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Hydrolysis of Neutral Substrates by Acetylcholinesterase*

R. M. Krupka

ABSTRACT: Evidence is presented to show that the same active center in a purified preparation of bovine erythrocyte acetylcholinesterase acts upon cationic and uncharged substrates. The pH dependence of V/K_m for hydrolysis of the two types of substrate differs. Experiments with acetylcholine show that the enzyme becomes inactive when an ionizing group of pK = 6.3 in the free enzyme is protonated, while experiments with phenyl acetate and isoamyl acetate indicate that another functional ionizing group in the free enzyme has a pK of 5.5. Thus two enzyme groups that ionize on the acid side of the pH optimum affect substrate hydrolysis. One (pK = 6.3) plays no essential role in substrate binding or in reaction of the enzyme–substrate complex, but when protonated, prevents cationic substrates from becoming bound to the active center. The other ionizing group (pK = 5.5) is an essential component of the catalytic mechanism.

Variations in the rates of enzymic reactions with pH may be due to ionizations of groups in the free enzyme which function in catalysis, but when the substrate bears a positive or negative charge, they may also result from ionizations that simply affect substrate binding. The latter groups may be revealed by comparison of the hydrolysis of neutral and charged substrates at different pH values. Before this comparison can be made, however, the same active center must be shown to act upon both types of substrate. This may be done in several ways. (1) Mixtures of the two substrates may be shown to compete for a single active site. (2) The dissociation constants of specific inhibitors may be shown to be the same with both types of substrate. (3) The same pH dependence for inhibition may be demonstrated with charged and neutral substrates.

Acetylcholine (AcCh),[1] the natural substrate of acetylcholinesterase (AChE), was chosen as a charged substrate, and phenyl acetate and isoamyl acetate as neutral substrates. Phenyl acetate is rapidly split by AChE, the rate-limiting step probably being deacetylation, as in the case of AcCh (Krupka, 1964). Isoamyl acetate is more slowly hydrolyzed, but is one of the best uncharged acetyl ester substrates derived from an aliphatic alcohol, since it sterically resembles AcCh (Mounter and Whittaker, 1950; Mounter and Cheatham, 1963).

Experimental Methods

The enzyme, supplied by Sigma Chemical Co. or Nutritional Biochemicals Corp., was a purified preparation from bovine erythrocytes. The two preparations had essentially the same kinetic properties. Spectrophotometric determinations of phenyl acetate hydrolysis were carried out in a Beckman DK2 recording spectrophotometer fitted with a thermostated cell holder, through which water at 26° was circulated. The reaction mixture was prepared as follows: to 5 ml of sodium phosphate buffer, 0.1 M, pH 7.5, were added various volumes (0.05–0.2 ml) of 0.517 M phenyl acetate in methanol[2] together with corresponding volumes of pure methanol, so that the total of methanol and phenyl acetate was 0.2 ml in all cases. In inhibited reactions 0.3 ml of 2.01×10^{-2} M AcCh was added. The volume

* From the Research Institute, Canada Department of Agriculture, London, Ontario, Canada. *Received March 30, 1965; revised January 24, 1966.* A preliminary report of this study has already appeared (Krupka, 1965).

[1] Abbreviations: AcCh, acetylcholine; AChE, acetylcholinesterase.

[2] Methanol (1–2%) caused only a small decrease (*ca.* 2–3%) in the maximum rate of AcCh hydrolysis and was accordingly used to solubilize the neutral substrates.

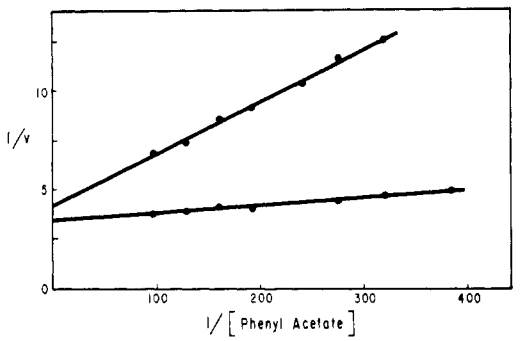

FIGURE 1: Lineweaver–Burk plot for rates of phenyl acetate hydrolysis in the presence (upper line) and absence (lower line) of 6.02×10^{-4} M AcCh. Initial rates were determined from the change in absorption at 270 mμ over a 6-min interval. Units of phenyl acetate concentration are molar.

was brought to a total of 8 ml with water, and 4 ml of the resulting mixture was removed and added to 1 ml of enzyme solution (14 mg of enzyme and gelatin/100 ml of water) in a cuvet. The remaining 4 ml was added to 1 ml of a solution containing 14 mg of gelatin/100 ml, and this solution was used as a control. The absorbance was followed at 270 mμ.

For the titrimetric determination of reaction rates (Krupka, 1963) in which the acid released during hydrolysis was titrated with 0.01 N NaOH, stock solutions of redistilled phenyl acetate, and isoamyl acetate in methanol were prepared immediately before use. The total volume of methanol and substrate in 10 ml of reaction mixture was kept constant at either 0.10 or 0.20 ml. The reaction mixtures contained 0.1 N NaCl and 0.04 M $MgCl_2$ and were sealed off to prevent CO_2 absorption or loss of substrates through evaporation. The temperature was maintained at 26°. Rates were corrected for buffering of the acetic acid product.

At substrate concentrations of $<6 \times 10^{-4}$ M the titration procedure was modified, in that a twin syringe assembly was used and the reaction volume was increased. One syringe contained 0.01 N NaOH and the other 0.01 N substrate. The two syringes were driven together, so that substrate was added to the reaction mixture as it was hydrolyzed, maintaining a steady concentration. Reaction volumes were increased to 20 or 100 ml at the lowest substrate concentrations, in order to keep the concentration of product low compared with substrate while the rate was being measured.

Competitive and noncompetitive inhibition constants, K_i and K_i', respectively, were determined according to the following relationships (Krupka, 1964)

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

and

$$K_i' = [I]/V_0(1/V - 1/V_0) \quad (2)$$

where [I] is the inhibitor concentration, "slope $(+I)$" and "slope $(-I)$" are the slopes of lines in $1/v \times 1/[S]$ plots in the presence and absence of inhibitor, or alternatively the intercepts in plots of $[S]/v \times [S]$ (see below). V and V_0 are the maximum velocities with and without inhibitor, respectively.

Ionization constants were determined by three methods, all based upon the simplified reaction scheme in which protonation of the free enzyme, E, and of the reactive enzyme–substrate complex, ES, gives inactive species, EH and EHS

$$E + H \overset{K}{\rightleftharpoons} EH \quad (3)$$

$$ES + H \overset{K_V}{\rightleftharpoons} EHS$$

The rate is then given by

$$1/v = (1/V)1 + [H]/K_V + K_m(1 + [H]/K)[S]) \quad (4)$$

and accordingly

$$K_m(\text{expt})/V(\text{expt}) = K_m(1 + [H]/K)/V \quad (5)$$

$$1/V(\text{expt}) = (1 + [H]/K_V)/V \quad (6)$$

Plots of log V(expt) and log $[V(\text{expt})/K_m(\text{expt})]$ *vs.* pH, therefore, reflect pK_V and pK, respectively, where K_m(expt) and V(expt) are the experimental values of K_m and V. In such plots, guide lines with slopes of 1 and 0 were drawn in the figure (Dixon, 1953), use being made of the fact that at the inflection point, where the lines intersect, the experimental curve is 0.3 log unit below the maximum. The pH corresponding to the inflection point is equal to the pK value of the ionizing group involved. In a second method, $K_m(\text{expt})/V(\text{expt})$ or $1/V(\text{expt})$ were plotted *vs.* [H], or alternatively $K_m(\text{expt})/V(\text{expt})[H]$ or $1/V[H]$ were plotted *vs.* 1/[H], and analysis was based on eq 5 and 6. In the third method rates of reaction were determined over a pH range, at a fixed substrate concentration that was either $\gtrless K_m$. In such experiments the enzyme concentration was increased in the pH range where the rates decline.[3] Plots were constructed of $[E]/v$ *vs.* $[H^+]$ and were analyzed according to eq 7 and 8

$$[E]_0/v = A(1 + [H]/K_V) \text{ for } [S] \gg K_m \quad (7)$$

and

$$[E]_0/v = B(1 + [H]/K) \text{ for } [S] \ll K_m \quad (8)$$

where A and B are constants, and where K and K_V are dissociation constants for ionizing groups in the free

[3] Linearity between reaction rate and enzyme concentration was demonstrated at pH 7.5.

enzyme and in enzyme–substrate intermediates, respectively (eq 3).

All experimental results were treated statistically and standard errors were determined by the method of least squares. Plots of $[S]/v$ *vs.* [S] have been shown by Dowd and Riggs (1965) to be preferable to those of $1/v$ *vs.* 1/[S]. Both types of plot were used to calculate constants and their standard errors, and were usually found to give very similar estimates. Most of the values given in the Results are based on the $[S]/v$[S] plot. For analysis based on eq 5–8, plots were constructed of K_m/V, $1/V$, and $[E]_0/v$ *vs.* $[H^+]$, as well as of $K_m/V[H^+]$, $1/V[H^+]$, and $[E]_0/v[H^+]$ *vs.* $1/[H^+]$. The latter correspond to the preferred $[S]/v$[S] plots, since the controlled variable appears in both coordinates. Again the estimates from the two plots were similar in all cases.

Experimental Results

Inhibition of Phenyl Acetate Hydrolysis by AcCh. The rates of phenyl acetate hydrolysis in 1% methanol solutions were determined by following the change in optical density at 270 mμ with substrate concentrations between 2.59×10^{-3} and 1.03×10^{-2} M, in either the presence or absence of 6.02×10^{-4} M AcCh. In experiments with AcCh, the rate was constant for approximately 6 min, but increased thereafter because of AcCh loss by hydrolysis. The reciprocals of the initial rates, measured as the changes in optical density over a 6-min interval, are plotted *vs.* 1/[S] in Figure 1. AcCh is seen to inhibit phenyl acetate hydrolysis in a manner which is largely competitive, but which includes a small noncompetitive component, as indicated by the decrease in the maximum velocity caused by AcCh. K_m for phenyl acetate and the competitive and noncompetitive inhibition constants for AcCh, K_i, and K_i', respectively, were calculated

$$K_m = 1.056 \pm 0.137 \times 10^{-3}\ \text{M}$$

$$K_i = 1.014 \pm 0.110 \times 10^{-4}\ \text{M}$$

$$K_i' = 2.65 \pm 1.08 \times 10^{-3}\ \text{M}$$

If AcCh inhibits phenyl acetate hydrolysis by combining with AChE, K_i should correspond to K_m for AcCh, and K_i' should be roughly equal to the constant for substrate inhibition, K_s. Rates of AcCh hydrolysis were determined using the hydroxylamine–$FeCl_3$ colorimetric assay of Hestrin (1949) under conditions identical with those above, except for the addition of pure methanol to the reaction mixture rather than phenyl acetate in methanol. K_m is low under these conditions, making the rates at suboptimal AcCh concentrations difficult to measure. Experiments at above-optimal substrate concentrations were interpreted on the basis of the following reaction scheme[4]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + \text{products} \qquad (9)$$

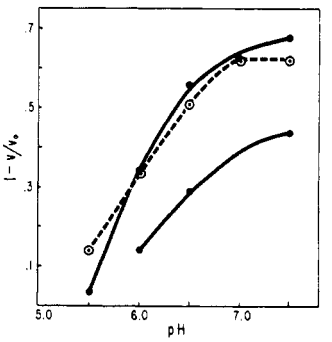

FIGURE 2: Relative inhibitions $(1 - v/v_0)$ of ester hydrolysis by tetramethylammonium chloride at pH 5.5–7.5, v and v_0 being rates in the presence and absence of inhibitor, respectively. Dashed line, 1.02×10^{-3} M AcCh, 1.76×10^{-2} M inhibitor. Upper solid line, 5.10×10^{-3} M phenyl acetate, 4.92×10^{-2} M inhibitor. Lower solid line, 5.10×10^{-3} M phenyl acetate, 1.64×10^{-2} M inhibitor.

$$ES + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES_2 \qquad (10)$$

The rate is given by

$$v = k_2[E]_0/(1 + K_m/[S] + [S]/K_s) \qquad (11)$$

where $K_m = (k_{-1} + k_2)/k_1$. At high substrate concentrations, where $K_m/[S] \ll 1$, eq 11 may be written

$$1/v = (1 + [S]/K_s)/k_2[E]_0 \qquad (12)$$

From a plot according to eq 12, the calculated value of K_s was $4.93 \pm 0.71 \times 10^{-3}$ M in fair agreement with K_i' above.

A second test involves measuring the optimum substrate concentration in phosphate buffer. By differentiation of eq 11, it may be shown that

$$[S]_{opt} = (K_m K_s)^{1/2} \qquad (13)$$

From K_i and K_i', $[S]_{opt}$ should be $5.18 \pm 1.20 \times 10^{-4}$ M. The experimental value agrees with this, being between 4 and 8×10^{-4} M.

Inhibition of Phenyl Acetate Hydrolysis by 3-Hydroxyphenyltrimethylammonium Iodide. The inhibition of phenyl acetate hydrolysis was studied by the titration method in the presence of 9.2×10^{-7}, 1.84×10^{-6}, and 3.59×10^{-6} M 3-hydroxyphenyltrimethylammonium iodide. The competitive inhibition constant, K_i, was $1.09 \pm 0.06 \times 10^{-6}$ M. For comparison, the inhibition

[4] Previous evidence indicated that substrate inhibition is due to formation of an inactive complex between the substrate and the acetyl enzyme (Krupka, 1963, 1964). For present purposes it is simpler, and more general, to make use of the scheme shown in (9) and (10), since the conclusions should then be valid, whatever the details of the inhibition mechanism.

TABLE I: Determination of pK and pK_V (Eq 7 and 8) for Phenyl Acetate, Isoamyl Acetate, and Acetylcholine from Rate Measurements at a Fixed Substrate Concentration.

Substrate	Concn (M)	[S]/K_m Range	pH Range	Methanol (%)	pK	pK_V
Phenyl acetate	5.14×10^{-5}	0.15–0.05	5.5–7.0	1.0	5.55 ± 0.05	
Phenyl acetate	8.84×10^{-3}	15.8–7.1	6.2–7.5	2.0		6.35 ± 0.02
Isoamyl acetate	5.07×10^{-4}	0.076–0.090	5.5–6.5	1.0	5.30 ± 0.05	
Isoamyl acetate	1.00×10^{-2}	1.5–1.8	5.5–7.0	2.0		5.50 ± 0.04
Acetylcholine Br	4.04×10^{-5}	0.21	6.3–7.5	0.0	6.27 ± 0.05	
Acetylcholine Br	2.02×10^{-3}	10.6	6.2–7.5	0.0		6.32 ± 0.04
Acetylcholine Br	2.02×10^{-3}	7.6	6.0–7.5	2.0		6.34 ± 0.04

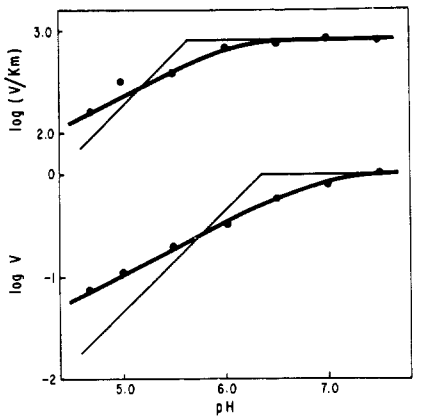

FIGURE 3: Plots of log V and log (V/K_m) *vs.* pH for hydrolysis of phenyl acetate in 1% methanol. The pK values of the enzyme's ionizing groups are given by the pH at which the observed enzyme constant becomes half-maximal (0.3 log unit below the maximum). pK values are 5.60 [log (V/K_m)] and 6.35 (log V). Guide lines of slopes 1 and 0 intersecting at this point have been drawn into the figure. If a single ionization determines the size of the experimental constants, points at low pH should fall along the line of unit slope.

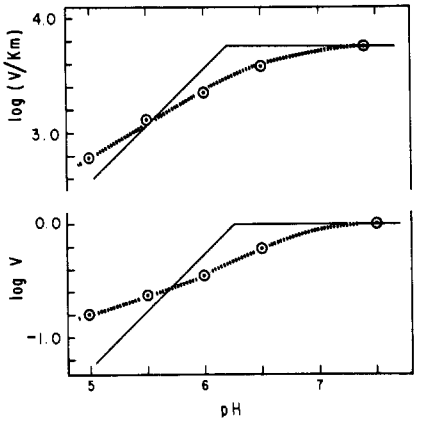

FIGURE 4: Plots of log V and log (V/K_m) for AcCh hydrolysis as functions of pH, as in Figure 3. The pK values are 6.20 [log (V/K_m)] and 6.26 (log V).

of AcCh hydrolysis was measured under similar conditions, *i.e.*, in a reaction solution containing 1% methanol, and in this case K_i was equal to $1.26 \pm 0.24 \times 10^{-6}$ M. In another experiment it was observed that 3.6×10^{-3} M 3-hydroxyphenyltrimethylammonium ion completely blocked the hydrolysis of phenyl acetate at pH values from 5.0 to 8.0.

pH Dependence of Tetramethylammonium Ion Inhibition. Fixed concentrations of phenyl acetate (5.10×10^{-3} M) and tetramethylammonium chloride (either 4.92×10^{-2} or 1.64×10^{-2} M) were incubated at pH values between 5.5 and 7.5 in the presence of salt and enzyme, and the rates were determined by titration of acid released. The relative inhibitions, $1 - v/v_0$, are plotted in Figure 2, where v and v_0 are rates in the presence and absence of inhibitor, respectively. For comparison, data for inhibition of hydrolysis of 1.02×10^{-3} M AcCh by 1.76×10^{-2} M tetramethylammonium chloride, in the absence of methanol, are also shown.

pH Dependence of Phenyl Acetate Hydrolysis. Rate measurements were carried out by titration at substrate concentrations between 1.5 and 5×10^{-3} M. The experimental values of log V (relative to a maximum value arbitrarily set at unity) and of log V/K_m are plotted *vs.* pH in Figure 3. The standard error of log V had an average value of 0.009 for the various determinations, and the average standard error of log (V/K_m) was 0.055. The values of pK_V and pK are found to be 6.35 and 5.60, respectively.

These constants were also determined by measuring rates over a pH range at a fixed substrate concentration, with analysis according to eq 7 and 8. The calculated pK values, together with standard deviations, are listed in Table I.

pH Dependence of Isoamyl Acetate Hydrolysis. The rates of isoamyl acetate hydrolysis were determined by titration at substrate concentrations between 2.5×10^{-3} and 1.0×10^{-2} M. The experimental values of

TABLE II: Isoamyl Acetate Hydrolysis at 26° in the Presence of 0.10 M NaCl, 0.04 M $MgCl_2$, and 2% Methanol (see Methods).[a]

pH	K_m	Rel V	pK	pK_V
7.5	$5.81 \pm 0.58 \times 10^{-3}$	1.000		
6.0	$5.50 \pm 0.57 \times 10^{-3}$	0.694		
5.5	$6.69 \pm 0.93 \times 10^{-3}$	0.531	5.58 ± 0.02	5.57 ± 0.10

[a] The maximum velocity of isoamyl acetate hydrolysis at pH 7.5, arbitrarily set at unity in the table, was 0.20 $\times$ V for AcCh at the same pH. Values of pK and pK_V were calculated on the basis of eq 5 and 6.

K_m and of V are shown in Table II together with the calculated values of pK, for ionization in E, and of pK_V, for ionization in ES. The constants were also calculated from rates at a fixed substrate concentration (eq 7 and 8), and these values are shown in Table I.

pH Dependence of AcCh Hydrolysis. For comparison with the experiments on neutral substrates, AcCh hydrolysis was measured by the titration method. V and V/K_m were determined in the absence of methanol over the pH range 5.0–7.5 (Figure 4). The average standard errors in log V and log (V/K_m) were 0.007 and 0.068, and pK and pK_V were found to be 6.26 and 6.20. These constants were redetermined from rates at a fixed substrate concentration (Table I). Determinations were also carried out in the presence of 2% methanol: at pH 7.5 and 6.0, K_m was $3.20 \pm 0.25 \times 10^{-4}$ M and $2.29 \pm 0.22 \times 10^{-4}$ M, respectively. The value of V at pH 7.5 relative to that at pH 6.0 was 2.76 ± 0.07. From these data, pK and pK_V were found to be 6.01 ± 0.12 and 6.29 ± 0.02, respectively. pK_V was also determined from rates at a fixed substrate concentration in 2% methanol (Table I).

Discussion

The agreement between the binding constants for AcCh as an inhibitor of phenyl acetate hydrolysis and as a substrate is good evidence that the same active center acts upon both esters. This conclusion is fortified by the observation of similar binding constants for reversible inhibition of phenyl acetate and AcCh hydrolysis by 3-hydroxyphenyltrimethylammonium ion. The latter is a remarkably potent and specific inhibitor of AChE from electric organ (Wilson and Quan, 1958) and bovine erythrocytes (Krupka, 1965), and is bound approximately 2000 times more strongly than choline and 120 times more strongly than phenyltrimethylammonium ion. The conclusion is also supported by the pH dependence of tetramethylammonium ion inhibition of phenyl acetate and AcCh hydrolysis, which is similar, even though hydrolysis of the two substrates is affected in a different manner by pH. In addition, the following evidence indicates that the over-all catalytic mechanism with neutral and charged substrates is the same. (1) Experiments on trimethylammonium ion inhibition of the hydrolysis of a series of acetyl ester substrates, including phenyl acetate and AcCh, showed that an intermediate acetyl enzyme was formed with all substrates, and also that the rate-limiting step in both phenyl acetate and AcCh hydrolysis was probably deacetylation (Krupka, 1964). (2) Wilson (1960) measured the relative rates of hydrolysis and hydroxylaminolysis in the presence of hydroxylamine, using AcCh and ethyl acetate as substrates. The enzyme was derived from the electric organ of the electric eel. The same ratio was found with both esters, indicating that water and hydroxylamine react with a common reaction intermediate formed with both substrates. The common intermediate must be an acetyl enzyme.

Estimates of pK and pK_V from pH effects on either V/K_m and V (eq 5 and 6) or rates at low and high substrate concentrations (eq 7 and 8) are in agreement. With phenyl acetate, values of 5.60 and 6.35 are obtained by the first method, and 5.55 ± 0.05 and 6.35 ± 0.02 by the second. With acetylcholine, the constants were 6.20 and 6.26 as against 6.27 ± 0.05 and 6.32 ± 0.04; and with isoamyl acetate 5.58 ± 0.02 and 5.57 ± 0.10 compared with 5.30 ± 0.05 and 5.50 ± 0.04. The experiments on AcCh hydrolysis in 2% methanol show that the alcohol does not cause a shift in the ionization constant. For example, pK_V is 6.32 ± 0.04 in pure water and 6.34 ± 0.04 in 2% methanol. In methanol, pK is 6.01 ± 0.12, and in water is 6.26 from V/K_m and 6.27 from rates at low substrate concentration.

Since different pK values are observed for ionizations in the free enzyme with neutral and cationic substrates, even though the same active center is involved in the hydrolysis of both, the conclusion seems unavoidable that two different ionizing groups affect the catalytic properties of AChE. Plots of V/K_m[5] for phenyl acetate and isoamyl acetate indicate that protonation of an

[5] It may be noted that effects of pH on V/K_m depend only upon ionizations in the free enzyme and not in enzyme–substrate intermediates. This conclusion is independent of assumptions regarding the number and kinds of enzyme–substrate intermediates (Krupka and Laidler, 1960).

ionizing group of pK 5.5, which is present in the free enzyme, results in loss of enzyme activity. The pH dependence of V/K_m for AcCh reflects the presence, also in the free enzyme, of a group of pK = 6.3. Tetramethylammonium ion binding is affected by the ionization of the latter group, whether the substrate is AcCh or phenyl acetate. The experiments therefore suggest that the group of pK = 6.3 prevents binding of cationic substrates and inhibitors, but is not otherwise involved in forming ES. Furthermore, this group cannot be required in the reaction of ES, *i.e.*, in acetylation, since in this case its ionization would be reflected in V/K_m for neutral substrates. On the other hand, the second ionizing group, pK = 5.5, is essential in the formation or reaction of ES, at least with neutral substrates.

To explain the pH dependence of V with various substrates, formation of an acetyl enzyme from the enzyme–substrate complex during the course of the reaction must be taken into account. This subject, as well as the function of the two ionizing groups, will be dealt with in the following paper (Krupka, 1966). Also deferred is discussion of the increase in V for phenyl acetate and AcCh hydrolysis above the expected values at pH 5.5–4.7 (Figures 3 and 4).

Acknowledgment

The technical assistance of Mr. F. Smeltzer is gratefully acknowledged.

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Chemical Structure and Function of the Active Center of Acetylcholinesterase*

R. M. Krupka

ABSTRACT: Acetyl ester substrates of acetylcholinesterase fall into two classes on the basis of the pH dependence of their maximum hydrolysis rates. The most rapidly hydrolyzed depend upon an ionizing group in the enzyme of pK = 6.3, while poorer substrates depend on a group of pK = 5.5. This and other evidence has led to the following conclusions regarding the active center.

Two basic groups are present which are catalytically active when unprotonated. One (pK = ~5.5) functions in acetylation and is apparently located at least 9 A from the anionic site, while the other (pK = 6.3) functions in deacetylation and is situated within 5 A of the anionic site. The anionic site undergoes an apparent neutralization when the group of pK = 6.3 is protonated. The latter thus bears a positive charge which repels cationic substrates or inhibitors from the active center. The anionic site itself is protonated at pH 4.0–4.5 and is, therefore, probably a side-chain carboxyl group. A fourth group (pK = 9.2), which functions catalytically in the protonated form, appears to be more than 10 A from the anionic site.

The active center of AChE[1] may be divided into two parts, an anionic site which attracts the positive charge in AcCh or cationic inhibitors, and some 5 A away, an esteratic site which catalyzes the hydrolysis of the ester linkage in a substrate (Wilson, 1960). There is evidence that groups ionizing in the range of pH 5–10 function in catalysis, and some of these are presumably constituents of the esteratic site. The pK values of these groups should be altered in the presence of a positive

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[1] Abbreviations: AChE, acetylcholinesterase; AcCh, acetylcholine bromide; ES, enzyme–substrate complex; EA, acetyl enzyme.