a useful enzyme for the detection or selective destruction of D-amino acids. Thus, although L-proline is oxidized at a significant rate, it was still possible to detect one part of D-proline in 15,000 parts of L-proline (Scannone *et al.*, 1964).

In recent years a number of other purified enzymes have been found to catalyze reactions at a rate which is several orders of magnitude lower than that of the reactions which they were previously known to catalyze. These include the formation of 2-pyrrolidone-5 carboxylic acid by glutamine synthetase (Krishnaswamy *et al.*, 1962), a number of transamination reactions catalyzed by aspartate β-decarboxylase (Novogrodsky and Meister, 1964a) and by glutamate aspartate transaminase (Novogrodsky and Meister, 1964b), the hydrolysis of acetylglycine ethyl ester by trypsin (Inagami and Murachi, 1963), the oxidation of α-aminovaleric acid and other amino acids by glutamic dehydrogenase (Bässler and Hammar, 1958; Struck and Sizer, 1960), and the hydrolysis of polyadenylic acid by pancreatic ribonuclease (Beers, 1960). These examples, as well as the present findings, indicate the necessity for caution in assigning "absolute specificity" to an enzyme. Although these "minor" activities may be of little physiological importance, they are significant in the understanding of the mechanism of the enzymatic reaction.

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Acetylcholinesterase: Trimethylammonium-Ion Inhibition of Deacetylation

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The noncompetitive inhibition of acetylcholinesterase by trimethylammonium ion is shown to result from binding of an inhibitor molecule to the acetyl enzyme intermediate, which is formed from the enzyme-substrate complex. The evidence for this is independent of any initial assumption that an acetyl enzyme is formed, and comes from studies of the inhibition of hydrolysis of a number of acetylcholine analogs, all acetyl esters, some hydrolyzed as rapidly as acetylcholine and others more slowly. The observed direct proportionality between the experimental noncompetitive inhibition constant and the maximum velocity indicates that the inhibitor adds to a common intermediate in the hydrolysis of these substrates. The only intermediate likely to be formed during the enzymic hydrolysis of all the acetyl esters is an acetyl enzyme. Other evidence shows that trimethylammonium ion can completely block deacetylation of the acetyl enzyme, and that the inhibition is reversible. The inhibitor is bound at the anionic site in the active center, as are other substituted ammonium ions. The experiments also suggest that deacetylation limits the rate of hydrolysis of acetylcholine, acetylthiocholine, and phenyl acetate.

Characteristic substrates of acetylcholinesterase (AChE)[1] contain a methyl-substituted ammonium ion, which probably becomes bound at the anionic site in the active center of the enzyme. Substituted ammonium ions should therefore compete with substrates for the enzyme and should inhibit in a purely competitive manner. Surprisingly, some compounds of this type

[1] Abbreviations used in this work: AChE, acetylcholinesterase; AcCh, acetylcholine; ES, enzyme-substrate complex; EA, acetyl enzyme.

are noncompetitive inhibitors. Examples are *cis*-2-dimethylaminocyclohexanol, investigated before (Krupka and Laidler, 1961a), and trimethylammonium ion, which has now been chosen for study because of the unusually large noncompetitive component in its inhibition (Wilson and Alexander, 1962). The present approach to the problem involves the use of many acetylcholine (AcCh) analogs as substrates and leads to conclusions which are independent of prior knowledge of the enzyme-reaction mechanism. By contrast, the previous study (Krupka and Laidler, 1961a) assumed the formation of an acetyl enzyme intermediate (EA) and the rate limitation of the deacetylation step in AcCh hydrolysis, since other work had indicated that AChE acted by such a mechanism (Wilson and Cabib, 1956). The present experiments are found to provide independent support for these assumptions.

Noncompetitive kinetics should be observed if an inhibitor can become bound to both the free enzyme and the enzyme-substrate complex (ES), and for this reason noncompetition is often regarded as evidence for different combining sites for the substrate and inhibitor. However, past experiments suggested the involvement of a single negatively charged site in AChE, for this site, though covered by the substrate in ES, would be available to the inhibitor in an acetyl enzyme. The latter (EA) may be formed from ES as a result of transacylation from the ester substrate to the enzyme, and would be subsequently hydrolyzed to re-form the free enzyme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[k_2]{C} EA \xrightarrow[k_3]{H_2O} E + A \qquad (1)$$

where C represents choline, A acetic acid, and S the substrate, AcCh.

In view of the possible operation of mechanism (1), we must decide whether trimethylammonium ion becomes bound to EA, if it is indeed a reaction intermediate, to ES, or to both. A second question to be faced is whether or not the inhibitor completely blocks further reaction of the enzyme intermediate to which it is bound. There are at least two ways of answering the first question. If it is known that an acetyl-enzyme intermediate is formed, the inhibition may be studied with two different substrates whose rate-limiting steps are acetylation (k_2) in one case and deacetylation (k_3) in the other. The formation of a complex between an inhibitor molecule and an enzyme intermediate can affect the kinetic behavior of a system only if the reaction of this particular intermediate limits the overall rate of substrate hydrolysis. Thus noncompetitive behavior is a sign that the inhibitor interferes with the reaction of a rate-limiting enzyme-substrate intermediate. Noncompetitive inhibition observed with a substrate for which deacetylation (k_3) is rate limiting, but not with a substrate for which acetylation (k_2) is rate limiting, would therefore indicate that the inhibitor becomes bound to the acetyl enzyme and blocks deacetylation.

Unfortunately, such experiments will be inconclusive unless there is definitive evidence for acetyl enzyme formation with both substrates and for the rate limitation of either acetylation or deacetylation. To avoid this difficulty, another approach may be made which does not depend on such prior knowledge. If an acyl enzyme intermediate is formed during the hydrolysis of all esters, then the same intermediate, an acetyl enzyme, should be formed with all acetyl esters. In this case there is a linear relationship between the maximum velocity, V, at which a substrate (an acetyl ester) is hydrolyzed and the steady-state concentration of EA, the reason being that V and [EA] depend in a similar way upon k_2. ($(V = k_2[E_0])/(1 + k_2/k_3)$ and $[EA] = k_2[E_0]/k_3(1 + k_2/k_3)$ at saturating substrate levels; k_3, the deacetylation constant, is the same for all acetyl esters.) If inhibitors combine with EA, but not with ES, the experimental noncompetitive inhibition constant (K_i' expt), measured at saturating substrate concentrations as usual, should be proportional to the concentration of EA, which in turn is proportional to V. Hence we predict that

$$K_i' \text{ (expt)} = \text{constant} \times V$$

This relationship will be derived more rigorously below. If an acyl enzyme intermediate is not universally formed, or if the inhibitor combines with other reaction intermediates such as ES, we would not expect to find a direct proportionality between K_i' (expt) and V.

The second question is to what degree trimethylammonium ion blocks further reaction of the intermediate to which it becomes bound. As shown before (Krupka, 1963), the answer may be obtained from the effect of the inhibitor on the optimum velocity (v_{opt}), the highest measurable reaction rate, which, because of substrate inhibition, is slightly lower than the extrapolated maximum velocity, V. In the presence of the inhibitor the optimum velocity declines, and if blocking is complete the relationship between the reciprocal of the optimum velocity and the inhibitor concentration is linear, but otherwise reaches a limiting upper value. The latter value of $1/v_{opt}$ is the reciprocal of the rate of reaction of the intermediate to which the inhibitor is bound, and this is the value we wish to determine. A second, and related, approach depends on the relationship between the inhibitor concentration and the maximum velocity (V) in the presence of an inhibitor. Again, a limiting lower value of the rate indicates that the complex between the reaction intermediate and the inhibitor is partially reactive.

Methods

The measurements of initial rates were carried out at 26° in a manner described before (Krupka, 1963). The purified enzyme from bovine erythrocytes was obtained from Nutritional Biochemicals Corp. 2-Methylaminoethyl acetate hydrochloride (mp 105°) was synthesized according to the procedure of Wilson (1954). Phenyl acetate and 2-dimethylaminoethyl acetate (Eastman, White Label) were redistilled before use. Trimethylamine hydrochloride and recrystallized AcCh bromide (mp 143°) were products of Eastman Organic Chemicals, and reagent-grade acetylthiocholine iodide was supplied by Light & Co., Colnbrook, England. The other AcCh analogs were synthesized by Dr. W. C. Dauterman (Mehrotra and Dauterman, 1963).

All reaction mixtures contained 0.1 M NaCl and 0.04 M $MgCl_2$. They were flushed briefly with CO_2-free air before initiation of the reaction by the addition of 1 ml of enzyme solution to 9 ml of a mixture containing the other components of the system. During the course of the reactions a stream of CO_2-free air was usually passed over the surface of the solution, a procedure which led to small losses of trimethylamine, with liberation of hydrogen ion in the solution. In such cases appropriate corrections were applied to the observed rates. In later experiments corrections were unnecessary, as the reaction vessel was closed off from the surrounding atmosphere after the initial flushing with CO_2-free air and addition of enzyme. This procedure was followed in experiments with phenyl acetate, which is volatile.

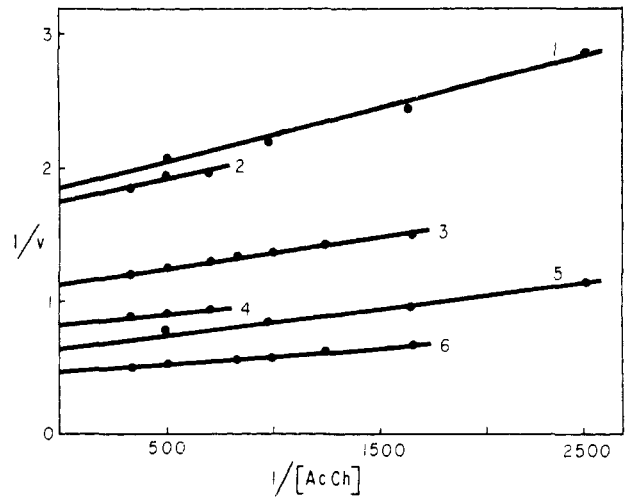


FIG. 1.—Inhibition of AcCh hydrolysis by trimethylammonium chloride. Lines 2, 3, 4, and 6 represent rates at *p*H 7.5 with inhibitor concentrations of 1.008×10^{-2} M, 5.04×10^{-3} M, 2.52×10^{-3} M, and zero, respectively. Lines 1 and 5 show experimental results at *p*H 6.5; in 1, the inhibitor concentration is 1.01×10^{-2} M, and in 5 it is zero.

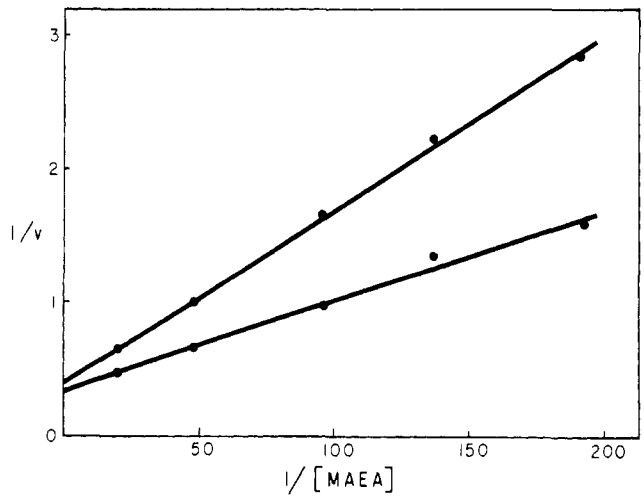


FIG. 2.—Inhibition of 2-methylaminoethyl acetate hydrolysis by trimethylammonium chloride, *p*H 6.5. The lower line shows the uninhibited rates, and the upper line the rates in the presence of 1.01×10^{-2} M inhibitor.

In experiments with substrates which are hydrolyzed slowly, the enzyme concentration was adjusted to give convenient reaction rates. The enzyme stock solutions were calibrated by determining the rates at which they hydrolyzed AcCh. Experimental rate measurements were analyzed by the least-mean-squares method.

Experimental Results

AChE-catalyzed Hydrolysis of AcCh, Acetylthiocholine, and Phenyl Acetate.—The rates of acetylthiocholine and AcCh hydrolysis were measured by titration with 0.01 N NaOH over a range of substrate concentrations, and their maximum velocities in $1/v \times 1/[S]$ plots were found to be in the ratio 1.11. However, thiocholine is partially ionized at *p*H 7.5 (Heilbronn, 1958b). A correction was determined by titrating the acid released during the rapid and complete hydrolysis of small quantities of the two substrates, the reaction mixture being the same as in the other experiments. The acid released in the hydrolysis of 1 mole of acetylthiocholine relative to 1 mole of AcCh was found to be in the ratio of 1.34, corresponding to a *pK* value for thiocholine of 7.8, in good agreement with the value given by Heilbronn. The maximum velocity of acetylthiocholine hydrolysis, relative to acetylcholine hydrolysis, is then 0.83.

A stock solution of phenyl acetate (0.5 M) was made up in methanol immediately before use, and 0.02–0.10 ml of this solution was included in a total of 10 ml of reaction mixture. In all runs the reaction solution contained a total of 0.10 ml of methanol and phenyl acetate–methanol solution, as well as 0.1 M NaCl and 0.04 M $MgCl_2$. The presence of methanol was found to reduce the maximum rate of AcCh hydrolysis by approximately 2%. The maximum velocities for phenyl acetate and AcCh were in the ratio 1.13.

Trimethylammonium-Ion Inhibition of Acetyl Ester Hydrolysis.—Eleven different substrates of AChE were examined, usually at *p*H 7.5. Nonenzymic hydrolysis of *N*-methylaminoethyl acetate and *N*-dimethylaminoethyl acetate is rapid above *p*H 7, and accordingly these esters were studied at *p*H 6.5. AcCh hydrolysis was followed at both *p*H 6.5 and 7.5. Plots ($1/v \times 1/[S]$) for trimethylammonium-ion inhibition of AcCh and 2-methylaminoethyl acetate hydrolysis are shown in Figures 1 and 2. From such plots for the hydrolysis of the other acetyl esters, V, K_m, and K_i' (expt) (see equation 7) were calculated and are listed in Table I.

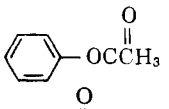
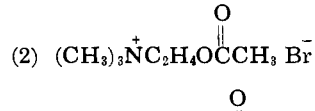
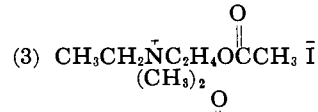
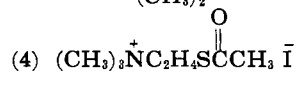
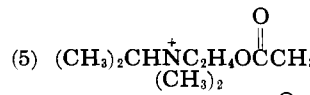
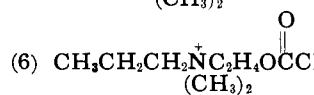
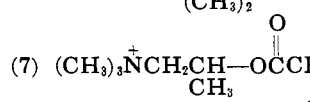
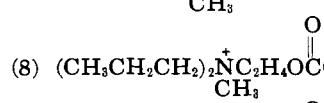
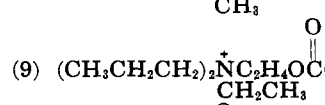
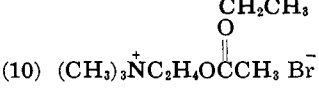
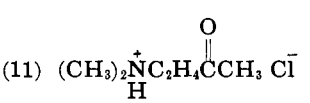
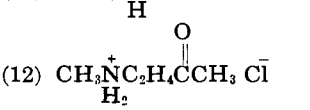
Reversibility of Trimethylammonium-Ion Inhibition.—The observation of widely different degrees of noncompetitive inhibition with different substrates (see Figs. 1 and 2, and Table I) makes it most improbable that trimethylammonium ion causes irreversible enzyme inactivation. As a further test of this possibility a solution containing the enzyme and inhibitor, together with salt and water (total 9 ml), was allowed to stand at 26°, *p*H 7.5, for 75 minutes before the addition of AcCh, after which the rate of hydrolysis was measured. The rate was also determined when the reaction was started by adding 1 ml of enzyme solution to a mixture containing the substrate, inhibitor, and salt. The two rates were the same within experimental error, showing that no progressive inactivation of the enzyme took place.

Effect of Trimethylammonium Ion on the Optimum Velocity.—The rate of AcCh hydrolysis was measured over a range of substrate concentrations either in the absence of inhibitor or in the presence of fixed concentrations of trimethylammonium ion between 5×10^{-4} and 10^{-2} M, and in this way the highest velocity obtained at a given inhibitor concentration (v_{opt}) was determined. Figure 3 shows a plot of $1/v_{opt}$ against the concentration of the inhibitor.

Effect of pH on Trimethylammonium-Ion Inhibition.—Bergmann and Shimoni (1952) concluded that the negative charge in the anionic site of AChE is neutralized at about *p*H 6, as shown by the declining effectiveness of quaternary-nitrogen inhibitors at low *p*H. In view of the marked differences in the inhibitions caused by trimethylammonium and tetramethylammonium ions (Krupka, 1963), it was of interest to compare the effects of *p*H on these substances. Figure 4 shows a plot of $1 - v/v_0$ against *p*H, v and v_0 being the rates at a fixed AcCh concentration in the presence and absence of the inhibitor, respectively. At low pH the inhibitions decline in an almost identical manner, suggesting that both inhibitors are bound at the same negatively charged site.

The Limiting Rate of AcCh Hydrolysis in the Presence of High AcCh Concentration.—The rate of AcCh hydrolysis was measured in the presence and absence of enzyme (*p*H 7.5, 26°) at AcCh concentrations as high as 0.5 M. From rate measurements at lower AcCh concentrations, V was calculated. The ratio of the rate

TABLE I
VALUES OF K_i' (expt), RELATIVE V, AND K_m FOR ACETYL ESTER SUBSTRATES OF AChE

No.	Substrate	pH	Concentration of Trimethylammonium Chloride (M)	1/v Intercept Relative to AcCh Value	K_i' (expt)	Relative V	K_m
(1)		7.5	0 3.67×10^{-3}	0.918 ± 0.018 1.876 ± 0.038	285	1.13	1.31×10^{-3}
(2)		7.5	0 5.04×10^{-3}	1.000 ± 0.023 2.395 ± 0.026	277	1.00	2.68×10^{-4}
(3)		7.5	0 5.23×10^{-3}	1.081 ± 0.017 2.381 ± 0.056	227	0.95	2.61×10^{-4}
(4)		7.5	0 5.04×10^{-3}	1.210 ± 0.024 2.441 ± 0.063	202	0.83	1.31×10^{-4}
(5)		7.5	0 5.23×10^{-3}	1.360 ± 0.039 2.730 ± 0.101	192	0.75	2.92×10^{-4}
(6)		7.5	0 5.23×10^{-3}	1.630 ± 0.028 3.055 ± 0.077	167	0.63	5.86×10^{-4}
(7)		7.5	0 1.317×10^{-2}	4.505 ± 0.219 8.080 ± 0.423	58	0.22	2.22×10^{-3}
(8)		7.5	0 1.317×10^{-2}	12.82 ± 0.26 16.12 ± 0.46	20	0.081	1.01×10^{-3}
(9)		7.5	0 2.092×10^{-2}	18.87 ± 0.37 25.32 ± 1.24	16	0.054	5.55×10^{-4}
(10)		6.5	0 1.007×10^{-2}	1.333 ± 0.041 3.550 ± 0.078	166	0.75	3.23×10^{-4}
(11)		6.5	0 1.043×10^{-2}	2.850 ± 0.240 5.260 ± 0.144	81	0.36	2.16×10^{-3}
(12)		6.5	0 1.008×10^{-2}	10.20 ± 0.42 12.80 ± 1.47	25	0.098	1.69×10^{-2}

at 0.5 M to V was slightly less than 0.1, in agreement with the work of Myers (1952) on human erythrocyte AChE.

DISCUSSION

The maximum velocities of AcCh, acetylthiocholine, and phenyl acetate hydrolysis are seen to be similar. By contrast, the rates of hydrolysis of these esters by OH, H^+, or imidazole vary widely (Heilbronn, 1958a,b; Bruice and Schmir, 1957). The simplest interpretation of these facts is that V is limited largely by the same reaction (deacetylation) with all three substrates. The low maximum velocity with 2-methylaminoethyl acetate indicates that deacetylation cannot be rate limiting here and that acetylation probably is. Since trimethylammonium-ion inhibition with the first three substrates is strongly noncompetitive, but is largely competitive with 2-methylaminoethyl acetate, it is likely that the inhibitor combines with the acetyl enzyme but not with ES. This conclusion was also reached for the inhibitor *cis*-2-dimethylaminocyclohexanol on the basis of similar evidence (Krupka and Laidler, 1961a).

Stronger support for this mechanism, as we have noted, can be obtained if the inhibition is studied with a number of substrates and if a direct proportionality can be shown to exist between the noncompetitive inhibition constant and the maximum velocity. This relationship may be derived as follows. If the inhibitor can form a complex with the free and the acetyl enzymes:

$$E + I \overset{K_i}{\rightleftharpoons} EI \tag{2}$$

$$EA + I \overset{K_i'}{\rightleftharpoons} EAI \tag{3}$$

and if the overall reaction scheme is written as in equation (1), the rate of hydrolysis in the steady state is

$$v = \frac{k_2[E_0]\bar{K}[S]}{1 + K_i[I] + \bar{K}[S]\{1 + (k_2/k_3)(1 + K_i'[I])\}} \tag{4}$$

where $\bar{K} = k_1/(k_{-1} + k_2)$ and where K_i and K_i' are association constants. In the presence of the inhibitor, the maximum velocity is

$$V = \frac{k_2[E_0]}{1 + (k_2/k_3)(1 + K_i'[I])} \tag{5}$$

and in the absence of the inhibitor it is

$$V_0 = \frac{k_2[E_0]}{1 + (k_2/k_3)} \tag{6}$$

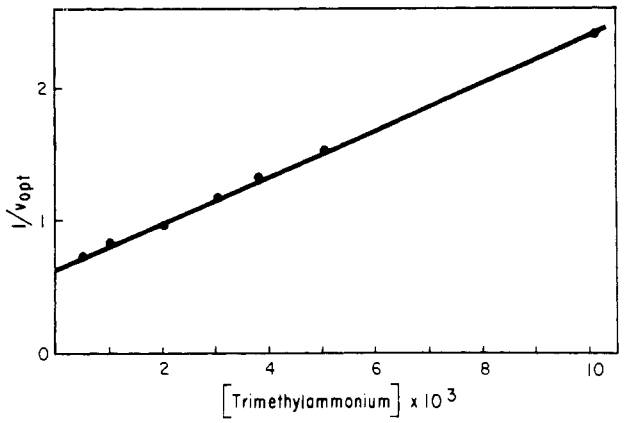


FIG. 3.—The effect of trimethylammonium ion on the optimum velocity (v_{opt}).

The experimental value of the noncompetitive binding constant for the inhibitor is obtained according to the following relations:

$$K_i' \text{ (expt)} = \frac{V_0(1/V - 1/V_0)}{[I]} = k_2K_i'/k_3(1 + k_2/k_3) = K_i'/(1 + k_3/k_2) \quad (7)$$

By comparing (6) and (7) it is seen that V_0 and K_i' (expt) depend in the same way upon the size of k_2, so that

$$K_i' \text{ (expt)} = \frac{K_i'V_0}{k_3[E]_0} \quad (8)$$

The deacetylation constant, k_3, should be the same for all acetyl esters, and equation (8) therefore predicts a linear relationship between K_i' (expt) and V_0. Precisely this relation is found experimentally (Fig. 5). The relation appears to be slightly different at *p*H 6.5 and 7.5. The close agreement between the predicted and experimental values for acetyl-DL-β-methylcholine is probably fortuitous, since only the D- isomer is hydrolyzed by AChE, while the L- isomer is a weak inhibitor (Hoskin and Trick, 1955; Hoskin, 1963).

As the data conform to equation (8), it follows that the ability of the inhibitor to block the reaction of either ES or an intermediate formed therefrom is directly dependent upon the rate of some common reaction step in acetyl ester hydrolysis. A common reaction implies the existence of a common intermediate, and it is therefore quite likely that the latter binds the inhibitor. The only possible common intermediate would appear to be an acetyl enzyme.

There are other possible mechanisms leading to noncompetitive inhibition, which involve the addition of the inhibitor to ES:

$$ES + I \overset{K_i''}{\rightleftharpoons} ESI \quad (9)$$

ES may break down directly to form the products of the reaction and the free enzyme,

$$ES \xrightarrow{k_2} E + P \quad (10)$$

or it may form an acetyl enzyme as in (1). The acetyl enzyme may or may not combine with the inhibitor. In the first case, where ES forms products directly (9 and 10), it can be shown that

$$K_i' \text{ (expt)} = V_0(1/V - 1/V_0)/[I] = K_i' \quad (11)$$

In (11), K_i' (expt) is independent of V_0. To explain the experiments on the basis of this scheme, we must make the unlikely assumption than an increase in the reactivity of ES (k_2) is accompanied by a proportionate increase in its ability to bind the inhibitor, even though

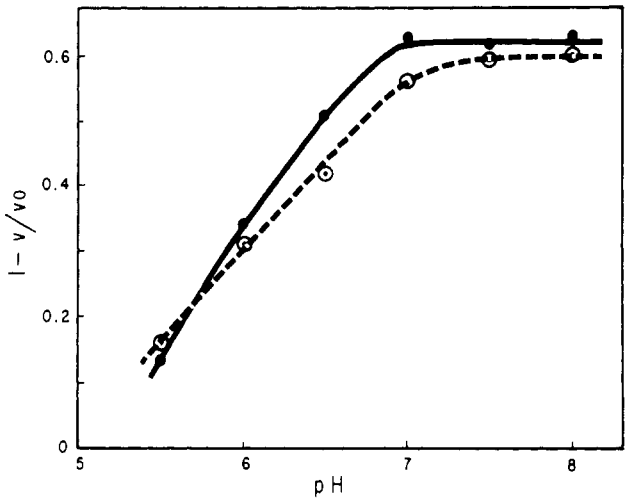


FIG. 4.—The effect of *p*H on the inhibitions caused by 1.03×10^{-2} M trimethylammonium chloride (dashed line) and 1.76×10^{-2} M tetramethylammonium chloride (solid line), at an AcCh concentration of 1.02×10^{-3} M. The expression $1 - v/v_0$ represents the degree of inhibition, which is seen to decline at low *p*H.

there is no parallel between V_0 or the binding of the inhibitor and the binding of the substrate (Table I). Such an interpretation is not warranted by our knowledge of the enzyme, and may be discounted in favor of the much simpler explanation involving addition of the inhibitor to the acetyl enzyme.

In the second case, where an acetyl enzyme is formed, and both EA and ES can combine with the inhibitor (1, 2, 3, and 9), the rate is given by

$$v = \frac{k_2[E]_0\bar{K}[S]}{1 + K_i[I] + \bar{K}[S]\{1 + k_2/k_3 + [I](K_i'' + k_2K_i'/k_3)\}} \quad (12)$$

$$\text{and } K_i' \text{ (expt)} = V_0(1/V - 1/V_0)/[I] = (K_i'' + k_2K_i'/k_3)/(1 + k_2/k_3) \quad (13)$$

Besides the situation where $K_i'' = 0$, which was considered earlier (4 and 7), two other distinct possibilities exist. In one, $K_i'' = K_i'$, and accordingly

$$K_i' \text{ (expt)} = K_i' \quad (14)$$

From (14) it is clear that K_i' (expt) should be independent of the rate at which the substrate is hydrolyzed. In the second situation, $K_i' = 0$ and

$$K_i' \text{ (expt)} = K_i''/(1 + k_2/k_3) \quad (15)$$

According to this, K_i' (expt) should become large when V_0 (and k_2) are small, and small when V_0 is large, which is the reverse of the experimental relationships. Obviously none of the mechanisms involving the addition of I to ES can account for the observed behavior.

We now wish to determine the effectiveness of trimethylammonium ion in blocking deacetylation, and this can be decided from its effect on the optimum velocity (v_{opt}) (Krupka, 1963). From the experiment reported above on substrate inhibition, we know that the binding of AcCh to some reaction intermediate reduces the rate to less than 0.1 of the maximum, so that for present purposes any reactivity of the substrate-inhibited intermediate may be neglected. Previously (Krupka and Laidler, 1961b) it was shown that if the substrate and inhibitor combine with EA, EAS being unreactive, the relationship between $1/v_{opt}$ and [I] is linear, provided that EAI does not react to form products and the free enzyme. If EAI is reactive,

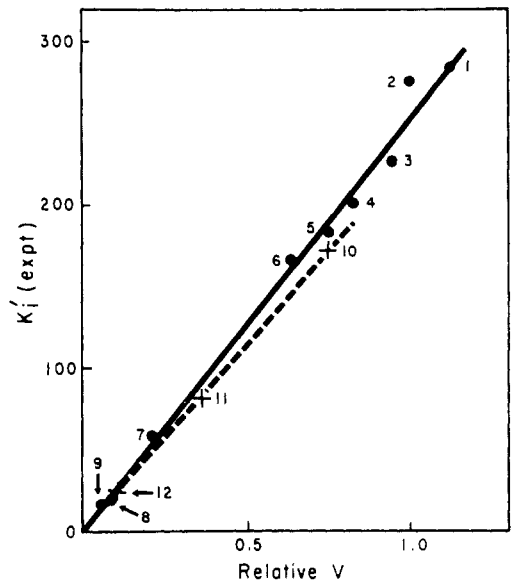


FIG. 5.—The relative maximum velocity, V, plotted against the experimental noncompetitive-inhibition constant for trimethylammonium chloride. The numbers in the figure refer to the acetyl ester substrates listed in Table I.

$1/v_{opt}$ reaches an upper limit as the inhibitor concentration rises. The effect of I on $1/v_{opt}$ does not depend upon the choice of this particular model for substrate inhibition, however. If S combines with ES and I with EA, or if both S and I combine with ES, the relations still hold. This is understandable because the rate at high substrate concentrations cannot be reduced below that at which EAI or ESI reacts to form products, and this residual activity is reflected in the upper limit $1/v_{opt}$, i.e., in a lower limit for v_{opt}. On the other hand, if EAI or ESI is completely unreactive, v_{opt} drops to zero at sufficiently high inhibitor concentrations. The experimental relationship between $1/v_{opt}$ and the concentration of trimethylammonium ion, shown in Figure 3, does not deviate significantly from linearity, indicating that EAI is unreactive.

Additional support for this conclusion is obtained from the data in Figure 1, and depends on the effect of the inhibitor upon V. The rationale is similar to that for the effect on v_{opt}. For inhibition by a substance which combines with E and EA (equations 2 and 3), where EAI can react to form products,

$$\mathrm{EAI} + \mathrm{H_2O} \xrightarrow{ak_3} \mathrm{E} + \mathrm{A} + \mathrm{I} \qquad (16)$$

and where hydrolysis of the substrate proceeds by the mechanism in (1), the rate is given by

$$v = \frac{k_2[\mathrm{E}_0]\bar{K}[\mathrm{S}]}{1 + K_i[\mathrm{I}] + \bar{K}[\mathrm{S}]\left\{1 + k_2/k_3\left(\frac{1 + K_i'[\mathrm{I}]}{1 + K_i[\mathrm{I}]}\right)\right\}} \qquad (17)$$

From this it can be shown that

$$1/(1/V - 1/V_0) = k_3[\mathrm{E}_0]\left\{\frac{a}{1-a} + \frac{1}{(1-a)K_i'[\mathrm{I}]}\right\} \qquad (18)$$

where V and V_0 are the maximum velocities in the presence and absence of the inhibitor. The values of $1/V$ in Figure (1) (the intercepts, pH 7.5) were utilized in a plot according to equation (18). The intercept was found to be zero, again indicating that EAI does not react. Previously it was shown that *cis*-2-dimethylaminocyclohexanol is also highly effective in blocking deacetylation, but that tetramethylammonium and choline ions are not (Krupka, 1963). Other experiments supporting these conclusions will be reported later, when the structural requirements for inhibition of deacetylation will be explored.

CONCLUSIONS

The experiments described here strongly support the following ideas: (1) There is a common step in the enzymic hydrolysis of all the acetyl esters studied. Trimethylammonium ion adds on to the common intermediate involved in this step, thereby causing noncompetitive inhibition. The common step is probably the deacetylation of an acetyl enzyme. (2) Deacetylation largely limits the rate of hydrolysis of AcCh, acetylthiocholine, and phenyl acetate. (3) Trimethylammonium ion forms an inactive complex with the free and acetyl enzymes (E and EA), but not with the enzyme-substrate complex (ES). (4) The inhibition does not involve irreversible enzyme inactivation. (5) Trimethylammonium and tetramethylammonium ions become less effective inhibitors as the pH is lowered, because both are bound at the anionic site, whose negative charge is neutralized below pH 6. In spite of their attachment to the same enzyme site, these ions cause markedly different inhibitions, which can be correlated with their effects on deacetylation: the latter reaction is blocked by the tertiary amine, which therefore inhibits noncompetitively, but is only partly blocked by the quaternary amine, which, accordingly, is a largely competitive inhibitor.

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