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Evidence for an Intermediate in the Acetylation Reaction of Acetylcholinesterase*

R. M. Krupka

ABSTRACT: Acetylation of acetylcholinesterase by the substrate methyl acetate was found to be controlled by an ionizing group in the enzyme of pK = 5.3. The reverse reaction (acetyl enzyme + methanol → methyl acetate), which was studied by hydrolyzing acetylcholine in the presence of 5% methanol and determining the ratio of hydrolysis to methanolysis, was controlled by another ionizing group, pK = 6.3. It follows that an intermediate, whose formation and decomposition are catalyzed by different enzyme groups, must occur in acetylation. In the direction of ester hydrolysis, the rate-limiting step is catalyzed by a group of pK = 5.3, and a subsequent fast step is catalyzed by a group of pK = 6.3. The latter group, which is adjacent to the enzyme's anionic site, also functions in the rate-limiting step of deacetylation.

Recently published evidence showed that different catalytic basic groups in AChE[1] function in the rate-limiting steps of each of the two main stages of substrate hydrolysis, acetylation of the enzyme, and deacetylation (Krupka, 1966a,b). The study of acetylation has now been carried further, with investigation of the pH dependence of the forward reaction, where an ester reacts with the enzyme to produce alcohol and an acetyl enzyme, compared with the back reaction, where the acetyl enzyme and alcohol react to form an

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[1] Abbreviations used: AChE, acetylcholinesterase; AcCh, acetylcholine bromide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

ester and free enzyme. The substrate chosen for study was methyl acetate, first because the rate-limiting step in its hydrolysis is expected to be acetylation, and second because it does not interact electrostatically with the basic group that functions in deacetylation, as do cationic substrates (Krupka, 1966b). The back reaction was carried out by allowing the enzyme to hydrolyze AcCh in the presence of methanol. Under these conditions an acetyl enzyme should be formed, which reacts either with water, as in normal substrate hydrolysis, or with methanol, producing methyl acetate. The latter is the reverse of acetylation with methyl acetate as substrate. The over-all reaction may be written as

$$\begin{array}{ccccc} E + S & \rightleftharpoons & ES & & \\ & & \downarrow{\scriptstyle ROH} & & \\ & & EA & \xrightarrow{H_2O} & E + CH_3COOH \\ & & \downarrow{\scriptstyle CH_3OH} & & \\ E + CH_3COOCH_3 & \rightleftharpoons & E{\cdot}CH_3COOCH_3 & & \end{array}$$

where E, ES, and EA stand for free enzyme, enzyme–substrate complex, and acetyl enzyme, respectively; $E{\cdot}CH_3COOCH_3$ is the complex formed with methyl acetate. If the substrate (S) is AcCh, ROH is choline. Formation of EA from ES (or $E{\cdot}CH_3COOCH_3$) is referred to as acetylation, and of acetic acid from EA as deacetylation. Experiments were also performed to show that the same active center in the enzyme preparation hydrolyzed AcCh and methyl acetate, and that solvent effects of methanol and methyl acetate, in the concentrations used, did not significantly influence the ionization of catalytic groups in the enzyme.

Materials and Methods

The enzyme was a purified preparation from bovine erythrocytes supplied by Sigma Chemical Co. Reagent grade methanol was refluxed over KOH and granular Zn and distilled. Reagent grades of methyl acetate and hexane were redistilled before use. DFP was a product of Boots Pure Drug Co., Ltd., Nottingham, England, supplied by Aldrich Chemical Co., Milwaukee, Wis. Acetylcholine bromide was obtained from Matheson Coleman and Bell.

All reactions were carried out in the presence of 0.10 N NaCl and 0.04 M $MgCl_2$ at 26°, unless otherwise noted. Acid released in ester hydrolysis was followed by automatic titration with 0.01 or 0.10 N NaOH, using a Radiometer TTTl titrator and titrigraph, with Radiometer G202C glass and K100 liquid-junction calomel electrodes. Ionization constants were calculated by procedures described before (Krupka, 1966a).

Inhibition by DFP. Rates of enzyme inactivation by DFP were determined as follows. The initial reaction mixture contained enzyme and 0.063 M methyl acetate, in a total volume of 10 ml, pH 6.5. The reaction vessel was loosely covered, and CO_2-free N_2, first bubbled through 1 l. of 0.5% methyl acetate, was emitted above the reaction solution. Under these conditions a constant rate of methyl acetate hydrolysis (as determined from automatic addition of 0.01 N NaOH to neutralize acid released) was observed over a period of at least 1 hr, and the blank rate, in the absence of enzyme, was negligible. After establishing the initial rate in the presence of enzyme, 0.20–0.30 ml of DFP solution was added from a syringe without interrupting the titration of acid released in hydrolysis. The final DFP concentration was of the order of 10^{-6} M. The reaction mixture, which was constantly stirred, was sampled immediately and then at intervals, without interrupting titration. The sample size was normally 0.1 ml, though 0.2 or 0.3 ml was taken when little activity remained. Each sample was added to a test tube containing 10 ml of water in an ice bucket. Under these conditions no changes in enzyme activity were observed in the samples over a period of several hours, though assays were usually performed within 15 min.

Rates of methyl acetate hydrolysis in the reaction solution were determined periodically from tangents to the recorded tracing of volume of added base *vs.* time and were corrected for volume changes. Enzyme activity in the samples withdrawn at intervals was determined with acetylthiocholine as substrate, using the colorimetric method of Ellman *et al.* (1961), modified slightly. The assay solution consisted of 2 ml of sodium phosphate buffer (0.1 M, pH 7.5), 0.4 ml of DTNB solution (99 mg of DTNB plus 37.5 mg of $NaHCO_3$ in 25 ml of phosphate buffer, 0.1 M, pH 7.0), 0.2 ml of acetylthiocholine iodide (4.34 g $l.^{-1}$), enzyme, and water to give a total volume of 5.6 ml. The reaction was run at room temperature, and optical densities at 412 mμ were read periodically on a Hitachi Perkin-Elmer Model 139 spectrophotometer.

Methanolysis. Reaction of methanol with the acetyl enzyme was measured quantitatively by comparison of relative rates of hydrolysis and methanolysis when the enzyme acts upon AcCh (2×10^{-3} M) in the presence of 5% methanol (1.24 M). The reaction mixture (10 ml) contained 0.10 M NaCl and 0.04 M $MgCl_2$. Hydrolysis was followed by use of an automatic recording titrator, which delivered 0.10 N NaOH to the reaction solution in amounts equivalent to acid released. It also delivered an equal volume of AcCh solution contained in a second, synchronously driven syringe (Heilbronn, 1958; Jensen-Holm, 1961), at a concentration of 0.10 N for experiments at pH 7.5. The substrate concentration was, therefore, constant during the run. At lower pH the AcCh concentration in the syringe was raised to compensate for the buffering action of product acetic acid. After hydrolysis of a predetermined quantity of AcCh equivalent to 0.5 ml of base (5×10^{-5} mole) the reaction was stopped by addition of 0.1 ml of 1.2 N HCl, bringing the pH to approximately 2.2, and the total volume to 11.1 ml. Five 0.5-ml samples were then withdrawn for ester determination using the NH_2OH–$FeCl_3$ test of Hestrin (1949). An 8-ml sample was withdrawn and extracted consecu-

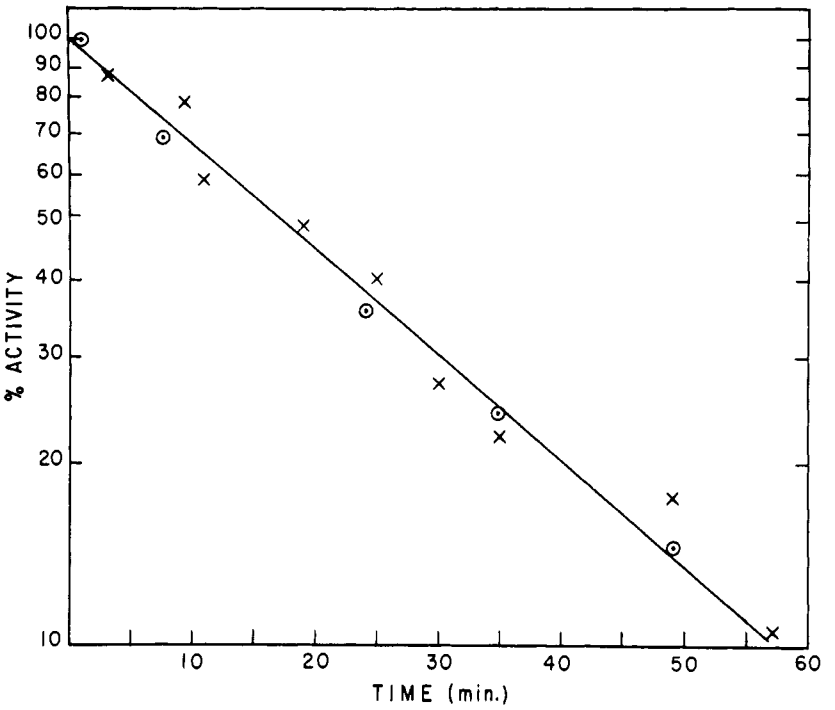


FIGURE 1: Percentage inactivation of acetylcholinesterase by diisopropylphosphorofluoridate at pH 6.5 determined with two substrates, acetylthiocholine (⊙) and methyl acetate (×).

tively with 10, 7, 7, 6, and 5 ml of hexane, using a separatory funnel. The combined hexane extracts were washed with 1 ml of water and then assayed for ester content using a modified Hestrin test. A freshly prepared 1:1 solution (2 ml) of 2.0 M $NH_2OH \cdot HCl$ and 3.5 N NaOH was added and shaken into the hexane layer for 5 min. Next, 1 ml of 25% concentrated HCl was added and shaking was continued for 1 min, followed by addition of 1 ml of $FeCl_3$ solution (0.37 M in 0.1 N HCl) with continued shaking for 1 min more. The optical density of the water layer was read on a Hitachi spectrophotometer at 540 mμ.

Several different controls were run. (1) The total AcCh concentration following addition of 0.5 ml of AcCh solution from the syringe, in the absence of enzyme-catalyzed hydrolysis, was determined by preparing 11.1 ml of solution containing all the ingredients of the reaction mixture after completion of the run, except enzyme, and assaying 0.5-ml samples for ester content. The quantity of AcCh hydrolyzed in the experiments was determined from the difference between this concentration and that found after completion of the enzymic reaction. (2) A solution was prepared identical with the reaction mixture except for addition of 0.1 ml of 1.2 N HCl before addition of AcCh (total volume 11.1 ml). Eight milliliters was extracted in the usual way with hexane and tested for ester content. The optical density in this test was low (comparable to that obtained when the assay procedure was applied with 35 ml of pure hexane) and was used as a blank to correct the corresponding optical density value for the reaction mixture. (3) To determine the expected recovery of methyl acetate in the assay procedure another solution similar to the reaction mixture was prepared in which acid was added before AcCh, and which contained 0.4–2 ml of a methyl acetate solution (0.1 ml of methyl acetate in 500 ml of water). As before, the final volume was 11.1 and 8 ml was extracted with hexane and assayed for ester. In addition Hestrin tests were carried out on 1.0-ml aliquots of the methyl acetate stock solution. The recovery was found to be approximately 80%. It was also shown that in the range of optical densities found in the methanolysis experiments there was a linear relation between the quantity of methyl acetate available for hexane extraction (up to 3.6 μmoles), and the resulting optical density in the NH_2OH–$FeCl_2$ test (up to 0.59).

Results

Methyl Acetate Hydrolysis. Hydrolysis rates were found to be proportional to the methyl acetate concentration up to at least 0.38 M, equivalent to 3% methyl acetate. A relatively low concentration (0.5% = 0.063 M) was chosen for the experiments, in order to minimize possible side effects of the organic solvent on the enzyme. This concentration is well below K_m.

To begin with, experiments were run to show that the same active center in the enzyme preparation acts upon methyl acetate and AcCh. One way of doing this is by measuring inhibition by 3-hydroxyphenyl-

trimethylammonium iodide, a highly specific inhibitor of acetylcholinesterase (Wilson and Quan, 1958; Krupka, 1965) and, therefore, a useful tool for distinguishing this enzyme from other esterases. Rates of hydrolysis of 0.063 M methyl acetate were determined with 1.82 or 0.91 $\times$ 10^{-6} M inhibitor, and inhibition constants were calculated from the formula

$$K_i = [I]/((v_0/v) - 1) \quad (1)$$

When [S] $\ll$ K_m, as it is here, this gives a good estimate of K_i, the dissociation constant for the enzyme–inhibitor complex. K_i was found to be 0.86 $\pm$ 0.08 $\times$ 10^{-6} M, compared with previously determined values for AcCh inhibition of 0.63 $\times$ 10^{-6} M in water and 1.26 $\times$ 10^{-6} M in 1% methanol.

Another approach is by determining rates of inactivation by DFP with two different assays, hydrolysis of methyl acetate or acetylthiocholine. The latter is a specific substrate of AChE (Koelle and Friedenwald, 1949; McOsker and Daniel, 1956; Krupka, 1964, 1966b). Enzyme activities in the two assays, as percentages of initial activities, were plotted against time (Figure 1), using the equation

$$\log (v/v_0) = -k[I]t/2.3 \quad (2)$$

which corresponds to the following reaction

$$E + I \xrightarrow{k} EI$$

Values of k were calculated by the method of least squares, and in five experiments in which the final DFP concentration ranged from 6.67 $\times$ 10^{-7} to 4.08 $\times$ 10^{-6} M the average value of k was 1.52 $\pm$ 0.05 $\times$ 10^4 l. mole^{-1} min^{-1} in the acetylthiocholine assay and 1.51 $\pm$ 0.12 $\times$ 10^4 l. mole^{-1} min^{-1} in the methyl acetate assay. With both assays eq 2 was obeyed at least as far as 90% inactivation, beyond which methyl acetate rate determinations become extremely imprecise.

Next, experiments were conducted on the pH dependence of methyl acetate hydrolysis. Reaction mixtures were prepared containing 0.063 M methyl acetate and enzyme in a total volume of 10 ml, and hydrolysis rates were measured by titration with 0.01 N NaOH at pH values between 5.5 and 7.5. Initial rates were corrected for blanks without enzyme and for the buffering action of product acetic acid. Plots were constructed of $1/v$ against [H$^+$], and from the ratio of slope and intercept pK values of 5.30 $\pm$ 0.084, 5.30 $\pm$ 0.080, and 5.16 $\pm$ 0.13 were calculated in three separate experiments.

To determine the solvent effect of this concentration of methyl acetate (0.063 M or 0.5%) on the pH dependence of AcCh hydrolysis, initial rates were measured over the pH range 5.5–7.5 with 2.0 $\times$ 10^{-3} M AcCh and enzyme at approximately 1% the concentration used in the previous experiment. Under these conditions hydrolysis of methyl acetate should be negligible since (1) its rate is only about 1% as fast as AcCh hydrolysis, and (2) whereas methyl acetate is well below the saturation level, the AcCh concentration is about ten times K_m. From a plot of $1/v$ against [H$^+$] in the pH range 6.0–7.5, the pK of the enzyme group controlling AcCh hydrolysis was found to be 6.27 $\pm$ 0.07, compared with 6.32 $\pm$ 0.04 in the absence of any organic solvent, and 6.34 $\pm$ 0.04 in 2% methanol (Krupka, 1966a). These data are shown in Figure 2.

Methanolysis. Following hydrolysis of 5 $\times$ 10^{-5} mole of AcCh in 5% methanol at pH 7.5, as described under Methods, the solution was extracted with 5 ml of petroleum ether (bp 39–53°), and the extract was subjected to gas chromatography using a column of 30% Carbowax 1500, with a helium flow of 50 cc/min and a temperature of 74°. A single peak was observed with the same retention time as methyl acetate (4.75 min), showing that this ester was a product of the reaction, as expected.

Using the procedures described above, the relative rates of hydrolysis and methanolysis were calculated for a number of experiments run at pH 5.5–7.5. The results are summarized in Table I, where the average value at pH 7.5 is arbitrarily set at unity. This was done by dividing all the ratios by the average ratio at pH 7.5.

$$R = \left(\frac{k_W[H_2O]}{k_M[CH_3OH]}\right)_{expt} \Big/ \left(\frac{k_W[H_2O]}{k_M[CH_3OH]}\right)_{av\ pH\ 7.5} \quad (3)$$

The calculated ratio of second-order rate constants for reaction at pH 7.5 of water and methanol, whose

TABLE I: Effect of pH on Partitioning of Acetyl Enzyme between Water and Methanol.[a]

	R = Constant[b] x (rate of hydrolysis)/(rate of methanolysis)			
	Predicted Values			
pH	B_2 Controls Methanolysis	B_1 Controls	Experimental Values	Average
7.5	1.00	1.00	0.97, 1.00, 1.00, 1.02	1.00 $\pm$ 0.02
7.0	0.89	1.00	0.89, 1.13	1.01 $\pm$ 0.12
6.5	0.67	1.00	0.99, 1.05	1.02 $\pm$ 0.03
6.0	0.47	1.00	0.98	
5.5	0.39	1.00	1.00, 1.00, 1.04, 1.06, 1.09	1.04 $\pm$ 0.04

[a] The rate ratio, R, is arbitrarily set at 1.00 at pH 7.5 (eq 3). B_1 is the basic group involved in the rate-limiting step in deacetylation (pK = 6.3), and B_2 is the group involved in acetylation (p$K \simeq$ 5.3). [b] Constant = (k_M[MeOH]/k_W[H_2O]) pH 7.5; k_W/k_M = 0.38.

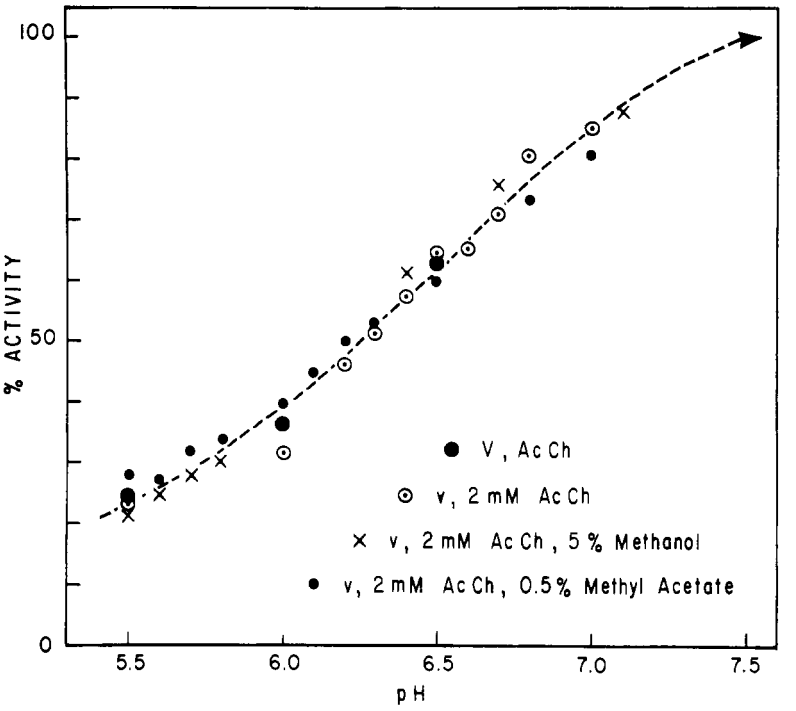


FIGURE 2: Percentage activity of acetylcholinesterase from pH 5.5 to 7.5; relative activity at pH 7.5 is set at 100% (arrow). The maximum velocity (V) in pure water, and rates of hydrolysis of 2 mM AcCh in pure water, 5% methanol, or 0.5% methyl acetate are shown. All reaction mixtures contained 0.10 N NaCl and 0.04 M $MgCl_2$.

concentrations were 52.8 and 1.24 M, respectively, was $k_W/k_M = 0.38$. Similar experiments at pH 7.5 and 5.5 were carried out in the presence of 3.81×10^{-2} M tetraethylammonium bromide to compare its effects on hydrolysis and methanolysis. At pH 7.5 the ratio (R) was 1.59, 1.76, 1.77; mean 1.71 ± 0.08. At pH 5.5 the values were 1.71, 1.73, 1.74, 1.78, 1.88; mean 1.77 ± 0.06.

The pH dependence of hydrolysis of 2×10^{-3} M AcCh in 5% methanol was determined in separate experiments, where initial rates were measured by titration of acid released with 0.01 N NaOH. The results are shown in Figure 2. Values between pH 6.4 and 7.5 were used to plot $1/v$ against $[H^+]$, from which a pK of 6.24 ± 0.03 was computed.

Discussion

The closely similar inhibition constants for 3-hydroxyphenyltrimethylammonium ion with methyl acetate and AcCh indicate that the same active center in the enzyme preparation acts upon both substrates. The *meta*-hydroxy substituent, but not the *ortho* or *para*, was shown to be complementary to the active site of AChE (Wilson, and Quan, 1958), and for this reason the inhibitor is far more potent than other substituted ammonium ions, including phenyltrimethylammonium (Krupka, 1965). It should, therefore, be relatively specific for AChE. The nearly identical rates of DFP inhibition of methyl acetate and acetylthiocholine hydrolysis also indicate that the same enzyme site hydrolyzes both substrates. The involvement of a single enzyme is attested further by the linearity of the plot of log activity against time (Figure 1). Nonlinearity should be observed if either substrate is attacked by two or more enzymes of differing susceptibility to DFP.

In discussing the experiments it will be helpful to refer to the reaction

$$E + CH_3COOCH_3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}CH_3COOCH_3 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EA + CH_3OH$$

$$E + CH_3COOH \xleftarrow[H_2O]{k_3} EA$$

Methyl acetate and the enzyme form a complex that is converted, in the "acetylation" reaction, to an acetyl enzyme (k_2). "Deacetylation" refers to hydrolysis of EA (k_3). Methanolysis (k_{-2}) is the reverse of acetylation. The discussion that follows is mainly concerned with the pH dependence of these reactions. Previous work with a series of acetyl ester substrates showed that the rate of acetylation depends on an ionizing group of pK = 5.2–5.5 (Krupka, 1966a,b). This group appears to be distant from the anionic site since its pK is much the same whether the substrate is neutral or a cationic AcCh analog. The positive

charge in the latter is bound at the enzyme's anionic site, while a catalytic site interacts with the ester bond in the substrate. On the other hand, deacetylation was shown to depend on the ionization of a group of pK = 6.3, and since bound cations strongly perturb its ionization it must be near the anionic site. Both groups are inactive when protonated; *i.e.*, acetylation and deacetylation rates decline as the pH falls. The ionization of both groups could be detected in a single enzyme species, the free enzyme, showing that two different ionizing groups are involved rather than a single group whose pK shifts as the enzyme–substrate complex is converted to the acetyl enzyme. For convenience the ionizing group of pK = 6.3 has been referred to as B_1 and that of pK = 5.2–5.5 as B_2. This convention will be followed here.

The present experiments indicate that the rate of methyl acetate hydrolysis is controlled by an ionizing group of pK = 5.3. As already noted the pK of the group involved in acetylation is similar, ranging from 5.2 to 5.5 with various substrates. It was shown before that the ionization constant of this group may be determined from the effect of pH on rates at substrate concentrations much below K_m with any *uncharged* substrate, or from rates at concentrations much above K_m with any substrate whose hydrolysis is rate limited at acetylation (Krupka, 1966a,b). Since methyl acetate is uncharged and present in the experiments at a concentration much below K_m, its hydrolysis should reflect the ionization of groups involved in acetylation. However, this is probably true at all concentrations, for the rate-limiting step in methyl acetate hydrolysis is likely to be acetylation, as the following considerations suggest. Among acetyl ester substrates, those having low maximum velocities of hydrolysis relative to AcCh must be rate limited at this step, assuming an acetyl enzyme is formed, as is likely (Wilson, 1960; Krupka, 1964, 1966b). This follows because deacetylation is a common step in hydrolysis and, therefore, differences in over-all rates must be due to rate limitations at the acetylation step. Isoamyl acetate (Krupka, 1966a) and butyl acetate (unpublished observations) are two such substrates, and methyl acetate, also an alkyl acetate and neutral, should be another, though its maximum velocity has not been determined because of weak attachment to the enzyme. It is to be concluded, therefore, that acetylation of AChE by methyl acetate, as by other substrates, depends upon an enzyme group of p$K \simeq 5.3$.

The failure of 0.5% methyl acetate and 5% methanol to alter the pH dependence of AcCh hydrolysis shows that neither solvent significantly influences ionizations in the enzyme. The AcCh concentration employed saturates the enzyme ($[S]/K_m \simeq 10$) and under these conditions the rate-limiting step is deacetylation, which is catalyzed by an ionizing group of pK = 6.3 (Krupka, 1966a,b). Hence, the ionization constant derived from experiments on methyl acetate hydrolysis (pK = 5.3) could not result from solvent effects on the enzyme, such as would lower the pK of the group involved in deacetylation by one full pK unit, but may be compared directly with constants for other substrates. Similarly, constants for the methanolysis reaction are directly comparable to those in pure water.

The experiments summarized in Table I show that methanolysis and hydrolysis have the same pH dependence and are controlled by an ionizing group with a pK of 6.3, for in this case their ratio should be independent of pH, as found. If, on the other hand, acetylation with methyl acetate and the reverse reaction, methanolysis of EA, have the same pH dependence, *i.e.*, are controlled by an ionizing group of pK = 5.3, then as the pH falls the rate of methanolysis should diminish less than the rate of hydrolysis (which depends on a group of pK = 6.3), and the ratio of hydrolysis to methanolysis should decrease. The expected change in ratio, relative to an arbitrary value of unity at pH 7.5, was calculated from the pH dependence of V for AcCh (a measure of the deacetylation rate) (Krupka, 1966b) and of methyl acetate hydrolysis. The ratios, listed in Table I (first column), decline from 1.0 at pH 7.5 to 0.39 at pH 5.5. It is obvious from the experimental values given in Table I that this mechanism, in which the same ionizing group controls acetylation in both the foreward and reverse directions, can definitely be ruled out.

The almost identical experimental ratios at pH 7.5 and 5.5 in the presence of tetraethylammonium ion indicate that the ionizing group involved in methanolysis, like that involved in hydrolysis, is adjacent to the anionic site. The ratio is given by the following expression

$$\frac{k_W[H_2O][EA] + ak_W[H_2O][EAI^+]}{k_M[CH_3OH][EA] + bk_M[CH_3OH][EAI^+]} \quad (4)$$

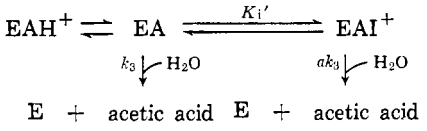
where EAI^+ is the complex formed between EA and tetraethylammonium ion (I^+), a is the relative reactivity of water toward EAI and EA, and b is the corresponding parameter for methanol. Dividing eq 4 by $(k_W[H_2O]/k_M[CH_3OH])$ (pH 7.5), the experimental ratio at pH 7.5 without tetraethylammonium ion, gives

$$R = \frac{[EA] + a[EAI^+]}{[EA] + b[EAI^+]} \quad (5)$$

In the experiments $[I^+] = 3.81 \times 10^{-2}$ M. As determined previously (Krupka, 1965), $a = 0.83$ and $K_i' = [EA][I^+]/[EAI^+] \simeq 7 \times 10^{-3}$ M. Therefore, $[EAI^+]/[EA] \simeq 5$. If methanol failed to react with EAI^+ ($b = 0$), R should equal 5, from eq 5, whereas the experimental value at pH 7.5 is only 1.7. Substituting 1.7 in the equation and solving for b gives a value of 0.4. According to this tetraethylammonium ion impedes reaction with methanol far more than with water, but does not prevent it.

Tetraethylammonium ion accelerates deacetylation at pH 5.0 and 5.5 (Krupka, 1966a,b). It does so by competing for the active center with a hydrogen ion adding to the catalytic basic group of pK = 6.3 in the enzyme. This group appears to be close to the anionic site

since a positive charge placed on either site prevents a second positive charge being placed on the other. Equivalent expressions are that addition of an ammonium ion to the anionic site lowers the pK of the adjacent ionizing group, making it a much stronger acid, and *vice versa* that addition of a positive charge to the basic group lowers the pK_i' of the anionic site, so that it binds I^+ much less strongly. These relations are illustrated in the reaction diagram



Three forms of the acetyl enzyme are shown: EA, the normal, reactive form; EAH^+, which is unreactive; and EAI^+, the complex with tetraethylammonium ion, which, as already noted, undergoes deacetylation almost as rapidly as EA (a = 0.83). At low pH the rate drops because the enzyme is largely in the form of EAH^+, but on addition of I^+ there is a competition between H^+ and I^+ for EA, and EAI^+ is formed. Conversion of EAH^+ to EAI^+ accelerates the reaction, since the former is unreactive. From the known values of a, K_i', and the decline at low pH in the maximum rate of AcCh hydrolysis, the experimental rate increase has been predicted with considerable accuracy.

If the pK of the ionizing group involved in methanolysis is not altered by cation binding at the anionic site, this reaction should not be accelerated at low pH by tetraethylammonium ion. Therefore, since hydrolysis is accelerated, R should rise as the pH falls. The hydrolysis rate at pH 5.5 should increase by a factor of 2.3, and therefore the ratio of hydrolysis to methanolysis at pH 5.5 should be 2.3 times that at pH 7.5; *i.e.*, $R = 2.3 \times 1.7 = 3.9$. The experimental ratio is the same at pH 7.5 and 5.5, showing that both methanolysis and hydrolysis must be accelerated to the same degree. Consequently, the group controlling methanolysis must be near the anionic site.

In summary, acetylation with methyl acetate as substrate (*i.e.*, conversion of E + methyl acetate to EA + methanol) is controlled by a group of p$K \simeq$ 5.3, as is acetylation with other acetyl esters, whether charged or uncharged. This group must be distant from the anionic site, since cations bound at the latter do not significantly alter its pK. The reverse reaction, methanolysis of EA to form E + methyl acetate, is controlled by a group of pK = 6.3 that must be adjacent to the anionic site, since bound cations strongly perturb its ionization. This same ionizing group also catalyzes deacetylation.

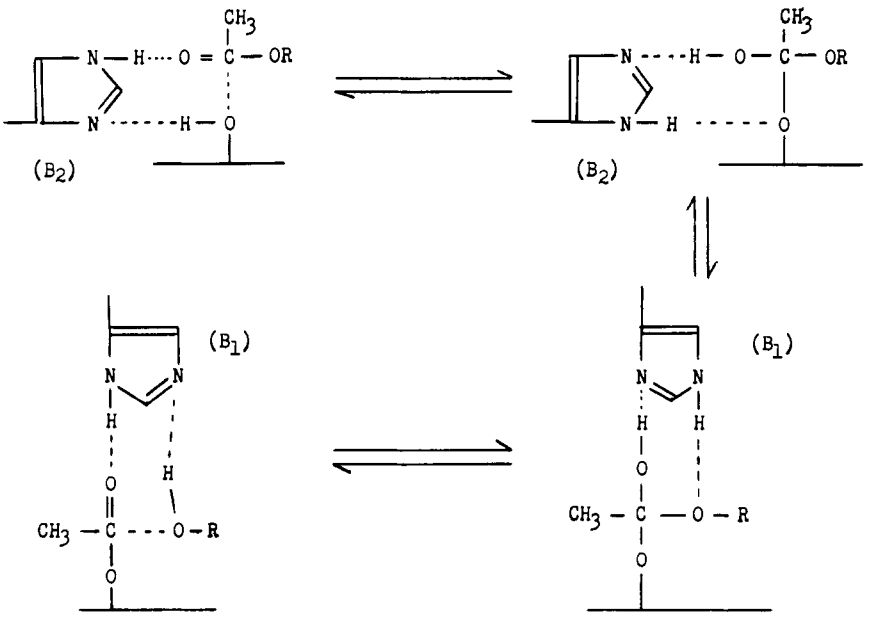
The significance of these findings is brought out in the light of evidence already referred to, which showed that two *different* ionizing groups are involved in acetylation and deacetylation, rather than a single group whose pK shifts as the enzyme–substrate complex is converted to the acetyl enzyme. Different groups must be involved because the ionization of both can be detected in a single enzyme species, the free enzyme (Krupka, 1966b). The simplest interpretation of the new experiments is, therefore, that an intermediate occurs in acetylation, and that its formation and decomposition are catalyzed by *different* enzyme groups. This intermediate may be less stable than EA, and if so the activation energies would be such that in both the forward and reverse directions the slow step would be its formation (from ES and EA) and the fast step its decomposition.

Earlier it was concluded, on the basis of the finding that different ionizing groups function in the rate-limiting steps of acetylation and deacetylation, that the detailed mechanisms of these reactions were different (Krupka, 1966b), implying that two distinct mechanisms are involved in reaction of the acetyl enzyme with water or with the alcohol derived from the substrate during hydrolysis. The new evidence unveils the possibility that acetylation and deacetylation are homologous reactions, involving the same catalytic groups. In the direction of ester hydrolysis the group of pK = 5.3, previously called B_2, functions in a slow step in acetylation and the group of pK = 6.3, called B_1, functions in a fast step. The latter also functions in a slow step in deacetylation, and if, by analogy with acetylation, B_2 then acts in a fast step, the mechanisms would be identical, and an alcohol would be mechanistically equivalent to water.

From analogies in the amino acid sequence of chymotrypsin, trypsin, and elastase, two histidine residues have been postulated to function catalytically in these enzymes (Walsh *et al.*, 1964; Smillie and Hartley, 1964). If their mechanisms are similar to that of AChE (as suggested by irreversible inhibition of all four enzymes due to reaction of organophosphorus inhibitors with an activated serine residue (Cohen *et al.*, 1959)) then the two basic groups in AChE could well be histidine residues, and this is not inconsistent with their pK values. In fact if two histidine residues do function catalytically in chymotrypsin, a case can be made for their acting in succession, rather than simultaneously. Rates of acylation and deacylation of chymotrypsin show a pH dependence between 5.0 and 7.5 related to the protonation of a single ionizing group, rather than two or more (Bender *et al.*, 1964). Simultaneous involvement of two histidines could still be explained if addition of a proton to one drastically altered the pK of the other. The histidines in chymotrypsin would then have to behave abnormally in acid–base titration, whereas their ionization is apparently normal (Marini and Wunsch, 1963; Yapel and Lumry, 1964). Therefore, simultaneous participation must be ruled out. An interpretation suggested before (Krupka, 1966b) is that one histidine residue functions in acylation and the other in deacylation. An alternative to this is that both function, in succession, in acylation and possibly also in deacylation. If so an intermediate must occur, as in AChE, whose formation and decomposition are catalyzed by different enzyme groups.

A mechanism based on the assumption that the two ionizing group of AChE (pK = 5.3 and 6.3) are histidine

SCHEME I



residues is shown in Scheme I. B_2, representing the group of pK = 5.3 and catalyzing the first step in the reaction with substrate (CH_3COOR), is regarded as permanently held near a serine hydroxyl, as illustrated in the upper half of the diagram. The two imidazole nitrogens of B_2 are shown simultaneously accepting and donating a proton, as suggested by Brestkin and Rozengart (1965) and Bender and Kézdy (1965), and the intermediate is represented as a tetrahedral compound, since such an intermediate probably occurs in nonenzymic ester hydrolysis (Bender, 1960). Because of its rigid emplacement near the serine hydroxyl, B_2 is presumably unable to maneuver into the required position for catalysis of the second stage of the reaction. Instead, the carbonyl oxygen is transferred from B_2 to another basic group, B_1, of pK = 6.3 (right side of diagram). The latter interacts with the carbonyl and ester oxygens in a manner analogous to the role of B_2 in the first step, and following this an acetyl enzyme is formed (lower half of diagram). When water enters, it can only interact with B_1, since B_2 is not in the required position. Deacetylation could then proceed by a mechanism that is the reverse of acetylation, but whether or not it does so must be decided by future experiments.

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