in the derivations that the substrate and the alternative substrate follow the same reaction pathway.

The employment of isotope competition for the purposes described above appear to increase the usefulness of the equations which predict the kinetic effects of alternative substrates. This would be particularly true in situations where good alternatives substrates are not available.

Experiments with radioactive and nonlabeled substrates appear to have another great virtue. Because the labeled and nonlabeled substrate must react at the same site on the enzyme, *i.e.*, they are competive, it is only necessary to do two kinetic experiments to make a choice of mechanisms from among those cited above. These experiments are of the type shown in Figures 2, 4, and 6 in which the varied substrate is the one that is not competitively inhibited.

References

Alberty, R. A. (1953), *J. Amer. Chem. Soc. 75*, 1928.
Alberty, R. A. (1958), *J. Amer. Chem. Soc. 80*, 1777.
Boyer, P. D. (1959), *Arch. Biochem. Biophys. 82*, 387.
Boyer, P. D., and Silverstein, E. (1963), *Acta Chem. Scand. 17, Suppl. 1*, S 195.
Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67*, 173.
Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67*, 104.
Crane, R. K. (1962), *Enzymes 6*, 58.
Dalziel, K. (1957), *Acta Chem. Scand. 11*, 1706.
Dalziel, K., and Dickinson, F. M. (1966), *Biochem. J. 100*, 34.
Fromm, H. J. (1964), *Biochim. Biophys. Acta 81*, 413.
Fromm, H. J. (1967), *Biochim. Biophys. Acta 139*, 221.
Fromm, H. J., and Nelson, D. R. (1962), *J. Biol. Chem. 237*, 215.
Fromm, H. J., and Zewe, V. (1962), *J. Biol. Chem. 237*, 3027.
Hammes, G. G., and Kochavi, D. (1962a), *J. Amer. Chem. Soc. 84*, 2069.
Hammes, G. G., and Kochavi, D. (1962b), *J. Amer. Chem. Soc. 84*, 2073.
Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.
Noat, G., Ricard, J., Borel, M., and Got, C. (1968), *Eur. J. Biochem. 5*, 55.
P-L Circular OR-10 (1961), P-L Biochemicals, Milwaukee, Wis.
Rudolph, F. B., and Fromm, H. J. (1969), *J. Biol. Chem. 244*, 3832.
Sund, H., and Theorell, H. (1963), *Enzymes 7*, 42.
Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127.
Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2*, 935.
Wratten, C. C., and Cleland, W. W. (1965), *Biochemistry 4*, 2442.
Zewe, V., Fromm, H. J., and Fabiano, R. (1964), *J. Biol. Chem. 239*, 1625.

Kinetic Studies on the Mechanism of Insect Acetylcholinesterase*

K. Hellenbrand† and R. M. Krupka‡

ABSTRACT: A purified preparation of acetylcholinesterase from the heads of houseflies was shown to contain a single active enzyme. Studies on transfer of the substrate acetyl group to water or methanol, as well as other evidence, indicated that an acetyl–enzyme intermediate is involved in the hydrolysis mechanism. Formation of this intermediate is probably the rate-limiting step in substrate hydrolysis. Similar maximum velocities for certain substrates having different leaving groups may depend on a common rate-limiting protein conformational change rather than slow reaction of a common intermediate. Substrate inhibition and noncompetitive inhibition were observed with all quaternary ammonium substrates and inhibitors tested and were due to interference with formation of acetyl–enzyme. The enzyme was shown to contain two cation binding sites, one of which attracts the substrate and the other an inhibitor. Binding at the second of these sites causes substrate inhibition and noncompetitive inhibition.

In vertebrate acetylcholinesterase, by contrast, a single anionic site is involved here, and these inhibitions are due to interference with breakdown rather than formation of acetyl–enzyme.

As the result of investigations beginning with those of I. B. Wilson and his collaborators, the mechanism of action of acetylcholinesterase (AChE)[1] from two vertebrate sources, the electric organ of the electric eel and bovine erythrocytes, is now understood in considerable detail (Wilson, 1960; Krupa, 1966a,b). This enzyme is particularly interesting because of its involvement in nerve transmission and because of the

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[1] Abbreviations used are: AChE, acetylcholinesterase; ACh, acetylcholine; ASCh, acetylthiocholine; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; ES, enzyme–substrate complex; EA, acetyl–enzyme intermediate.

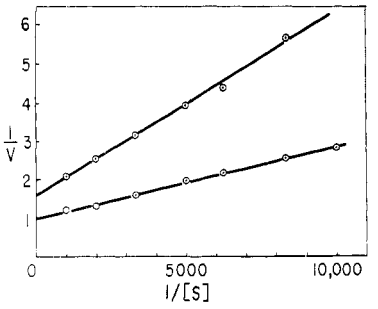


FIGURE 1: Inhibition of phenyl acetate hydrolysis by 10^{-5} M 3-hydroxyphenyltrimethylammonium ion. Upper and lower lines, with and without inhibitor, respectively.

question of its relationship to the ACh receptor site of nervous tissue. Here we wish to report a study of cholinesterase from another class of organisms, the invertebrates. Although the substrate specificities of several invertebrate enzymes have been investigated, as well as their relative inactivation rates by organophosphate and carbamate inhibitors (van Asperen, 1960; Dauterman and Mehrotra, 1963; Smissaert, 1964; Metcalf and Fukuto, 1967; Hellenbrand, 1967; O'Brien, 1966; Smissaert *et al.*, 1970) in no case does the detailed mechanism of action appear to have been established.

The source of enzyme used in this investigation was the heads of houseflies, which are rich in nervous tissue (Dauterman *et al.*, 1962). Fly-head cholinesterase has now been found to differ from the vertebrate enzyme in several notable respects. One is the existence of a second anionic site, which is involved in substrate inhibition and noncompetitive inhibition. The demonstration of this site clarifies certain puzzling observations made with the vertebrate AChE.

This paper deals with two basic questions: whether an acetyl–enzyme intermediate is involved in catalysis, and if so whether the formation or breakdown of this intermediate is normally the slower step in substrate hydrolysis. Evidence for the formation of an acetyl–enzyme intermediate was obtained from observations on the transfer of the acyl residue of an ester substrate to water, methanol, or choline. The second question was tackled by observing the effects of temperature, pH, and inhibitors on the maximum velocity of hydrolysis for several substrates. The details of the pH study will be presented in a later communication.

In preliminary experiments it was shown that a single enzyme in the purified extract catalyzes hydrolysis of the various substrates. This was done in two ways, by comparing the rates of hydrolysis of two substrates either alone in the reaction solution or together, and by measuring competitive inhibition constants with several different substrates and a single specific reversible inhibitor. The substrates used were all acetyl esters. This simplifies the analysis, for should an acyl–enzyme intermediate occur, it will be identical with every substrate. Some of the substrates were cationic, like ACh, and others uncharged; some were hydrolyzed as rapidly as ACh and some more slowly.

Materials

The enzyme was purified by a method that has already been described (Hellenbrand, 1967). Acetylcholine bromide (AcCh), acetylthiocholine iodide (ASCh), choline chloride, trimethylammonium chloride, tetramethylammonium iodide, tetraethylammonium chloride, tetrapropylammonium bromide, tetrabutylammonium iodide, and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were reagent grade and in some cases were recrystallized before use. The iodides of *N,N,N*-dimethyl-*n*-propylaminoethyl acetate, *N,N,N*-dimethyl-*n*-butylaminoethyl acetate, and *N,N,N*-diethyl-*n*-butylaminoethyl acetate were synthesized according to the method of Mehrotra and Dauterman (1963). Pure 3-hydroxyphenyltrimethylammonium iodide was a gift from S. Voerman, Wageningen, Netherlands. Reagent grade isoamyl acetate and phenyl acetate were redistilled before use. Methanol was refluxed over Zn and KOH and redistilled.

Methods

Acid released in ester hydrolysis was measured by automatic titration with 0.01 N NaOH at constant pH. A Radiometer TTT1 titrator and titrigraph equipped with Radiometer G202C glass and K100 liquid-junction calomel electrodes were employed. All reactions were carried out in the presence of 0.1 N NaCl and 0.04 M MgCl at 26° and pH 7.5 unless otherwise specified. The reaction mixture was kept under CO_2-free nitrogen to prevent CO_2 uptake. Control experiments were done both in the absence of enzyme, to allow for nonenzymic hydrolysis, and in the absence of substrate, to establish that the enzyme preparation contained no acid-forming material.

In some experiments with ASCh or phenyl acetate the formation of thiocholine or phenol was determined spectrophotometrically, and at the same time acid release was measured by titration. The procedure in these experiments was as follows. Release of acid during phenyl acetate hydrolysis was followed at constant pH in a reaction mixture with a total volume of 20 ml. Both at the beginning and end of the reaction period two samples, each 3 ml, were withdrawn and added rapidly to 0.6 ml of a solution containing sodium phosphate buffer and tetrapropylammonium bromide to stop the reaction. Similarly, in ASCh hydrolysis, which was followed titrimetrically in a total volume of 10 ml, two 1-ml samples were withdrawn at the beginning and end of the reaction period, and were added to 4 ml of a solution containing sodium phosphate buffer, tetrapropylammonium bromide, and DTNB. In both cases the final pH was 7.3 and the final solutions contained 0.01 M tetrapropylammonium bromide and 0.1 M sodium phosphate buffer. The final DTNB concentration was 1.4×10^{-4} M. The amount of phenol formed was determined spectrophotometrically by its absorption at 270 mμ, and the product of thiocholine reaction with DTNB was determined at 412 mμ according to the method of Ellman *et al.* (1961).

Studies were also carried out at several fixed temperatures between 10 and 30°. The pH was 8.0 and the temperature was held constant within 0.05°.

Since phenyl acetate and isoamyl acetate are rather insoluble in water, 1 or 2% methanol was added to the reaction mixture to increase their solubility. When ACh and phenyl acetate hydrolysis were to be compared the same volume of methanol was added for both.

All experiments were treated statistically, the method of Wilkinson (1961) being used whenever it was applicable.

TABLE I: Competitive and Noncompetitive Constants (K_i and K_i', see eq 1) for 3-Hydroxyphenyltrimethylammonium Ion Inhibition of the Hydrolysis of Various Substrates at pH 7.5.

Substrate	K_i	K_i'
Acetylcholine	$7.15 \pm 1.25 \times 10^{-6}$	$1.59 \pm 0.64 \times 10^{-4}$
N,N,N-Dimethylbutylaminoethyl acetate	$7.10 \pm 1.02 \times 10^{-6}$	$1.31 \pm 0.14 \times 10^{-4}$
N,N,N-Diethylbutylaminoethyl acetate	$7.06 \pm 0.46 \times 10^{-6}$	$2.70 \pm 0.05 \times 10^{-5}$
Phenyl acetate	$6.63 \pm 0.52 \times 10^{-6}$	$1.64 \pm 0.13 \times 10^{-5}$
Isoamyl acetate	$8.02 \pm 1.46 \times 10^{-6}$	$1.46 \pm 0.54 \times 10^{-5}$

TABLE II: Maximum Velocities and K_m Values for Various Substrates with Fly-Head and Bovine Erythrocyte AChE at pH 7.5.

Substrate	Fly Head		Bovine Erythrocyte[a]	
	V[b]	K_m	V[b]	K_m
Acetylcholine	1.00	$4.87 \pm 0.47 \times 10^{-5}$	1.00	2.68×10^{-4}
ACh + 2% MeOH	1.05	$1.21 \pm 0.16 \times 10^{-4}$		
Acetylthiocholine	0.85	$4.32 \pm 1.23 \times 10^{-5}$	0.83	1.31×10^{-4}
Phenyl acetate + 2% MeOH	0.92	$3.99 \pm 0.20 \times 10^{-4}$	1.13	1.31×10^{-3}
Dimethyl-*n*-propylaminoethyl acetate	1.00	$4.56 \pm 0.69 \times 10^{-5}$	0.63	5.86×10^{-4}
Dimethyl-*n*-butylaminoethyl acetate	0.66	$9.90 \pm 1.39 \times 10^{-5}$		
Diethyl-*n*-butylaminoethyl acetate	0.30	$2.53 \pm 0.18 \times 10^{-4}$	0.16	9.30×10^{-4}
Isoamyl acetate + 2% MeOH	0.36	$8.24 \pm 2.35 \times 10^{-3}$	0.20	5.81×10^{-3}

[a] Data from Krupka (1964, 1966a,b). [b] V with ACh is arbitrarily set at unity.

However, we often refer to Lineweaver–Burk plots ($1/v$ *vs.* $1/[S]$) on account of their familiarity, and in spite of their statistical inferiority to several other plots (Dowd and Riggs, 1965). When the slope of a Lineweaver–Burk plot is cited, it is actually calculated from the ratio K_m/V determined in the Wilkinson analysis. Its intercept is the reciprocal of the maximum velocity, V. For certain other kinds of data an unweighted least-squares analysis was appropriate.

Results

Enzyme Activity in the Presence of Two Substrates. To show that a single esterase acted in the enzyme preparation, enzyme activity was determined in the presence of two substrates, ACh and phenyl acetate. The relative values for the hydrolysis rates were 100.0 ± 0.59 for ACh at a concentration of 1.0×10^{-3} M, 81.0 ± 0.29 for phenyl acetate at 2.0×10^{-3} M, and 103.0 ± 0.29 for both substrates present together at these same concentrations.

Inhibition by 3-Hydroxyphenyltrimethylammonium Iodide. Inhibition by 3-hydroxyphenyltrimethylammonium is of the mixed competitive and noncompetitive type with all substrates examined. An example is shown in the Lineweaver–Burk plot in Figure 1, where the inhibitor is seen to increase both the slope and intercept, *i.e.*, both K_m and V, though not to the same extent.

The competitive (K_i) and noncompetitive (K_i') inhibition constants can be calculated from the following relationships (Krupka, 1965)

$$K_i = [I] \Big/ \left(\frac{\text{slope } (+I)}{\text{slope } (-I)} - 1 \right)$$
$$K_i' = [I] \Big/ \left(\frac{V}{V_i} - 1 \right) \quad (1)$$

where slope (+I) and slope (−I) stand for the slopes in these plots either in the presence or absence of inhibitor, and similarly V_i and V are the values of the maximum velocity with and without inhibitor. K_i can be shown to directly represent the dissociation constant for the complex formed from the inhibitor and the free enzyme, while K_i' is a weighted mean for binding of inhibitor to all forms of enzyme–substrate complex in the reaction (Krupka and Laidler, 1961a).

Inhibition constants for several acetyl substrates are listed in Table I.

Kinetics of Substrate Hydrolysis. The relative maximum velocities (V) and the Michaelis constants (K_m) are listed in Table II for several charged and uncharged acetyl–ester substrates. The maximum velocity for ACh has been arbitrarily set at unity. For comparison the values for AChE from bovine erythrocytes are also shown. Substrate inhibition of fly-head AChE is observed with all cationic substrates (Figure 2).

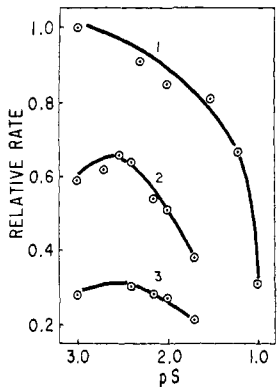


FIGURE 2: Inhibition by high concentrations of cationic substrates. The maximum velocity of ACh hydrolysis is set at unity. The three curves represent rates for ACh (1), *N,N,N*-dimethyl-*n*-butylaminoethyl acetate (2), and *N,N,N*-diethyl-*n*-butylaminoethyl acetate (3).

Temperature Dependence of the Maximum Velocity. The maximum velocities of ACh and phenyl acetate hydrolysis were determined at pH 8.0 in 2% methanol at temperatures from 10 to 30°. The results are shown in an Arrhenius plot (Figure 3). Phenyl acetate appears to be hydrolyzed a little faster than ACh at low temperatures, and a little slower at higher temperatures. Because these measurements were made at the optimum pH it is unlikely that the temperature dependence of acid–base dissociation constants affects the observations.

Transfer of Substrate Acetyl Group to Methanol. During hydrolysis the ester bond of the substrate is ruptured and a new bond is formed between the acyl moiety and water. The new bond can also form with alcohol, if present, producing an ester instead of an acid. Experimentally, the relative amounts of acid and ester may be calculated from the total acid released and the total substrate lost, since the difference between these quantities equals the quality of ester formed. This is shown in the following reactions

$$E + CH_3\overset{O}{\overset{\|}{C}}—OR \rightleftharpoons E\cdot CH_3\overset{O}{\overset{\|}{C}}—OR$$

$$E\cdot CH_3\overset{O}{\overset{\|}{C}}—OR + H_2O \overset{k_w}{\rightleftharpoons} E + ROH + CH_3\overset{O}{\overset{\|}{C}}—O^- + H^+ \quad (2)$$

$$E\cdot CH_3\overset{O}{\overset{\|}{C}}—OR + CH_3OH \overset{k_m}{\rightleftharpoons} E + ROH + CH_3\overset{O}{\overset{\|}{C}}—OCH_3$$

where $E\cdot CH_3COOR$ represents the enzyme–substrate complex and CH_3OH is an alcohol (methanol).

The rates of acid formation (d[A]/dt) and substrate depletion (d[S]/dt) are given by the following equations

$$\frac{d[A]}{dt} = k_w[H_2O][E\cdot CH_3\overset{O}{\overset{\|}{C}}—OR] \quad (3)$$

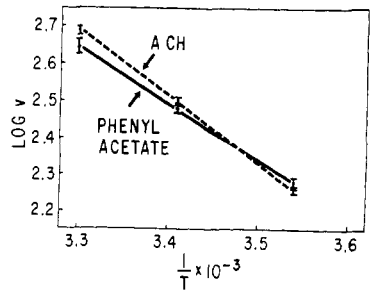


FIGURE 3: Arrhenius plot of log V against the reciprocal of the temperature for ACh and phenyl acetate. The pH in these experiments was 8.0.

$$\frac{-d[S]}{dt} = k_w[H_2O][E\cdot CH_3\overset{O}{\overset{\|}{C}}—OR] + k_m[CH_3OH][E\cdot CH_3\overset{O}{\overset{\|}{C}}—OR] \quad (4)$$

and the ratio of substrate lost ($-\Delta$[S]) to acid formed (Δ[A]) in a given period of time is found from their ratio

$$\frac{-d[S]/dt}{d[A]/dt} = 1 + \frac{k_m[CH_3OH]}{k_w[H_2O]} = -\frac{\Delta[S]}{\Delta[A]} \quad (5)$$

In our experiments different methods were used for measuring these quantities (spectrophotometry as against acid–base titration), and their values were initially found in different units. The required conversion factor was determined by measurement of substrate loss and acid production in the absence of methanol, where the two measurements must be equivalent. If their ratio is designated as $[S/A]_0$, and the ratio in the presence of methanol is $[S/A]_M$, then

$$\frac{-\Delta[S]}{\Delta[A]} = \frac{\left[\frac{S}{A}\right]_M}{\left[\frac{S}{A}\right]_0}$$

The ratio of reaction velocities with methanol and water is then calculated from eq 5.

In reactions carried out in 25% methanol with either ASCh or phenyl acetate (2 mM) as substrate the ratio of methanolysis to hydrolysis (v_m/v_w) was found to be 0.55 ± 0.076 for ASCh and 0.56 ± 0.032 for phenyl acetate. The ratio of the rate constants, (k_m/k_w), is therefore 3.76.

In these experiments the final samples for analysis were taken when at very most 10%, and usually no more than 5% of the substrate had been consumed. The final concentration of methyl acetate must therefore have been less than $^1/_{20}$ that of phenyl acetate or ASCh. As the K_m values for the latter substrates are low in relation to their concentrations (Table II), while K_m for methyl acetate is so high that no sign of saturation is seen at these concentrations, methyl acetate could not be hydrolyzed by the enzyme to any significant extent.

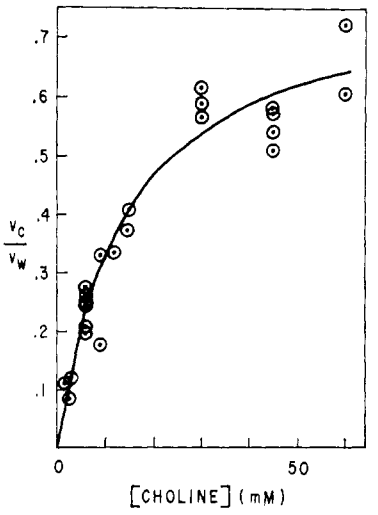


FIGURE 4: Partition of the acetyl group of ASCh between choline and water (v_c/v_w) in relation to choline concentration.

Transfer of Substrate Acetyl Group to Choline. Choline, like methanol, can be acetylated in the presence of enzyme and substrate, and the ratio of acetyl group transfer to choline and water may be measured using the methods described in the last section. The ester produced is now ACh instead of methyl acetate. Such experiments were carried out with ASCh as substrate and with several different concentrations of choline, either alone or in the presence of tetraethylammonium, tetrapropylammonium, or tetrabutylammonium ions. The ratio of reaction rates with choline and water (v_c/v_w) was 0.33 ± 0.032 with 10^{-2} M choline. With the same choline concentration the ratio was 0.16 ± 0.02 in the presence of 3×10^{-3} M tetraethylammonium, 0.15 ± 0.02 with 1×10^{-3} M tetrapropylammonium, and 0.11 ± 0.01 with 3×10^{-4} M tetrabutylammonium iodide. As will be shown later these lowered ratios show that quaternary ammonium ions do not prevent acid formation.

As the choline concentration rose, the rate of ACh formation relative to hydrolysis was observed to increase and approach a maximum, showing that choline forms a complex with the enzyme before being acetylated (Figure 4). The half-saturation constant for choline is roughly 9×10^{-3} M.

Effects of Inhibitors on the Maximum Velocity. The experiments described above provide evidence than an acetyl–enzyme intermediate is formed in the reaction (see Discussion). We now wish to decide if formation or breakdown of this intermediate is the rate-limiting step in hydrolysis of ACh and phenyl acetate, and the effects of cationic inhibitors on the maximum hydrolysis rates can be helpful in this regard. Such inhibitors could conceivably have different effects with these substrates. They should compete for the enzyme's anionic site with ACh, a cation, but not necessarily with phenyl acetate, which lacks the positive charge involved in this attachment. Thus if reaction of the enzyme–substrate complex—*i.e.*, acetylation—is rate limiting, the effect of cationic inhibitors on V might be different with different substrates. However if deacetylation is rate limiting the effects should be identical, since the same intermediate, an acetyl–enzyme, is involved with all substrates. The effects of choline on V were therefore determined with ACh, phenyl acetate, and ASCh (Figure 5).

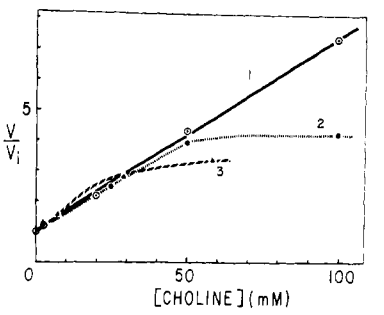


FIGURE 5: Effects of choline on the maximum velocity of substrate hydrolysis. V/V_i is the ratio of uninhibited and inhibited rates, V and V_i, respectively. Curve 1, ACh; 2, ASCh; 3, phenyl acetate.

With ACh, the maximum velocity is seen to fall continuously as the inhibitor concentration rises, but with the other substrates it reaches a minimum and then falls no more. Therefore the reaction blocked by the inhibitors cannot be the common step in hydrolysis of all the substrates, deacetylation, but rather the step that differs, acetylation. The plateaus observed with ASCh and phenyl acetate reflect a partial blockade of this step. Judging by the linear plot for ACh the blockade here is virtually complete.

Although a more complete pH study is to be reported later, it will be helpful at this stage to demonstate the effects of pH on the maximum velocities of ACh and phenyl acetate hydrolysis. These experiments are a logical extension of those just reported, with inhibitory hydrogen ions replacing quaternary ammonium compounds. In the experiments K_m and V and their standard errors were estimated at different pH values. A more precise measurement of the variation of V with pH was obtained by determining rates at a fixed substrate concentration well above K_m, and correcting these rates by multiplication by $1 + K_m/[S]$. Since $[S] \gg K_m$, errors in K_m lead to a relatively small error in V. A plot of the results is shown in Figure 6. The calculated pK_a value for ACh based on points from pH 8.5 to 6.4 is 6.47 ± 0.052, and that for phenyl acetate from measurements at pH 7.8 to 6.7 is 6.81 ± 0.033. These calculations depend on the relationship $1/V$ (expt) = $(1 + [H]/K_a)/V$ (Krupka, 1966a). A limited pH range was chosen because the behavior departs from a simple ionization curve. The causes of this departure will be discussed in a later communication.

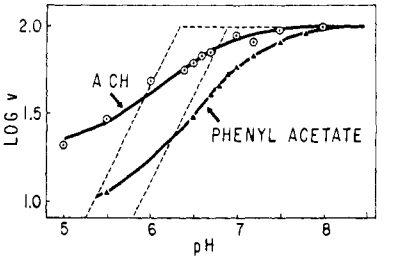


FIGURE 6: pH dependence of log V for ACh and phenyl acetate. Both curves are normalized to give $V = 100$ at high pH. Actually V for phenyl acetate is 12% lower than that for ACh, so that points for the former substrate would otherwise be slightly lower.

Discussion

Evidence for a Single Enzyme in the Preparation. The question of whether hydrolysis of the two rather dissimilar substrates, ACh and phenyl acetate, is catalyzed by one enzyme or two can be decided by comparing hydrolysis rates for the substrates singly and together. If there are two enzymes, one acting exclusively on phenyl acetate and the other on ACh, the total hydrolysis rate with both substrates will obviously be the sum of the rates at which each is hydrolyzed alone. If there is only one enzyme the substrates will have to compete for a single active site, making each an inhibitor of the other, and the total velocity will never exceed the maximum velocity of the better substrate. Intermediate cases are also possible, for example, where one enzyme acts on both substrates, the other on only one. Again the combined rates of hydrolysis with two substrates must exceed the rate expected for a single enzyme. Saturating concentrations of both substrates should be used in this experiment, preferably equally saturating concentrations, which maximize the difference between the one-enzyme and the two-enzyme systems.

If only one enzyme is present the rate may be calculated from the following equation

$$v = \frac{V_1\frac{[S_1]}{K_{m_1}} + V_2\frac{[S_2]}{K_{m_2}}}{1 + \frac{[S_1]}{K_{m_1}} + \frac{[S_2]}{K_{m_2}}} \qquad (6)$$

where S_1 and S_2 are two substrates, and subscripted V and K_m are their rate parameters. When both ACh and phenyl acetate were present the relative velocity was found to be 103.0 ± 0.29. The calculated figure is very close to this, 98.8 ± 2.97, whereas for two enzymes the calculated rate is 181. It follows that both substrates are mainly hydrolyzed by the same enzyme.

Further evidence for a single enzyme is seen in the similarity of the competitive inhibition constants (K_i) for 3-hydroxyphenyltrimethylammonium ion with all substrates (Table II). These constants are for binding of inhibitor to free enzyme, and if a single enzyme is present their values should be identical. If there are two enzymes with different substrate specificities the constants could differ widely, since this inhibitor has a high affinity for AChE (Wilson and Quan, 1958; Krupka, 1965).

Formation of an Acetyl–Enzyme Intermediate. Several lines of evidence support acetyl–enzyme formation by fly-head AChE. Such an inference is often drawn from the observation of irreversible inhibition by organophosphate and carbamate inhibitors. These compounds probably form a covalent bond with the active center of the fly-head enzyme since inhibition is not reversed by dilution, and since substrates afford protection (van Asperen, 1960), and also because reactivation of inhibited enzyme can be achieved by treatment with 2-pyridine aldoxime methiodide (Mengle and O'Brien, 1960). This reagent contains a nucleophilic group and has an affinity for the active center of vertebrate AChE (Ginsburg and Wilson, 1957). Still better evidence came from studies with a series of esters of *N*-methylcarbamate and *N,N*-dimethylcarbamate where spontaneous reactivation was observed, the rate of which depended only on the carbamyl and not the alcohol moiety of the inhibitor (Hellenbrand, 1967). This argues strongly that the inactive enzyme is a carbamyl–enzyme derivative capable of being slowly hydrolyzed to regenerate native enzyme, and the inhibitors may therefore be regarded as exceedingly poor substrates. This suggests that good substrates may react through a similar pathway, with an acyl–enzyme intermediate, corresponding to carbamyl–enzyme, formed initially, followed by its hydrolysis to give acid and free enzyme.

Additional evidence for such an intermediate comes from our measurements of methanolysis. The relative rates at which water and methanol react with the substrate, forming acetic acid and methyl acetate, were the same whether the substrate is phenyl acetate or ACh. This is difficult to explain unless reaction of a common intermediate is involved, which could only be an acetyl–enzyme.

The Rate-Limiting Step in Substrate Hydrolysis. Since an acetyl–enzyme is probably formed in the course of substrate hydrolysis, it must be decided whether its formation or breakdown is the slow step. The interpretation of K_m, of the pH dependence of the maximum velocity, of substrate inhibition, and of noncompetitive inhibition all depend on the nature of the rate-limiting step.

The substrates are all acetyl esters and should form the same acetyl–enzyme intermediate, with deacetylation a common step in hydrolysis. In the case of substrates having low maximum velocities compared with ACh it is obvious that acetylation must be slower than deacetylation. With the best substrates, such as ACh and phenyl acetate, the question is much more difficult to decide. Evidence bearing on this will now be considered.

The fact that substrates as structurally unlike as ACh and phenyl acetate have similar maximum velocities (Table I) suggests that the same reaction is rate limiting with both, which would be deacetylation. Noncompetitive inhibition, *i.e.*, a decreased maximum velocity in the presence of a reversible inhibitor, would then be presumed to follow from a blockade of this reaction. However, this cannot possibly be true, considering the completely different effects of inhibitor on the maximum velocities of hydrolysis with ACh, ASCh, and phenyl acetate (Figure 5). The inhibitors certainly affect different reactions with each substrate, arguing that the effects can only be on acetylation. From this it appears that acetylation may be rate limiting with all the substrates.

This conclusion is supported by the different pH dependence of the maximum velocities for ACh and phenyl acetate (Figure 6). The calculated pK_a values are 6.47 ± 0.052 for ACh and 6.81 ± 0.033 for phenyl acetate. The difference, like the difference in shape of the curves, is outside experimental error, and suggests that the maximum velocities with these substrates are controlled by different reactions.

Temperature Dependence of the Maximum Velocity. According to the Arrhenius law, which may be expressed as: $\log k = A - E/RT$, two factors determine the rate constant k: A, which is an entropy factor, and E, the activation energy. Identical rate constants for two dissimilar reactions at a given temperature could result from different but balancing A and E terms. In this case Arrhenius plots would clearly distinguish the reactions, since the slopes and intercepts would differ.

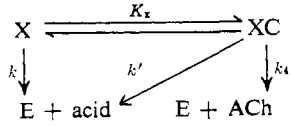
It was in the hope of deciding whether different reactions limited hydrolysis of the best substrates that the experiments reported in Figure 3 were carried out. The Arrhenius plots for ACh and phenyl acetate are seen to be closely similar,

though there is some hint of intersection of two lines of slightly different slope. Assuming however that the two lines really coincide, the most obvious interpretation would be that the same reaction—deacetylation—controls V with both substrates. Strong objections to this interpretation have already been raised, and it is possible that nearly identical activation energies for acetylation at the optimum pH could result from a rate limitation at some process involving the enzyme alone, such as a conformational change. This process might become rate limiting only with the best substrates. A similar suggestion was made on the basis of experiments with chymotrypsin and alkaline phosphatase (Barman and Gutfreund, 1966).

Noncompetitive Inhibition and Substrate Inhibition. Noncompetitive inhibition is seen with all substrates and inhibitors, and substrate inhibition with all cationic substrates. Since the slow step in hydrolysis is certainly acetylation with the poorer substrates and probably with the better ones too, inhibition must result from blockade of this step. This is confirmed by the radically different noncompetitive inhibitions of ACh, ASCh, and phenyl acetate hydrolysis (Figure 5). Cationic inhibitors and substrates must therefore become bound to the enzyme–substrate complex. This came as a surprise to us, since the enzyme's anionic site should be occupied by the quaternary nitrogen group of the first substrate molecule and should be unavailable for attachment of a second. A second cation binding site is therefore immediately suggested. This topic will be pursued in a later paper.

Binding of Choline to Enzyme–Substrate Complex and Acetyl–Enzyme. The choline saturation curve seen in transacetylation measurements (Figure 4) presumably depends on addition to the acetyl–enzyme (EA), whereas reductions in the maximum velocity of substrate hydrolysis depends on addition of choline to the enzyme–substrate complex (ES), as seen above. The binding constants for these two measurements can therefore differ, whereas if only one intermediate is formed during catalysis, ES but not EA, identical binding constants would be found. It appears that the constants differ by a factor of 2, as shown by the following analysis.

First consider the transacetylation reaction. Designating the enzyme intermediate involved as X, choline as C, and XC the complex, the relevant reactions may be written as follows



(7)

The rate constants for reaction of X and XC with water to form acid and free enzyme are k and k', respectively, and k_4 is the constant for reaction of X with choline to produce ACh. The rate of formation of acid is given by

$$v_w = k[X] + k'[XC] \quad (8)$$

$$= [X](k + k'[C]/K_x) \quad (9)$$

and the rate of formation of ACh by

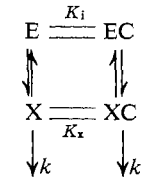
$$v_c = k_4[XC] = k_4(X)\frac{[C]}{K_x} \quad (10)$$

It follows that

$$\frac{v_c}{v_w} = \frac{k_4[C]/k'}{[C] + kK_x/k'} \quad (11)$$

The half-saturation constant, found to be approximately 9×10^{-3} M, therefore equals kK_x/k'.

For comparison, an equation for noncompetitive inhibition may be derived on the basis of the following reaction diagram



(12)

X now represents the intermediate involved in the rate-limiting reaction step, and k and k' have the same significance as before. It is possible to neglect k_4 because formation of ACh does not contribute to the measured rate of acid production. It can be shown that the maximum velocity in the presence of inhibitor is given by

$$V_i = [E_0](k + k'[C]/K_x)/(1 + [C]/K_x) \quad (13)$$

from which

$$V/V_i = (1 + [C]/K_x)/(1 + [C]k'/kK_x) \quad (14)$$

This equation explains the observations shown in Figure 5. If k' equals zero, V/V_i is a linear function of inhibitor concentration. If $k' = k$ or if $K_x = \infty$ the inhibitor will have no effect on the maximum velocity. If k' is between zero and k, V/V_i will approach an upper limit, equal to k/k', as the inhibitor concentration rises.

The plateaus observed with ASCh and phenyl acetate are therefore readily interpreted. The ratio k'/k is approximately 0.30 for phenyl acetate and 0.24 for ASCh. With ACh there is no sign of bending, indicating that $k'/k < 0.13$.

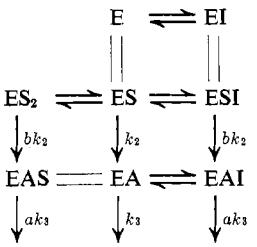
The value of kK_x/k' may now be estimated from the curve in Figure 5 for choline and ATC. When $[C] = kK_x/k'$, $V/V_i = (1 + k/k')/(1 + 1) = 2.6$ (see eq 14). That is, kK_x/k' is equal to the choline concentration at which $V/V_i = 2.6$, and this is bound to be approximately 2.7×10^{-2} M. The difference between this estimate and the first (9×10^{-3} M) is probably outside experimental error.

Effect of Cationic Inhibitors on Deacetylation. In studies with erythrocyte AChE it was shown that tetrapropylammonium and larger cations may become bound to the acetyl–enzyme intermediate and block deacetylation (Krupka, 1965). With fly-head AChE such ions become bound to the acetyl–enzyme but are apparently less effective in blocking deacetylation. The evidence is as follows. First, the saturation curve seen for choline in the transacetylation reaction (Figure 4) shows, as was noted above, that choline forms a complex with the acetyl–enzyme. It also shows that this complex can be hydrolyzed, releasing acetic acid, since otherwise there would be no limit to the increase in v_c/v_w at high choline concentrations (*i.e.*, v_w would fall to zero). The maximum of about 0.65 indicates that even with choline bound hydrolysis is

faster than acetylation of choline. A second type of evidence is the decreased v_c/v_w ratios in the presence of cationic inhibitors. These should displace choline from the acetyl–enzyme and diminish ACh formation (v_c). If at the same time they prevent water from reacting, the v_c/v_w ratio will be unchanged. The decreased ratio indicates that they have less effect on hydrolysis of the acetyl–enzyme than on acetylation of choline.

Summary

Cationic substrates and inhibitors become bound to the enzyme–substrate complex and block its reaction, accounting for noncompetitive and substrate inhibition. A second intermediate formed during substrate hydrolysis, the acetyl–enzyme, is not involved in these inhibitions, though choline and other cations form a complex with it. The following reaction scheme summarizes the detailed findings.



where E, ES, and EA represent free enzyme, enzyme–substrate complex and acetyl–enzyme. ES_2 is a complex containing two-substrate molecules, while ESI contains one substrate and one inhibitor molecule. Complexes between acetyl–enzyme and substrate or inhibitor are represented by EAS and EAI. With some substrates, such as ACh, ESI may be unreactive, *i.e.*, $b = 0$, while with others ESI reacts, though at a rate somewhat slower than ES; for example, $b = 0.3$ with phenyl acetate and 0.24 with ASCh. EAI is reactive even with relatively large cations such as tetrabutylammonium, but the exact value of a has not been determined. The simplifying assumption is made in the diagram that ES_2 and ESI react at the same rate (bk_2), as do EAS and EAI (ak_3). Noncompetitive and substrate inhibitions result from a blockade at the acetylation step. Acetylation is rate limiting with the poorer substrates, and probably with the best as well, though the similarity in Arrhenius plots for ACh and phenyl acetate makes the latter conclusion less certain.

A striking contrast has shown up between fly-head and vertebrate AChE, which deserves a brief comment at this time. Substrate inhibition and noncompetitive inhibition of vertebrate AChE involve the acetyl–enzyme, for deacetylation is blocked when a substrate or inhibitor molecule is bound to this intermediate (Wilson and Cabib, 1956; Krupka and Laidler, 1961b; Wilson and Alexander, 1962; Krupka, 1964). The enzyme–substrate complex is not involved, and cations do not appear to become bound to it. In the fly-head enzyme the situation is reversed. Here cations readily bind to ES to block its reaction, and the result is noncompetitive or substrate inhibition. The possibility exists that substrate inhibition is accidental in vertebrate AChE, for in EA the anionic site is vacant, following release of choline in acetylation. Cations should therefore become bound to this intermediate and could block deacetylation, the step that is rate limiting with ACh as substrate. With fly-head AChE on the other hand the slow step in ACh hydrolysis appears to be acetylation, and according to this argument there should be no substrate inhibition. Evidence cited above that cations bound to EA do not block deacetylation would tend to reinforce this view. Nevertheless strong substrate inhibition is found. Its mechanism probably depends on the existence of a second anionic site in fly-head AChE, which makes it possible for two-substrate molecules to be bound to the enzyme at the same time. This apparent evolution of two different mechanisms may suggest that substrate inhibition is biologically functional. It will be interesting to see whether divergent mechanisms of this sort are also found in other vertebrates and invertebrates.

References

Barman, T. E., and Gutfreund, H. (1966), *Biochem. J. 101*, 411, 460.

Dauterman, W. C., and Mehrotra, K. N. (1963), *J. Ins. Physiol. 9*, 257.

Dauterman, W. C., Talens, A., and van Asperen, K. (1962), *J. Ins. Physiol. 8*, 1.

Dowd, J. E., and Riggs, D. S. (1965), *J. Biol. Chem. 240*, 863.

Ellman, G. L., Courtney, D., Andres, V., and Featherstone, F. M. (1961), *Biochem. Pharmacol. 7*, 88.

Ginsburg, S., and Wilson, E. B. (1957), *J. Amer. Chem. Soc. 79*, 481.

Hellenbrand, K. (1967), *J. Agr. Food Chem. 15*, 825.

Krupka, R. M. (1964), *Biochemistry 3*, 1749.

Krupka, R. M. (1965), *Biochemistry 4*, 429.

Krupka, R. M. (1966a), *Biochemistry 5*, 1983.

Krupka, R. M. (1966b), *Biochemistry 5*, 1988.

Krupka, R. M., and Laidler, K. J. (1961a), *J. Amer. Chem. Soc. 83*, 1445.

Krupka, R. M., and Laidler, K. J. (1961b), *J. Amer. Chem. Soc. 83*, 1448.

Mehrotra, K. N., and Dauterman, W. C. (1963), *J. Neurochem. 10*, 119.

Mengle, D. C., and O'Brien, R. D. (1960), *Biochem. J. 75*, 201.

Metcalf, R. L., and Fukuto, T. R. (1967), *J. Agr. Food Chem. 15*, 1022.

O'Brien, R. D. (1966), *Annu. Rev. Ent. 11*, 369.

Smissaert, H. R. (1964), *Science 143*, 129.

Smissaert, H. R., Voerman, S., Oostenbrugge, L., and Renooy, N. (1970), *J. Agr. Food Chem. 18*, 66.

van Asperen, K. (1960), *Biochem. Pharmacol. 3*, 136.

Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.

Wilson, I. B. (1960), *Enzymes 1*, 501.

Wilson, I. B., and Alexander, J. (1962), *J. Biol. Chem. 237*, 1323.

Wilson, I. B., and Cabib, E. (1956), *J. Amer. Chem. Soc. 78*, 202.

Wilson, I. B., and Quan, C. (1958), *Arch. Biochem. Biophys. 73*, 131.