comp}$ on simultaneous acetylcholine hydrolysis can be observed in terms of the inhibition arising from the substrate and the inhibitor acting both individually and in combination. An experiment of this type is shown in Figure 3, where 1-naphthyl acetate and PyrCHNOH, the substrate–inhibitor system described in Figure 1, act as inhibitors during acetylcholine hydrolysis. The competitive inhibition component arising from the presence of both inhibitors is more than 20 times greater than the competitive inhibition component of either inhibitor individually. Essentially no uncompetitive inhibition component is observed. The inhibitors appear to act synergistically to give a much greater inhibition than the sum of their individual contributions.

A second example of such synergistic inhibition of acetylcholine hydrolysis is shown in Figure 4, where the two

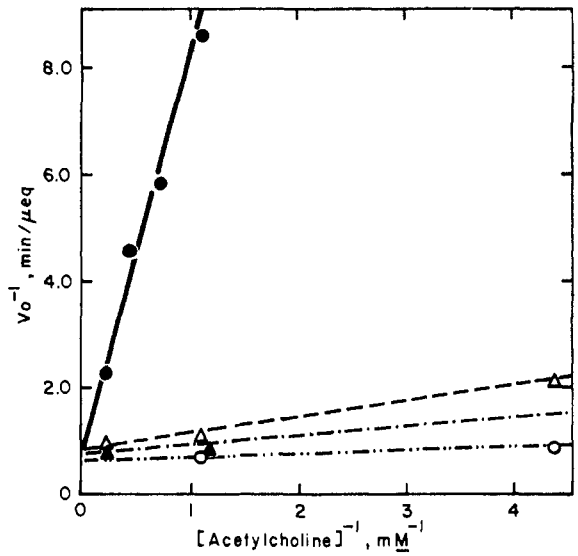


FIGURE 3: Inhibition of acetylcholine hydrolysis by 2-PyrCHNOH and 1-naphthyl acetate acting both individually and in concert. (O---O) No inhibitors; (△---△) plus 0.225 mM 2-PyrCHNOH; (▲-·-▲) plus 0.865 mM 1-naphthyl acetate; (●—●) plus 0.255 mM 2-PyrCHNOH and 0.865 mM 1-naphthyl acetate. The observed velocities were corrected for the expected proton release resulting from 1-naphthyl acetate hydrolysis and the v_0 plotted here is the result of acetylcholine hydrolysis only. The solid line was calculated from a least-squares analysis of the data in which both inhibitors were present; broken lines were calculated from the parameters listed in Tables II, III, and IV using eq 2 as described in the Methods section.

TABLE V: K_{uncomp}^{I} for Inhibition of MeQnl-7-OAc Hydrolysis by Various Naphthalene Derivatives.[a]

Inhibitor	K_{uncomp}^{I}	K_{comp}	K_{comp}/K_{uncomp}^{I}
1-Naphthyl acetate	0.0394 ± 0.0021	0.77 ± 0.12	19.4
1-Naphthol	0.312 ± 0.022	1.73 ± 0.53	5.5
1-Naphthyl-amine	0.543 ± 0.056	1.30 ± 0.34	2.4
N-Acetyl-1-naphthyl-amine	*b*	2.10 ± 0.43	<1
2-Naphthyl-amine	0.79 ± 0.23	2.8 ± 1.8[c]	3.5
N-Acetyl-2-naphthyl-amine	*b*	2.5 ± 1.0[d]	<1

[a] Parameters were calculated as outlined in the Methods section. Units are mM. [b] No significant uncompetitive inhibition was observed at inhibitor concentrations used to measure K_{comp}. [c] Competitive inhibition was significant only at the 85% confidence level. [d] Competitive inhibition was significant only at the 90% confidence level.

inhibitors are 1-naphthol and PyrCHNOH. Here both the competitive and the uncompetitive inhibition components are enhanced by the inhibitors acting in concert.

Synergistic inhibition of acetylcholine hydrolysis is observed only when ligands from group C and group D simultaneously act as inhibitors. In addition to the stringent structural requirements for group D inhibitors noted in Table V above, group C is also highly selective, as shown by the inclusion of acetoxy and carbamoxy but the exclusion of hydroxyquinolinium compounds (Table III; also see Table VIII below).

Substrate Inhibition. Inhibition of substrate hydrolysis may occur at high concentrations of substrate in the absence of any other inhibitor. Substrate inhibition by acetylcholine has been observed by many workers, and substrate inhibition by the *N*-methylacetoxyquinolinium iodides has been reported by Prince (1966). Values of the substrate inhibitions constants were obtained in this study as outlined in the Methods section and are given in Table IV. Substrate inhibition by MeQnl-7-

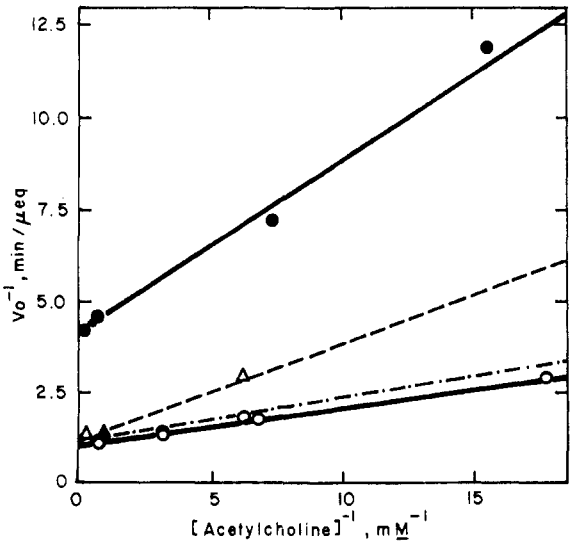


FIGURE 4: Inhibition of acetylcholine hydrolysis by 2-PyrCHNOH and 1-naphthol acting both individually and in concert. (O—O) No inhibitors; (△---△) plus 0.109 mM 2-PyrCHNOH; (▲-·-▲) plus 0.416 mM 1-naphthol; (●—●) plus 0.109 mM 2-PyrCHNOH and 0.416 mM 1-naphthol. Solid lines were calculated from a least-squares analysis of the data shown; broken lines were calculated from the inhibition parameters listed in Tables II, III, and IV using eq 2.

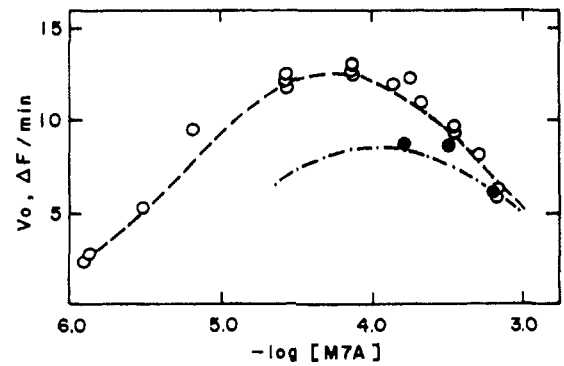


FIGURE 5: Substrate inhibition by MeQnl-7-OAc in the presence and absence of acetylcholine. (O) MeQnl-7-OAc alone; broken line was calculated from eq 2 using the kinetic parameters listed in Table IV. (●) MeQnl-7-OAc plus 0.42 mM acetylcholine; broken line was calculated from eq 3 and eq 15A in the Appendix (with [I] = 0) using Tables III and IV. Above 1 mM MeQnl-7-OAc the v_0 values approach a constant value.

OAc is shown in Figure 5. Roughly the same substrate optimum is observed in the presence or absence of acetylcholine.

Acetylcholine as an Inhibitor of Other Acetic Acid Ester Substrates. The replot graphs for the slopes and intercepts of $1/v_0$ *vs.* 1/[MeQnl-7-OAc] plots at various acetylcholine concentrations are shown in Figure 6. Each point has been normalized to the corresponding value in the absence of acetylcholine. The curvature of the points in both cases is significant at the 0.95 confidence level. At high concentrations of acetylcholine the replots become linear in acetylcholine concentration. No curvature is seen in the reciprocal plots themselves, either in the presence or in the absence of acetylcholine. Observations on the enzyme-catalyzed hydrolysis of MeQnl-7-OAc have been extended to substrate concentrations as low as 10^{-7} M, where the velocity is about 1.5% of the maximum velocity, without deviation from linearity in the reciprocal plots. Curvature is seen in similar replots of the inhibition of MeQnl-5-OAc and 1-naphthyl acetate by acetylcholine, although in the latter case the curvature is less pronounced and the points do not deviate from the least-squares line within experimental error.

The curvature of the replot lines is unique to acetylcholine as the inhibitor and indicates that K_{comp} and K_{uncomp}^{I} for acetylcholine are concentration dependent (see eq 14A in the Appendix). Since the acylation step k_2 is rate limiting for MeQnl-7-OAc hydrolysis (see Table VI), this result suggests that a parallel acylation pathway for MeQnl-7-OAc is available with an enzyme species formed in the presence of acetylcholine.[5] A similar result is observed during inhibition by acetylcholine of the acylation of acetylcholinesterase with the corresponding dimethylcarbamic acid ester, MeQnl-7-CxNMe$_2$ (Rosenberry and Bernhard, 1972)[2].

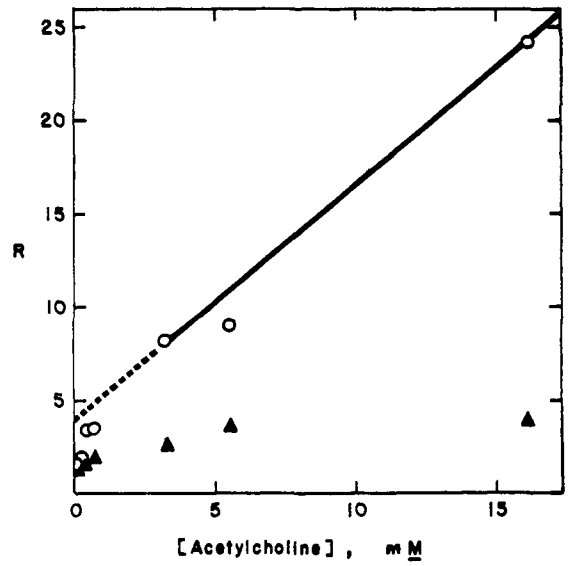


FIGURE 6: Replot data for inhibition of the enzyme-catalyzed hydrolysis of MeQnl-7-OAc by acetylcholine. Each point is a normalized slope (○) or intercept (▲) obtained from a reciprocal plot of v_0^{-1} *vs.* [MeQnl-7-OAc]$^{-1}$ at a fixed acetylcholine concentration (see Methods section). (○) R is the ratio of the slope of the reciprocal plot at a fixed [I] to the slope of the reciprocal plot in the absence of acetylcholine inhibitor; (▲) R is a similar ratio of the intercepts of the reciprocal plots. An additional observation was made at [I] = 92 mM; R_0 = 167 and $R_▲$ = 4.65. The line is calculated from a (weighted) least-squares analysis of the R_0 data for [I] > 3 mM.

Discussion

Analysis of Ligand Interactions. A major object of the present study was to demonstrate that the acetylcholinesterase active sites for dimethylcarbamic acid ester and acetic acid ester substrates are identical according to competitive inhibition criteria. The consistency of the competitive inhibition constants listed in Table II from one acetic acid ester to another for all inhibitor ligands provides strong evidence for a common substrate binding site for this varied group of acetic acid esters. A comparison of the constants to that obtained for the same inhibitor during enzyme reaction with the dimethylcarbamic acid ester MeQnl-7-CxNMe$_2$ (Rosenberry and Bernhard, 1971) further extends this consistency and strongly supports a common active site for both substrate classes.

A second aspect of this research was to examine the possibility that enzyme acylation could be inhibited in an uncompetitive fashion. A general model which includes this possibility has been presented in Scheme I. According to this model, an uncompetitive inhibition constant which is independent of ligand concentration can only arise if the rate-determining catalytic process (either acylation or deacylation of the enzyme) is blocked completely by occupancy of ligand at a modifier site.

As Krupka and Laidler (1961a) have pointed out, one can determine whether the modifier site is affecting acylation and/or deacylation by a comparison of the K_{uncomp}^{I} values obtained for a single inhibitor with a series of acetic acid ester substrates. An examination of the expression for K_{uncomp}^{I} derived from Scheme I (eq 3) indicates that, in general, no simple relationship exists between a series of acetic acid esters (RX, RY, . . .) and the respective values of K_{uncomp}^{I} obtained with a single inhibitor. If, however, the uncompetitive inhibition arises *solely* from the binding of inhibitor to acyl-enzyme, then the extent of *uncompetitive* inhibition of the series of acetic acid ester hydrolyses (for which k_3 are identical but k_{cat} vary) will be proportional to the fraction of enzyme acylated, given by k_{cat}/k_3. The results on the uncompetitive inhibition constants for the inhibitor MeQnl-5-OH obtained from a series of acetic acid esters are shown in Table VI. $(K_{uncomp}^{I})^{-1}$ is proportional to k_{cat} over the 25-fold range of k_{cat} values. A similar relationship appears to hold for other inhibitors in group B of Table III and for the uncompetitive inhibition components arising from trimethylammonium ion inhibition of acetic acid ester hydrolysis (Krupka, 1964). For these inhibitor ligands, all uncompetitive inhibition components can be accounted for by ligand binding to the modifier site in the acetyl-enzyme which *totally* inhibits deacylation. Occupancy of the modifier site by a ligand in the putative preacetylation complexes E·(RX)(I^m) or E·(I)(RX^m) (see Scheme I) either does not occur or else does not affect acetylation.

Substrate inhibition at high substrate concentrations (*e.g.*, Figure 5) has also been considered to result from an inhibition of enzyme deacylation associated with an inactive ER·(RX^m) complex (Krupka and Laidler, 1961a; Wilson and Alexander, 1962). This possibility is included in the model in Scheme I, where substrate inhibition may result from sub-

[5] The data in Figure 6 can be fit quantitatively by the general rate expression (eq 14A in the Appendix) if the parallel pathway is Ck_2 in Scheme I. In this case $1 < M_0 < M^j$ and acetylcholine would interact with the modifier site. Since this interaction is ruled out by other data (see eq 6f), the parallel pathway involved cannot be accounted for by the models presented in this paper.

TABLE VI: Inhibition of Acetic Acid Ester Hydrolysis by MeQn1-5-OH.[a]

Acetic Acid Ester	Rel k_{cat}[b]	K_{uncomp}^{I}, μM	(Rel k_{cat})·(K_{uncomp}^{I}), μM
Acetylcholine	1.00 ± 0.035	6.7 ± 0.7	6.7 ± 1.2
1-Naphthyl acetate	0.65 ± 0.028	6.4 ± 1.1	4.2 ± 0.92
MeQn1-7-OAc	0.057 ± 0.0035	58 ± 23	3.3 ± 1.5
MeQn1-5-OAc	0.046 ± 0.010	113 ± 36	5.2 ± 2.8

[a] Parameters were calculated as outlined in the Methods section. [b] These values are averages of a number of experiments (see Table IV). The standard deviation listed is the standard deviation of the mean. The k_{cat} values have been normalized to a value of 1.00 for acetylcholine.

strate binding to the modifier site with consequent inhibition of acylation and/or deacylation; hence substrate inhibition is proposed to be analogous to uncompetitive inhibition. A test to determine whether substrate inhibition results solely from a block of deacylation has been carried out using the substrates acetylcholine and MeQn1-7-OAc. The K_{uncomp}^{S} resulting from substrate inhibition by MeQn1-7-OAc (RX) alone is compared to the uncompetitive inhibition component K_G (eq 16A in the Appendix) which arises from the inhibition of hydrolysis of acetylcholine (RY) by MeQn1-7-OAc. If both these inhibition phenomena result from an ER·(MeQn1-7-OAc^m) complex in which deacylation is blocked, it follows (see eq 16A in the Appendix) that eq 5

$$\frac{k_{cat}^{RY}}{k_{cat}^{RX}} = \frac{K_{uncomp}^{S=RX}}{K_G^{I=RX,S=RY}} \quad (5)$$

obtains provided that the data are taken under conditions in which [RX] $\ll K_{uncomp}^{S=RY}$. The value observed for $k_{cat}^{RY}/k_{cat}^{RX}$ is 17.5 ± 1.2 (Table VI); that observed for $K_{uncomp}^{RX}/K_G^{RX,RY}$ is 20.0 ± 7.9 (Tables III and IV). The predicted relationship holds.

Since there has been no evidence in the data presented to this point for a modifier site except in the acetyl-enzyme, it could be argued that the modifier site is simply the active site in the acetyl-enzyme (Krupka and Laidler, 1961b). This proposal is reconsidered in the discussion of synergistic inhibition (below). The above data, both on the uncompetitive inhibition by inhibitors of group B of Table III and on substrate inhibition by MeQn1-7-OAc, offer compelling evidence that an inhibitory complex is formed with a catalytic intermediate *common* to all three acetic acid esters. According to the model in Scheme I, this intermediate is the acetyl-enzyme.

In contrast to the results described above for group B inhibitors, unexpectedly large uncompetitive inhibition components are observed in particular instances, for example, with inhibitors of group C (Table III) when MeQn1-7-OAc is the substrate or with inhibitors of group D when 1-naphthyl acetate is the substrate. These large components do not result from the formation of a complex of inhibitor with the acetyl-enzyme or another intermediate common to all acetic acid ester substrates, since the uncompetitive inhibition components derived for these same inhibitors when acetylcholine is the substrate is much smaller. According to Scheme I, this result indicates that the modifier site and the active site are simultaneously binding the substrate and inhibitor in an E·(RX)(I^m) or an E·(I)(RX^m) ternary complex in which acetylation is completely blocked. The fact that these uncompetitive inhibition components are much larger than the competitive inhibition components (Table III and V) indicates that the binding of the inhibitor in the ternary complex is several times tighter than its binding at either the active site or the modifier site in the free enzyme.

One test for the formation of a ternary complex is to measure the inhibition of enzyme-catalyzed acetylcholine hydrolysis by the simultaneous addition of both compounds involved in the proposed complex. The ternary complex should contribute to the competitive inhibition of acetylcholine hydrolysis (see eq 15A in the Appendix). An experiment is shown in Figure 3, where 1-naphthyl acetate and 2-PyrCHNOH are both present during acetylcholine hydrolysis. The compounds act *synergistically* to competitively inhibit the enzyme to a much greater extent than the sum of their individual competitive inhibition components. This result may be compared quantitatively to the one predicted from the model by focusing on the quantity R_S, defined as the ratio of the slope of the reciprocal plot of v_0^{-1} *vs.* $[RX]^{-1}$ in the presence of both 1-naphthyl acetate and 2-PyrCHNOH to the slope of the reciprocal plot in the absence of inhibitors, where [RX] is the concentration of acetylcholine substrate. From eq 15A in the Appendix, it follows that R_S is given by eq 6. In the present example, I = 2-PyrCHNOH and RY =

$$R_S = 1 + \frac{[I]}{K_{comp}^{I,S=RX}} + \frac{[RY]}{K_{app}^{RY}}\left[1 + \frac{[I]}{K_{uncomp}^{I,S=RY}}\right] \quad (6)$$

1-naphthyl acetate. A comparison of the calculated and observed values of R_S is given in Table VII. The agreement of the observed value with that of the model is satisfactory. The fact that I and RY combine to give a synergistic competitive inhibition component rather than a synergistic uncompetitive inhibition component supports the argument that I and RY form a ternary complex either with the free enzyme or with some enzyme–substrate intermediate which cannot be generated from acetylcholine (*i.e.*, *not* the acetyl-enzyme). According to Scheme I this ternary complex occurs with the free enzyme and is either E·(I)(RY^m) or E·(RY)(I^m). The quantitative agreement in Table VII further indicates that acetylcholine does not interact at the modifier site to displace either ligand.

The synergistic inhibition of acetylcholine hydrolysis observed with the inhibitors 1-naphthol and 2-PyrCHNOH (Figure 4) has both a competitive and an uncompetitive component. However, a direct quantitative comparison of the R_S arising from the competitive component to the value predicted by the inhibition constants for the inhibitors acting individually is not possible by the methodology described since the interaction of 1-naphthol and 2-PyrCHNOH with the enzyme is detected solely by its effect on substrate reactivity. If it is assumed that 1-naphthol has the same affinity for the E·(2-PyrCHNOH) complex as it has for the E·(MeQn1-7-OAc) complex (Table III; also see the discussion of

TABLE VII: Comparison of R_S Obtained by Different Methods.[a]

Inhibitors of Acetylcholine Hydrolysis			R_S Calcd from Individual Inhibition Studies	R_S Obsd for Simultaneous Inhibition
[2-PyrCHNOH], mM	[1-Naphthyl Acetate], mM	[1-Naphthol], mM		
0.255	0.865		96 ± 23	121 ± 17
0.109	0.094		6.9 ± 1.1	7.3 ± 0.4
0.109		0.416	4.9 ± 0.7[b]	4.6 ± 0.3
0.109		0.693	6.4 ± 1.0[b]	6.2 ± 1.7

[a] R_S observed is defined above eq 6 in the text; and R_S calculated is given in eq 6 using values for the inhibition constants given in Tables II, III, and IV. [b] These values were calculated assuming that 1-naphthol has the same affinity for the E·(2-PyrCHNOH) complex as it has for the E·(MeQn1-7-OAc) complex, as given in Table III (see Discussion section).

Table VIII), the experimental R_S values can be compared with the theoretical (eq 6) as shown in Table VII. A quantitative agreement is obtained, again supporting the model.

The model as written in Scheme I cannot lead to the synergistic uncompetitive inhibition component observed in Figure 4, since the possibility of simultaneous binding of two inhibitors to an enzyme–substrate intermediate in the general case involves three ligand binding sites on the enzyme. If, however, the acetyl-enzyme can bind a ligand at both the active site and the modifier site to give an $ER\cdot(I_1)(I_2^m)$ complex, this extended version of the model can accommodate the observation of synergistic uncompetitive inhibition.

The data on inhibitor–ligand interactions with the enzyme may be summarized as follows. The striking internal consistency of the synergistic competitive inhibition components with the inhibition constants in Tables II and III supports the contention that an $E\cdot(I_1)(I_2^m)$ complex is formed by certain pairs of inhibitors.[6] This complex, whose formation is extremely sensitive to structural modifications in either inhibitor, cannot be simply the result of simultaneous binding of two inhibitors to different sites, since the apparent affinity of at least one of the inhibitors is increased by the binding of its synergist by a factor of between 2 and 20 (Table V). 1-Naphthol and 2-PyrCHNOH act as a synergistic inhibitor pair. Since neither of these compounds is known to react with the enzyme, covalent bonds between inhibitor and enzyme are not required for the formation of the $E\cdot(I_1)(I_2^m)$ complex. It is noteworthy that 1-naphthol but not 1-naphthyl acetate can form an apparent $ER\cdot(I_1)(I_2^m)$ complex.

Proposed Mechanism of Ternary Complex Formation. Several investigators have emphasized that the active site of acetylcholinesterase is composed of esteratic and anionic subsites (Nachmansohn and Wilson, 1951; Župančič, 1967). The anionic subsite is assumed to bind the cationic portion of a charged molecule, while the esteratic subsite binds an electrophilic group like the carbonyl carbon and leads to acylation during catalysis. On the basis of a variety of observations, it appears that the two subsites can be occupied simultaneously by distinct molecules. Inhibition studies by Krupka and Laidler are most simply interpreted by a model which involves the binding of inhibitor to the anionic site after the formation of the acetyl-enzyme at the esteratic site as noted above (Krupka and Laidler, 1961a; and Table VI). Wilson (1967) has contended that the observed acceleration of the rates of enzyme reaction with carbamoyl or methanesulfonyl fluorides by tetraethylammonium ion is due to a simultaneous binding of the cation to the anionic site and the acyl fluoride to the esteratic site. Several other cationic compounds also can bind to enzyme carbamoylated at the esteratic site and thereby either accelerate or reduce the decarbamoylation rate (Kitz *et al.*, 1970; Rosenberry and Bernhard, 1971); when such compounds which accelerate the rate contain nucleophilic groups they apparently function as acceptors in transcarbamoylation reactions (Roufogalis and Thomas, 1969). However, these examples involve only very small acylating groups at the esteratic site. From the inferred dimensions of the active site (Krupka and Laidler, 1961b), it seems likely that for two molecules the size of 1-naphthyl acetate and MeQn1-7-OAc to simultaneously occupy the active site a change in enzyme conformation would be required.

Two observations suggest that naphthalene derivatives which act in synergistic inhibition are bound to the esteratic subsite rather than to a less specific hydrophobic area. One is the specificity of 1-naphthyl acetate as a synergist, particularly in comparison to *N*-acetyl-1-naphthylamine (Table V); the other is that 1-naphthol (but not 1-naphthyl acetate) can form an apparent $ER\cdot(I_1)(I_2^m)$ complex. 1-Naphthyl acetate is presumably prevented from forming an $ER\cdot(I_1)(I_2^m)$ complex because the acetylated group (probably a serine hydroxyl; Wilson, 1966) at the esteratic site occupies part of the 1-naphthyl acetate binding site.

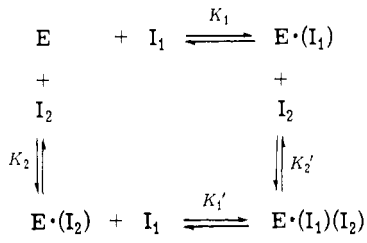
It appears then that the naphthalene synergist binds to the esteratic subsite and that the anionic subsite binds the aromatic cation. There appears to be no need to postulate a modifier site physically distinct from the total active site. One cannot, however, rule out the possibility that synergists are bound to the esteratic and anionic portions of distinct catalytic sites in light of the report that acetylcholinesterase is an oligomeric protein (Leuzinger *et al.*, 1969) which contains either three or four active sites (Rosenberry *et al.*, 1972). Our observation that MeQn1-7-OAc hydrolysis follows a hyperbolic saturation curve over a wide range of substrate concentrations (below the substrate optimum) and a previous report of the failure to find homotropic cooperative interactions in reactions catalyzed by the purified enzyme (Changeux, 1966) argue strongly against modifier function *via* allosteric subunit interactions. Moreover, the model of Scheme I is consistent with the data presented herein only if

[6] No attempt has been made to rigorously define the molecular prerequisites of any of the groups listed in Tables II and III, particularly those involved in ternary complex formation with the enzyme (groups C and D). The compounds listed should only be considered examples.

TABLE VIII: K_2' Values Reflecting the Binding of the Naphthalene Derivative in the Ternary Complex.[a]

I_1	I_2: 1-Naphthyl Acetate	I_2: 1-Naphthol
MeQn1-7-OAc	39 ± 2	310 ± 20
MeQn1-7-CxNMe$_2$	42 ± 4[b]	570 ± 150[c]
2-PyrCHNOH	37 ± 12	350 ± 120[d]

[a] Units are μM. Ternary complex formation is expressed according to the following scheme:

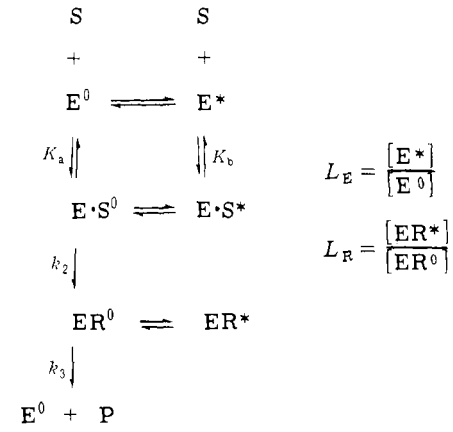


K_2' is related to the other binding constants by the thermodynamic equality $K_1K_2' = K_1'K_2$. Values of K_2' were obtained from Tables II and III with the assumption that the ternary complex $E\cdot(I_1)(I_2)$ was the only enzyme species contributing significantly to the uncompetitive inhibition component. [b] Rosenberry and Bernhard (1972).[2] [c] Rosenberry and Bernhard (1971). [d] This value is calculated from the data in Figure 4, and its agreement with the other K_2' values for 1-naphthol is a restatement of the conclusions reached in Table VII concerning ternary complex formation with 2-PyrCHNOH and 1-naphthol.

no $E\cdot(I_1)(I_1^m)$ complex is formed, and one would expect such complexes if separate catalytic sites were involved. More likely, the synergists bind to the esteratic and anionic sites of a single catalytic site and thereby interact favorably with one another either *via* a conformational change or *via* attractive van der Waal's interactions.

Further examination of data presented in Tables II and III strongly supports the involvement of a conformational change in the synergistic interaction. The naphthalene binding site in the ternary complex is the same regardless of which group C ligand is bound at the anionic subsite. This is shown in Table VIII, where the affinities of either 1-naphthyl acetate or 1-naphthol are shown for each of three E·(I) complexes, where I is a group C compound. The affinity of the naphthalene derivative is the same regardless of which I is present, even though this affinity is quite different from that for the free enzyme (Table V). This consistency is striking in view of the stringent structural requirements placed upon inhibitors which form the synergistic complex; *e.g.*, the acetoxy and the dimethylcarbamoxy but not the hydroxyquinolinium compounds participate in the synergistic interaction. It is unlikely that van der Waal's interactions between inhibitors in the synergistic complex could simultaneously account for the specificity requirements of ligands in group C and for the constant affinities which naphthalene derivatives show in forming the complex. These observations, however, are nicely in accord with a conformational change specific to the group C ligands which defines a new binding site for the group D naphthalene derivatives. Little direct intermolecular interaction between the synergists would then be necessary.

SCHEME II: Proposed Modification of the Model in Scheme I to Include an Enzyme Conformational Change.[a]



[a] Free acetylcholinesterase is assumed to consist of two forms, E^0 and E*, which are in equilibrium. No separate modifier site is proposed in this modification of the model, but the two subsites of the active site are allowed to bind compounds independently.

Data suggesting conformational equilibria in acetylcholinesterase have been reported previously (Changeux, 1966; Kitz and Kremzner, 1968; Kitz *et al.*, 1970). A simple enzymic system involving both enzyme–substrate intermediates and a conformational equilibria is shown in Scheme II. Assuming equilibration among the reversibly formed species E^0, E*, $E\cdot S^0$, and E·S* during catalytic turnover, it follows that the velocity of the reaction according to the Scheme II is given by eq 7, where L_E, K_a, and K_b are as defined in Scheme II. It follows that for such a system, K_{app} for a single substrate is equal to K_{comp} for that substrate as an inhibitor, and K_{uncomp}[1] for an inhibitor is a simple weighted average of the inhibitor binding constants to all ES and EA species. Thus, this scheme is not distinguishable from that of the original model (Scheme I) by steady-state inhibition studies, and it may easily be expanded to include the ternary complexes proposed in Scheme I with either enzyme conformation.

$$v_0 = \frac{d[P]}{dt} = \frac{1}{k_{cat}(E_0)}\left[1 + \frac{K_{app}}{[S]}\right] \quad (7)$$

$$k_{cat} = \frac{k_3}{1 + L_R + \frac{k_3}{k_2}\left[1 + \frac{L_E K_a}{K_b}\right]} \quad (8)$$

$$K_{app} = \frac{[1 + L_E]K_a}{1 + \frac{k_2}{k_3}[1 + L_R] + \frac{L_E K_a}{K_b}} \quad (9)$$

The formation of the synergistic complex can be discussed in terms of Scheme II. The choice of this model is somewhat arbitrary, since the data do not permit this conformational equilibrium model to be distinguished from one involving an inhibitor-induced conformational change (Koshland *et al.*, 1966). The diverse results reported above can all be accommodated to this scheme in the following manner. Acetylcholine, flaxedil, and the cations in group B of Table III are proposed to bind preferentially to the predominant confor-

mations E^0 and ER^0 while the aromatic quaternary ions in group C bind preferentially to the E* and ER* conformations. Unlike the E^0 conformer, the E* conformer has separated the anionic and esteratic portions of a single catalytic site to the extent that a molecule of a member of group D, *e.g.*, 1-naphthyl acetate or 1-naphthol, can bind to the esteratic subsite, while a molecule of a member of group C, *e.g.*, MeQn1-7-OAc or 2-PyrCHNOH, is bound to the anionic subsite. The group D naphthalene derivatives bind more tightly to the esteratic subsite in E* than in E^0, perhaps because a more hydrophobic environment is created by its separation from the anionic subsite, but are not hydrolyzed because the E* conformation is inactive. The ER* conformation also has separated the anionic and the esteratic subsites, allowing 1-naphthol to bind in the vicinity of the esteratic site in the ER·(2-PyrCHNOH)* complex.

Relationship of Acetylcholinesterase to Acetylcholine Receptor. The potential significance of a conformational equilibrium among acetylcholinesterase subunits depends on their role in the cholinergic membrane. Inhibitors of acetylcholinesterase show a general similarity to compounds pharmacologically active on the "acetylcholine receptor," an apparent protein which mediates chemically induced depolarizations of this membrane (Roepke, 1937; Nachmansohn, 1965). Models proposing the identity of enzyme and receptor have been offered for many years (Župančič, 1953; Cohen *et al.*, 1955; Belleau, 1964), and Changeux (1966) postulated a common protein which could undergo conformational transitions. While the concept of conformational transitions in the receptor had been introduced earlier (Nachmansohn, 1955), Changeux's model further equated a distinct enzyme conformer with the depolarized nerve membrane state and another conformer with the polarized membrane state. This proposal was based on experiments which demonstrated that flaxedil, an inhibitor of membrane depolarization (receptor inhibitor) in physiological systems, reverses enzyme inhibition by decamethonium and other compounds which depolarize the membrane (receptor activators). More recently Kitz *et al.* (1970) have found that a strong correlation exists between receptor inhibitors and compounds which accelerate decarbamoylation in the isolated enzyme. Receptor activators were found either to have little effect upon, or to inhibit, decarbamoylation.

A similar, although less exact, correlation obtains in this study between quaternary ammonium ions which inhibit the receptor in the isolated electroplax preparation (Schoffeniels, 1957; Higman *et al.*, 1964) and those which participate in synergistic inhibition of the enzyme. The inhibitors in group C, proposed in Scheme II to bind preferentially to the E* conformation, are receptor inhibitors (Bartels and Rosenberry, unpublished observations); acetylcholine and decamethonium in group B,[7] which in Scheme II bind preferentially to the E^0 conformation, are well-known receptor activators (Nachmansohn, 1965). The correlation does not hold for flaxedil, a receptor inhibitor which does not participate in synergistic inhibition of the enzyme. Flaxedil, however, is a trivalent cationic compound in which little charge delocalization occurs, and it may inhibit the receptor by a mechanism different from that of the monovalent aromatic cations in group C. Enzyme inhibitors which apparently act only at the esteratic site have little effect on receptor activity (see below); and the naphthalene derivatives in group D, presumed to bind to the esteratic site in Scheme II, show no receptor activity. Furthermore, the naphthalene derivatives in combination with group C inhibitors have no synergistic effects on the receptor (E. Bartels and T. Rosenberry, unpublished observations).

Several experiments directed toward the characterization of the receptor have tended to discount the identity of enzyme and receptor. A few inhibitors were found to have drastically different affinities for the receptor and for partially purified enzyme in solution (Webb, 1965). Such evidence alone is insufficient to distinguish between the two species, however, because solubilization of the enzyme from the membrane may alter its conformation and its affinity for certain ligands. Similar conformation-dependent reactivities have been found with dehydrogenases (Kirschner *et al.*, 1971; MacQuarrie and Bernhard, 1971; Kirschner *et al.*, 1966) and with aspartate transcarbamylase (Changeux *et al.*, 1968). Other experiments have clearly distinguished receptor activity from enzyme *catalytic activity* in the intact cell and in membrane fragments. Irreversible enzyme acylating agents which presumably block the esteratic site have no effect on receptor activity (Podleski, 1967); sulfhydryl reducing and alkylation agents block receptor activity but have no affect on enzyme activity (Karlin, 1967; Karlin and Winnik, 1968); and diazonium compounds irreversibly inactivate both receptor and enzyme but at different rates (Kasai and Changeux, 1971). Furthermore, recent experiments with snake neurotoxins, which bind with apparently high specificity to the receptor both *in vivo* and in tissue homogenates (Changeux *et al.*, 1970) have demonstrated that a component interacting with neurotoxins may be both chromatographed distinct from enzyme activity (Miledi *et al.*, 1971; Meunier *et al.*, 1971b) and physically separated from enzyme activity to about 80% efficiency (Meunier *et al.*, 1971a).

The two species, enzyme and receptor, show several similarities in their interactions with classes of ligands. Despite this apparent functional homology, the species appear to be separate and distinct proteins.

Appendix

To solve the rate equations arising from the modifier-site model in Scheme I it is assumed both that the acylation step k_2 and the deacylation step k_3 are effectively irreversible and that all reversibly linked enzyme species are virtually at their equilibrium concentrations with respect to one another. It is further assumed that experimental conditions are such that the enzyme concentration is several orders of magnitude smaller than the concentrations of either substrates or inhibitors (which remain effectively constant throughout the period of measurement of product formation). Under these conditions all the rate equations may be summed to give the cumulative rate expression shown in eq 1A.

$$E \times L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E\text{-}R \times N \qquad \begin{matrix} X^- \uparrow \\ ROH + H^+ \downarrow \end{matrix} \qquad (1A)$$

[7] The other two inhibitors in group B are the hydroxyquinolinium compounds which at pH 7.0 act as receptor inhibitors. However, a significant pH dependence of the effect was observed which indicated that the major contribution to the receptor inhibition arose from an extremely pronounced inhibition by the protonated form of the hydroxyquinolinium ion (Podleski and Nachmansohn, 1966). The receptor activity of the zwitterionic forms of the ions, the predominate forms at pH 7.0, was thus obscured. The enzyme inhibition by these compounds showed little variation over the same pH range.

$$k_{12} = \frac{k_2 M_0[RX]}{K_S L} \quad (2A)$$

$$k_{21} = \frac{k_3 N_0}{N} \quad (3A)$$

The physical interpretation of the partitions L and N has been described earlier (Rosenberry and Bernhard, 1971). The total concentration of enzyme active sites $[E_0] = L[E] + N[ER]$. These partitions for Scheme I are given in eq 4A–6A.

$$L = M^O + \frac{[RX]M^S}{K_S} + \frac{[I]M^I}{K_I} \quad (4A)$$

$$M^J = 1 + \frac{[RX]}{K_S{}^{m,J}} + \frac{[I]}{K_I{}^{m,J}} \quad (5A)$$

$$N = 1 + \frac{[RX]}{K_S{}^R} + \frac{[I]}{K_I{}^R} \quad (6A)$$

The rate partitions M_0 and N_0 are given in

$$M_0 = 1 + \frac{A[RX]}{K_S{}^{m,S}} + \frac{C[I]}{K_I{}^{m,S}} \quad (7A)$$

$$N_0 = 1 + \frac{a[RX]}{K_S{}^R} + \frac{c[I]}{K_I{}^R} \quad (8A)$$

When the steady state is obtained the rate expression in eq 1a is solved to give the rate of product formation v_0 as shown in

$$v_0 = \frac{[E_0]}{\frac{1}{k_{12}} + \frac{1}{k_{21}}} \quad (9A)$$

For eq 9A to conform to the experimentally observed form of eq 2 the rate partitions M_0 and N_0 are set equal to unity as described in the Methods section. The inhibition and rate constants in eq 2 are then straightforwardly defined as coefficients of the appropriate ligand concentration variables in eq 9A.

$$\frac{1}{k_{cat}} = \frac{1}{k_2}\left[1 + \frac{k_2}{k_3} + \frac{K_S}{K_S{}^{m,O}}\right] \quad (10A)$$

$$K_{app} = \frac{k_{cat}K_S}{k_2} \quad (11A)$$

$$\frac{1}{K_{comp}} = \frac{1}{K_I} + \frac{1}{K_I{}^{m,O}} \quad (12A)$$

$$\frac{1}{K_{uncomp}{}^S} = \frac{k_{cat}}{k_2 K_S{}^{m,S}} + \frac{k_{cat}}{k_3 K_S{}^R} \quad (13A)$$

The corresponding equation for $K_{uncomp}{}^I$ is given in eq 3.

If two acetic acid esters are being hydrolyzed simultaneously and the hydrolysis of one, RX, can be observed independently of that of the other, RY, then the forward rate in the cumulative rate expression (eq 1A) is the sum of the k_{12}'s for each ester substrate. It follows that the steady-state rate of X formation is given by eq 14A, where the partitions are expanded beyond the expressions in eq 4A–8A to include terms for the second substrate RY. If it is assumed both that the rate partitions $M_0{}^{RX} = M_0{}^{RY} = N_0 = 1$ as suggested above from the data on individual substrate hydrolysis and that each substrate concentration is kept far below the corresponding value of $K_{uncomp}{}^S$ for that substrate, eq 14A may be written as shown in eq 15A for a system containing the two substrates plus an inhibitor I.

$$\left[\frac{d[X]}{dt}\right]^{-1} = \frac{1}{k_2[E_0]}\left[\frac{N}{N_0}\left(\frac{k_2{}^{RX}}{k_3} + \frac{k_2{}^{RY}K_{RX}[RY]M_0{}^{RY}}{k_3 K_{RY}[RX]M_0{}^{RX}}\right) + \frac{K_{RX}L}{[RX]M_0{}^{RX}}\right] \quad (14A)$$

$$\left[\frac{d[X]}{dt}\right]^{-1} = \frac{1}{k_{cat}{}^{RX}[E_0]}\left[1 + \frac{[I]}{K_{uncomp}{}^I} + \frac{[RY]}{K_G} + \frac{K_{app}{}^{RX}}{[RX]}\left(1 + \frac{[I]}{K_{comp}{}^I} + \frac{[RY]}{K_{app}{}^{RY}}\left[1 + \frac{[I]}{K_{uncomp}{}^{I'}}\right]\right)\right] \quad (15A)$$

where $K_{uncomp}{}^{I'}$ is the constant obtained for uncompetitive inhibition of RY hydrolysis by I. All the parameters in eq 15A are defined identically with those for the hydrolysis of each substrate individually (eq 4A–8A) except for K_G, which is an extension of an uncompetitive inhibition constant that includes a term for the binding of RX to acyl-enzyme formed from RY (eq 16A).

$$\frac{1}{K_G} = \frac{k_{cat}{}^{RX}}{k_2{}^{RX}K_{RY}{}^{m,RX}} + \frac{K_{app}{}^{RX}}{K_{RY}K_{RX}{}^{m,RY}} + \frac{k_{cat}{}^{RX}}{k_3 K_{RY}{}^R} + \frac{k_{cat}{}^{RY}K_{app}{}^{RY}}{k_3 K_{app}{}^{RY}K_{RX}{}^R} \quad (16A)$$

The possibility that certain uncompetitive inhibition data arise solely from inhibitor binding to the acyl-enzyme is investigated in the Discussion section. If this condition obtains the expression above for $K_{uncomp}{}^I$, $K_{uncomp}{}^S$, and K_G are simplified by setting all $1/K_J{}^m$ equal to zero. The relationship in eq 5 follows trivially from this simplification if the last term in the expression for $1/K_G$ (eq 16A) is negligible. This term is negligible for the particular case posed above eq 5, since $k_{cat}{}^{RX}K_{app}{}^{RY}/k_{cat}{}^{RY}K_{app}{}^{RX} \approx 1$ from the values in Tables IV and VI and the term then is less than or equal to $1/K_{uncomp}{}^{S=RY}$.

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