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

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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.

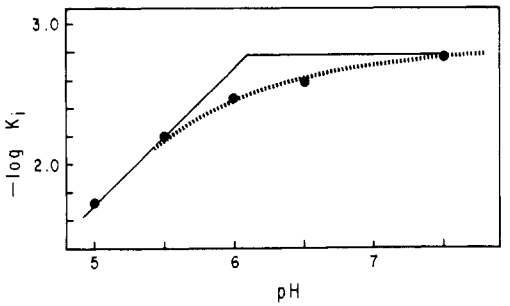

FIGURE 1: Plot of pK_i *vs.* pH for competitive inhibition of AcCh hydrolysis by tetramethylammonium chloride. The pK of the ionizing group which affects binding, corresponding to the pH at which pK_i is 0.3 unit below its maximum value, and shown by the point of intersection of the guide lines of slopes 1 and 0, is 6.17 ± 0.14 (see Experimental Results).

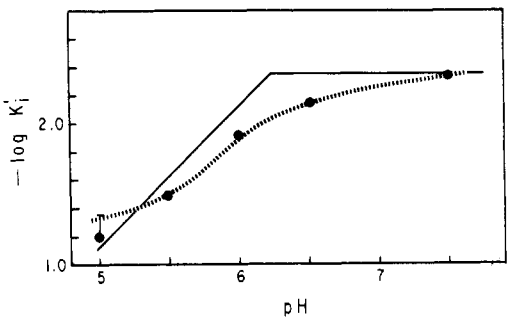

FIGURE 2: Plot of pK_i' *vs.* pH for noncompetitive inhibition of AcCh hydrolysis by trimethylammonium chloride. The pK value of the ionizing group affecting binding is 6.25 ± 0.05. The point at pH 5.0 shows the experimentally determined constant, and the bar indicates a correction (see Experimental Results, 1).

charge, such as that in an inhibitor or substrate molecule held at the anionic site, and the size of this effect should reflect the distance between the ionizing group and the bound cation. Experiments based on this idea have been performed with a view to answering the following questions: (1) What causes the apparent neutralization of the anionic site at pH 6? (2) How close to the anionic site are the three ionizing groups implicated in the catalytic process (pK = 5.5, 6.3, and 9.2)? (3) What roles do the two basic groups (pK = 5.5 and 6.3) play in the two phases of hydrolysis, acetylation and deacetylation? As will be seen, answers to these questions are provided by the experiments.

Experimental Methods

Rates of hydrolysis of ester substrates were determined by automatic titration of acid released with 0.01 N NaOH (Krupka, 1966). The reaction mixture, at 26°, contained 0.10 M NaCl and 0.04 M $MgCl_2$. Purified bovine erythrocyte AChE was obtained from Sigma Chemical Co. or Nutritional Biochemicals Corp. The two enzyme preparations had essentially the same kinetic properties. 4-Trimethylaminobutyl acetate iodide, dipropylmethylaminoethyl acetate iodide, and diethylbutylaminoethyl acetate iodide were generously donated by Dr. W. C. Dauterman of the Pesticide Residues Laboratory, North Carolina State College. *N*-Methylaminoethyl acetate was synthesized according to the method of Wilson (1954). Other chemicals were of reagent grade. Methods of analyzing kinetic data were given in the preceding paper (Krupka, 1966).

Experimental Results

(1) Tetramethylammonium and Trimethylammonium Ion Inhibition. The first experiments to be described concern the effects of pH changes on the binding of two cationic inhibitors, tetramethylammonium and trimethylammonium ions. With tetramethylammonium chloride, an approximately linear relationship was demonstrated, at a fixed AcCh concentration (1.0×10^{-3} M), between the reciprocal of the reaction rate and the inhibitor concentration up to 0.15 M. This shows that inhibition is due to binding of a single inhibitor molecule to the enzyme, as expected in ordinary competitive inhibition. Competitive inhibition constants in AcCh hydrolysis were determined at pH values from 5 to 7.5, and the average standard error of pK_i was found to be 0.059 log unit. The results are plotted in Figure 1. Binding depends on the ionization state of an enzyme group of pK = 6.17 ± 0.14, as determined from a plot of K_i *vs.* $[H^+]$.

In trimethylammonium ion inhibition the relation between $1/v$ and [I] was not linear, the inhibitor being relatively less effective as its concentration rose above 0.01 M at pH 7.5 or >0.02 M at pH 5.0. This did not alter inhibition constants at pH 6–7 where inhibitor concentrations were comparatively low, but at pH 5.0, where the concentration was higher (0.042 M) the association constant should be low. A correction was determined from rates at pH 5.0 with 0.002 M AcCh, $1/v$ being plotted as a function of [I]. A straight line was drawn through the points for low inhibitor concentrations to give the extrapolated rate at 0.042 M trimethylamine, which was 1.16 times the experimental value. As trimethylammonium ion inhibition follows noncompetitive kinetics, the ratio of rates in the presence and absence of inhibitor is independent of substrate concentration. The maximum velocity in the presence of inhibitor may therefore be corrected directly and substituted into eq 2 of the preceding paper to give K_i', the noncompetitive inhibition constant. In the case of trimethylamine hydrochloride, this constant is a measure of binding to the acetyl enzyme. K_i' is shown as a function of pH in Figure 2, with a correction of the experimental result at pH 5.0. The average standard error of pK_i' was 0.027. Binding is affected by an ionizing group in the enzyme of pK = 6.25 ± 0.05 (calculated from a plot of K_i' *vs.* $[H^+]$ for pH 5.5–7.5).

TABLE I: Hydrolysis Constants for AcCh Analogs.[a]

Substrate	pH	Rel V	K_m	pK	pK_V
Acetylthiocholine iodide	7.5		$1.032 \pm 0.273 \times 10^{-4}$		
	5.0		$1.899 \pm 0.352 \times 10^{-4}$		
Dimethylaminoethyl acetate hydrochloride	6.5		$2.06 \pm 0.22 \times 10^{-3}$		
	5.0		$2.34 \pm 0.47 \times 10^{-3}$		
Methylaminoethyl acetate hydrochloride	6.5	1.000	$1.724 \pm 0.184 \times 10^{-2}$	5.90 ± 0.10	5.20 ± 0.03
	5.5	0.630	$2.925 \pm 0.141 \times 10^{-2}$		
	5.0	0.405	$5.424 \pm 0.567 \times 10^{-2}$		
Dipropylmethylaminoethyl acetate iodide	7.5	1.000	$1.053 \pm 0.077 \times 10^{-3}$		
	6.0	0.930	$2.594 \pm 0.115 \times 10^{-3}$	6.25 ± 0.07	
	5.5	0.710	$2.714 \pm 0.179 \times 10^{-3}$	5.98 ± 0.06	5.10 ± 0.10
Diethylbutylaminoethyl acetate iodide	7.5	1.00	$8.30 \pm 0.57 \times 10^{-4}$	5.64 ± 0.06	5.51 ± 0.02
	5.5	0.50	$9.57 \pm 1.04 \times 10^{-4}$		
Trimethylaminobutyl acetate iodide	7.5	1.000	$2.94 \pm 0.30 \times 10^{-3}$		
	6.0	0.820	$4.17 \pm 0.33 \times 10^{-3}$	5.88 ± 0.09	
	5.5	0.510	$8.83 \pm 0.62 \times 10^{-3}$	5.64 ± 0.09	5.46 ± 0.07

[a] The maximum velocity at the highest pH studied is arbitrarily set at unity. With dipropylmethylaminoethyl acetate and trimethylaminobutyl acetate pK values have been computed from data for pH 7.5 together with pH 6.0 or 5.5 (see Discussion).

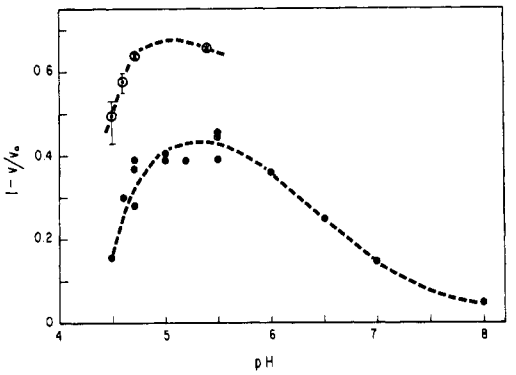

FIGURE 3: Inhibition of AcCh hydrolysis by betaine as a function of pH. The expression $1 - v/v_0$ reflects inhibitory strength, v and v_0 being hydrolysis rates in the presence and absence of inhibitor, respectively. In the upper and lower curves the betaine concentrations are 1.02×10^{-1} and 3.43×10^{-2} M, respectively, and the concentration of AcCh is 1.0×10^{-3} M in all cases. The points on the upper curve correspond to the average of several determinations and the bars connected with each point show the maximum spread of calculated values based on the highest and lowest v and v_0 at each pH.

(*2*) *Betaine Inhibition.* As discovered by Bergmann and Shimoni (1952) and confirmed by the experiments described above, inhibition by cations declines at pH <6, suggesting a neutralization of the anionic site. Neutralization could be caused by addition of a proton to the anionic site itself, which may be a carboxylate anion. Alternatively, it might result from protonation of a neighboring group, which then, being positively charged, could repel inhibitory cations from the anionic site. To distinguish between these mechanisms, the binding of the zwitterion, betaine, was studied. Relative inhibitions ($1 - v/v_0$) of the hydrolysis of 1.0×10^{-3} M AcCh by betaine (3.43×10^{-2} or 1.02×10^{-1} M) are plotted in Figure 3, v and v_0 being rates in the presence and absence of inhibitor, respectively. Inhibition is maximal at pH 5–6 and declines at higher and lower pH values.

(*3*) *Hydrolysis of Cationic Substrates.* The pH dependence of the hydrolysis of six cationic substrates was studied. Estimates of K_m, V, pK, and pK_V are listed in Table I. Other estimates of pK and pK_V were obtained from rate measurements made over a pH range at a single substrate concentration, which was either $\gtrless K_m$. These values are given in Table II. In the case of acetylthiocholine, the observed rates were corrected for ionization of the product thiocholine, whose pK_a was taken to be 7.8 (Krupka, 1964). pK and pK_V refer to ionizations in the free enzyme and in rate-limiting enzyme–substrate intermediates, respectively (Krupka, 1966).

(*4*) *Effect of Tetraethylammonium Ion at Low pH.* The maximum velocity of AcCh hydrolysis, which probably reflects the rate of deacetylation, is dependent upon the ionization state of a group of pK = 6.3 (Krupka, 1966). To determine the nearness of this group to the anionic site, inhibition by tetraethylammonium bromide was studied at pH 5.0 and 5.5. Previous work has shown that this inhibitor may become bound at the anionic site without greatly hindering deacetylation (Krupka, 1965a). If the ionizing group

TABLE II: Determination of pK and pK_V for AcCh Analogs from Rate Measurements at a Fixed Substrate Concentration.

Substrate	Concn (M)	$[S]/K_m$ Range	pH Range	pK	pK_V
Acetylthiocholine	2.08×10^{-3}	17.5–15.9	6.2–7.5		6.18 ± 0.05
Dimethylaminoethyl acetate	5.40×10^{-4}	0.25	5.7–7.0	5.85 ± 0.03	
Dimethylaminoethyl acetate	9.23×10^{-3}	4.2	5.0–6.5		5.50 ± 0.04
Methylaminoethyl acetate	4.87×10^{-4}	0.023–0.028	5.9–7.0	5.90 ± 0.03	
Methylaminoethyl acetate	3.27×10^{-1}	5.9–15.3	5.0–5.9		5.15 ± 0.07
Dipropylmethylaminoethyl acetate	1.22×10^{-4}	0.077–0.116	6.0–7.5	6.20 ± 0.03	
Dipropylmethylaminoethyl acetate	1.107×10^{-2}	3.7–10.5	5.4–7.5		5.20 ± 0.06
Diethylbutylaminoethyl acetate	1.07×10^{-4}	0.12	6.0–7.5	6.25 ± 0.06	
Diethylbutylaminoethyl acetate	5.35×10^{-3}	6.9–5.5	5.5–7.5		5.50 ± 0.08

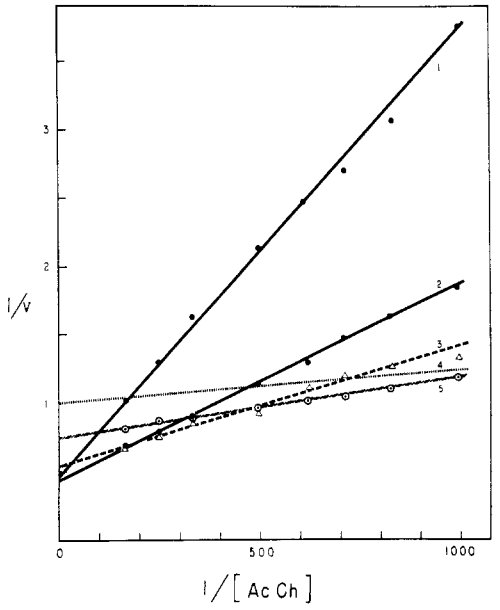

FIGURE 4: Effects of tetraethylammonium bromide on rates of AcCh hydrolysis at pH 5.0. Units of AcCh concentrations are molar, and the maximum velocity in the absence of tetraethylammonium is arbitrarily set at unity (line 4). Each line corresponds to hydrolysis rates in the presence of a fixed concentration of the cation: (1), 4.78×10^{-2} M; (2), 1.90×10^{-2} M; (3), 9.56×10^{-3} M; (4), no inhibitor; (5), 4.75×10^{-3} M. The experimental ratios V_0/V are (1) 0.522 ± 0.030; (2) 0.461 ± 0.013; (3) 0.559 ± 0.021; (4) 0.789 ± 0.024.

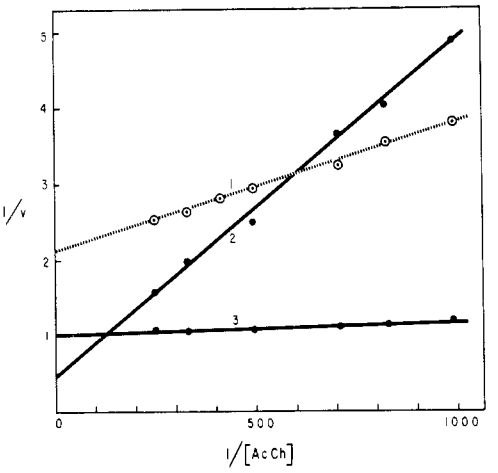

FIGURE 5: Effects of tetraethylammonium and tetrapropylammonium bromides on rates of AcCh hydrolysis at 5.5. Units as in Figure 5. Line 1, 2.48×10^{-3} M tetrapropylammonium; line 2, 4.76×10^{-2} M tetraethylammonium; line 3, no inhibitor. V_0/V with tetraethylammonium is 0.47 ± 0.10.

is near the anionic site, the inhibitor should compete with protons for the active center, and by displacing them should increase the maximum velocity at low pH. The results are shown in Figures 4 and 5. A similar experiment at pH 5.5 is included with tetrapropylammonium bromide (Figure 5), which is known to block deacetylation when bound to the acetyl enzyme (Krupka, 1965a). Inhibition by this ion is seen to be of the noncompetitive type, and similar behavior was observed at pH 5.0, as well as at higher pH.

Experiments at pH 5.0 with and without 1.43×10^{-2} M tetraethylammonium bromide were carried out with three other substrates: V_0/V was found to be 0.463 ± 0.064 with phenyl acetate (in reaction mixtures containing 1% methanol), 0.542 ± 0.078 with acetylthiocholine iodide, and 1.047 ± 0.254 with diethylbutylaminoethyl acetate iodide.

(5) *Inhibition by Tetrapropylammonium Bromide.* Competitive and noncompetitive inhibition constants, K_i and K_i', were measured at high pH with 3.13×10^{-4} M tetrapropylammonium bromide. The substrate was AcCh. K_i was found to be $1.51 \pm 0.35 \times 10^{-4}$ and $1.38 \pm 0.22 \times 10^{-4}$ M at pH 8.0 and 9.5, respectively, and K_i' was $2.14 \pm 0.21 \times 10^{-4}$ and $2.06 \pm 0.16 \times$

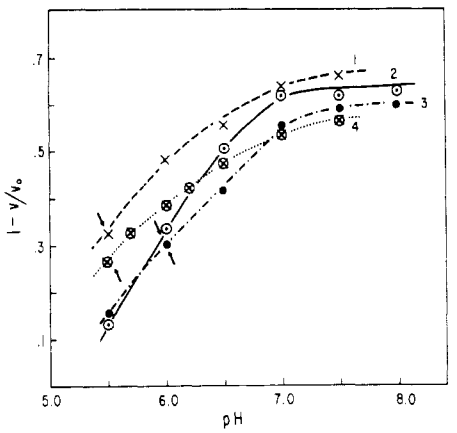

FIGURE 6: Relative inhibitions ($1 - v/v_0$) of AcCh (1.02×10^{-3} M) hydrolysis by 4.36×10^{-4} M tetra-*n*-propylammonium bromide (1), 1.76×10^{-3} M tetramethylammonium chloride (2), 1.03×10^{-2} M trimethylammonium chloride (3), and 1.36×10^{-4} M tetra-*n*-butylammonium iodide (4), where v and v_0 are rates with and without inhibitor. The arrows mark the pH where inhibition is half maximal.

10^{-4} M at the same pH values. Thus, no change in binding constants is apparent at high pH. In a second experiment, rates of hydrolysis of 1.01×10^{-3} M AcCh were measured in the presence and absence of 3.13×10^{-4} M tetrapropylammonium bromide. In this experiment NaCl and $MgCl_2$ were omitted from the reaction mixture in order to accentuate effects of electrostatic interactions at the enzyme surface. The relative inhibitions, $1 - v/v_0$, v and v_0 being the rates with and without inhibitor, were 0.761, 0.776, and 0.779 at pH 8.0, 9.0, and 9.5, respectively. Again, if high pH alters the binding of cations, the effect must be small.

(*6*) *Relative Inhibitions by Trimethyl-, Tetramethyl-, Tetrapropyl-, and Tetrabutylammonium Ions.* Inhibitions of AcCh hydrolysis (1.02×10^{-3} M) by tetra-*n*-propylammonium bromide (4.36×10^{-4} M) and tetra-*n*-butylammonium iodide (1.36×10^{-4} M) were compared with those caused by tetramethylammonium chloride (1.76×10^{-3} M), trimethylammonium hydrochloride (1.03×10^{-2} M) in the pH range 5.5–8.0. Initial rates were measured with or without inhibitor (v and v_0, respectively) and the relative inhibitions, $1 - v/v_0$, were plotted *vs.* pH (Figure 6). With the first two cations inhibition became half-maximal at pH 5.5, but with the second pair, at pH 6.0. It was also shown that 1.00×10^{-2} M NaBr or NaI had no significant effect on the rate of AcCh hydrolysis (2.0×10^{-3} M) when added to a reaction mixture containing 0.10 M NaCl.

Discussion

pH Dependence of the Maximum Velocity. Hydrolyses catalyzed by AChE probably proceed in three steps, as follows (Wilson, 1960; Krupka, 1964)

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

where E is the enzyme, ES the enzyme–substrate complex, EA an acetyl enzyme, and C and A the alcohol and acid components of the ester substrate (S), respectively. From the steady-state treatment of this scheme, $K_m = (k_{-1} + k_2)/k_1 (1 + k_2/k_3)$ and $V = k_2[E]_0/(1 + k_2/k_3)$, V being the maximum velocity. Also, if E, ES, and EA may be protonated to form inactive species, EH, EHS, and EHA, with ionization constants K, K', and K'', respectively, then K can be calculated from the pH dependence of V/K_m, and a weighted mean of K' and K'', given by $(k_2 + k_3)/(k_3/K' + k_2/K'')$, can be calculated from the pH dependence of V (Krupka and Laidler, 1960a). These relationships are quite general, and do not depend on the particular assumptions made about the kinds of enzyme–substrate intermediates.

There is evidence that with the most rapidly hydrolyzed substrates, AcCh, acetylthiocholine, and phenyl acetate, the rate-limiting step is probably deacetylation ($k_2 > k_3$) (Krupka, 1964). With acetyl ester substrates having a lower maximum velocity, the limiting rate must be acetylation ($k_2 < k_3$). This follows since a common intermediate, an acetyl enzyme, is probably formed during hydrolysis, and the rate of reaction of this intermediate must be the same for all the substrates. Hence pH effects on V for AcCh, acetylthiocholine, and phenyl acetate hydrolysis may be regarded primarily as effects on the rate of deacetylation, but with slowly hydrolyzed acetyl ester substrates V should be a measure of the acetylation rate. With the first group of substrates, the relations between pH and V should, if the argument is correct, be very similar, and possibly different from the pH dependence of V with substrates having a low V compared with AcCh.

The experiments bear out this idea. The pK_V value for hydrolysis of phenyl acetate or AcCh is *ca.* 6.3 (Krupka, 1966). Furthermore, at pH 5.0 and 5.5, the values of V for both substrates increase above that expected from ionization of a single group. A possible explanation for this deviation will be discussed later. For acetylthiocholine, $pK_V = 6.18$ (Table II). With more slowly hydrolyzed acetyl ester substrates, namely methylaminoethyl acetate, dimethylaminoethyl acetate, dipropylmethylaminoethyl acetate, diethylbutylaminoethyl acetate, and 4-trimethylaminobutyl acetate, pK_V varies between *ca.* 5.2 and 5.5 (Tables I and II), and in isoamyl acetate hydrolysis, pK_V is 5.50 (Krupka, 1966). Values of pK' and pK'' for neutral and charged substrates are summarized in Table III. It may be noted that values of pK_V calculated from log V (Table I) or from rates at high substrate concentration (Table II) are equivalent, as found before (Krupka, 1966).

Binding of Cations in Relation to Ionization of the First Basic Group (pK = 6.3). The anionic site becomes neutralized at pH values below 6, as shown by weakened binding of cationic inhibitors such as tetramethylammonium ion (Figure 1). The pK of the group which

TABLE III: Summary of Data on pH Dependence of Hydrolysis with Neutral and Cationic Substrates.[a]

Substrate	Rel V (pH 7.5)	Rate-Limiting Step	pK	pK'	pK''
Phenyl acetate	1.13	EA → E + A	5.55		6.35
Acetylcholine	1.00	EA → E + A	6.27		6.32
Acetylthiocholine	0.83	EA → E + A			6.18
Isoamyl acetate	0.20	ES → EA	5.5	5.50	
Dimethylaminoethyl acetate	0.36	ES → EA	5.85	5.50	
Methylaminoethyl acetate	0.098	ES → EA	5.90	5.20	
Diethylbutylaminoethyl acetate	0.164	ES → EA	6.25	5.50	
Dipropylmethylaminoethyl acetate	0.054	ES → EA	6.20	5.20	
4-Trimethylaminobutyl acetate	0.019	ES → EA	5.88	5.46	

[a] pK, pK', and pK'' refer to ionizations in E, ES, and EA, respectively.

affects binding is *ca.* 6.17. One explanation is that the anionic site is a carboxyl group having an exceptionally high pK because of charge interactions in the protein.[2] Another is that a neighboring group of pK = 6.17 becomes positively charged when protonated and then repels cations from the anionic site. The experiments on betaine inhibition rule out the first hypothesis, since the zwitterion is most tightly held to the enzyme when the anionic site has been "neutralized" (Figure 3). This behavior cannot be explained if neutralization is due to addition of a proton to a carboxylate anion, but is expected if a neighboring group becomes positively charged. Betaine presumably aligns itself so that its carboxyl group is attracted to the latter, while its ammonium ion is bound at the anionic site.

At pH values under 5.0 the binding of betaine decreases markedly. The carboxyl group of betaine should not add on a proton until a much lower pH is reached, in the neighborhood of 2.0, and the decline may, therefore, be due to protonation of an enzyme group of pK = 4.0–4.5. The anionic site is, therefore, probably a side-chain carboxyl, which should have a pK in this range (Tanford, 1962).

What is the distance between the anionic site and the group of pK = 6.2 responsible for the apparent neutralization of the anionic site? We know from the data in Figure 1 that pK_i for tetramethylammonium ion is shifted at least 1.2 units when the neighboring group is protonated, and, therefore, according to the calculations of Webb (1963), the groups should be no more than 5.5 A apart.[3] The distance is consistent with the relatively low binding strength of betaine. From the data in Figure 3, a competitive inhibition constant of 9.5 $\times 10^{-3}$ M was calculated for betaine at pH 5.5 according to the formula

$$K_i = [I]/(v_0/v - 1)(1 + [S]/K_m) \qquad (2)$$

Betaine is, therefore, less strongly bound than tetramethylammonium ion, whose minimum K_i value at pH 7.5 is smaller than this by a factor of *ca.* 5. The inhibitor's comparatively weak binding may be explained if the net attraction to the enzyme surface involves repulsive as well as attractive forces; in addition to attractions between the cation and anion centers of betaine, on one hand, and the anionic site and protonated group in the enzyme, on the other, there may be repulsion between like charges in the inhibitor and enzyme. Such interactions are expected if the two charged groups in the enzyme are as close together as those in betaine, and measurements of Stuart–Briegleb molecular models indicate that this distance is between 4.0 and 5.5 A.

In general, the pK values for ionizations in the free enzyme, as calculated from experiments with cationic substrates, are >6. For AcCh, pK = 6.27 (Krupka, 1966), and with dipropylmethylaminoethyl acetate and diethylbutylaminoethyl acetate, pK is 6.20 and 6.25 (Table II). In the case of the latter substrates, as well as trimethylaminobutyl acetate, noncoulombic forces appear to play a significant part in binding, and as a result K_m/V drops less sharply with declining pH than is expected from purely electrostatic attraction. Theoretically, the binding strength should be reduced when ionization occurs, and should achieve a new steady level at low pH (Webb, 1963). In this situation the calculated pK is smaller when based on data for lower pH, as shown in Table I. The best estimates of pK are, therefore, obtained for pH ≥ pK, as in the experiments

[2] Carboxyl groups with abnormally high pK values (7.3) have been reported in β-lactoglobulin (Tanford, 1962).

[3] Webb's calculations apply for 37.5° and 0.16 ionic strength, and are based on the assumption that the ionic groups are hydrated. The results were consistent with shifts in pK_a values in various dicarboxylic and amino acids due to a neighboring charge. In the present study the temperature (26°) and ionic strength (0.22) were slightly different. The apparent distance between charged groups depends on the dielectric constant of the intervening medium, and in the case of proteins this region could consist of polar or nonpolar groups, so that the true distance may differ from those estimated from shifts in pK. Such complications have been ignored in the present interpretation of data.

recorded in Table II. Noncoulombic attraction probably accounts for differences in the pH dependence of inhibition by tetrapropyl- and tetrabutylammonium ions as compared with tetramethyl- and trimethylammonium ions (Figure 6). The two larger ions are bound 10 and 18 times more strongly than tetramethylammonium (Krupka, 1965a), showing the importance of hydrophobic attraction (Belleau and Lacasse, 1964). With these larger ions, the pK of the ionizing group appears to be lowered by *ca.* 0.5 pH unit. With secondary and tertiary amine substrates (methylamino- and dimethylaminoethyl acetates) pK is slightly below 6.0 (5.90 and 5.85), as calculated from rates at low substrate concentration (Table II).

In summary, all the quaternary amine substrates, as well as tetramethylammonium ion, appear to reflect an ionization in the free enzyme above pH 6. The neutral substrates, phenyl acetate and isoamyl acetate, do not reflect this ionization, but show instead an ionization at pH ~5.5 (Table III). The simplest interpretation is that binding of cations in general, whether substrates or inhibitors, is hindered by ionization of a group of pK ~ 6.3 located near the anionic site. This group cannot be essential in acetylation, since its ionization would then show up with neutral substrates. On the other hand, the group in the free enzyme of pK = 5.5 must be involved in either the formation or reaction of ES; the values of pK' for acetylation (Table III) show that it plays an essential role in the latter process. The slightly lower pK values with secondary and tertiary amine substrates are unexplained at present.

Catalytic Role of the First Basic Group (pK = 6.3). Protonation of a group of pK = 6.3 prevents deacetylation of the acetyl enzyme, and the possibility therefore arises that this is the same group that ionizes in the free enzyme. As shown before (Krupka, 1964), the noncompetitive inhibition constant for trimethylammonium ion, K_i', is a measure of binding to the acetyl enzyme and should, therefore, be sensitive to charged groups in the neighborhood of the anionic site in EA. Figure 2 shows that pK_i' declines at low pH, from which a pK value of 6.25 ± 0.05 is calculated. The similarity to the pK of the catalytic basic group in EA suggests that the same group may be involved in all three processes, catalysis in EA and neutralization of the anionic site in E and EA. This idea was confirmed by experiments with tetraethylammonium bromide at pH 5.0 and 5.5, as will now be shown.

According to previous evidence, cations can become bound to the anionic site in the acetyl enzyme and can then interfere to varying degrees with deacetylation (Krupka, 1964, 1965a). Tetrapropylammonium ion almost completely blocks deacetylation, but tetraethylammonium has little effect on this reaction. As seen above, such ions are not bound to the anionic site when the neighboring group is protonated, and conversely a proton should not add on to the neighboring group once an inhibitory cation becomes attached to the anionic site. Hence tetraethylammonium ion should add to the acetyl enzyme at low pH without blocking deacetylation and should then prevent protonation of the neighboring group. If the latter functions in catalysis, the inhibitor should, therefore, increase the maximum velocity of substrate hydrolysis. On the other hand, tetrapropylammonium ion, which blocks deacetylation, should cause no activation. These predictions are borne out by experiment (Figures 4 and 5) and, as shown below, the increase in V due to tetraethylammonium ion is in quantitative agreement with expectations.

If V for AcCh hydrolysis is a measure of the deacetylation rate,

$$V \simeq k_3[\mathrm{EA}] \tag{3}$$

and if protons and inhibitory ammonium ions (I) compete for the acetyl enzyme, forming either EHA, which is unreactive, or EAI, which is reactive in the case of tetraethylammonium ion, though unreactive in the case of tetrapropylammonium, the following equilibria apply

$$\mathrm{EA} + \mathrm{H} \overset{K''}{\rightleftharpoons} \mathrm{EAH} \tag{4}$$

$$\mathrm{EA} + \mathrm{I} \overset{K_i'}{\rightleftharpoons} \mathrm{EAI} \tag{5}$$

and for such a reaction scheme, the maximum velocity is given by

$$V \simeq \frac{k_3(1 + a[\mathrm{I}]/K_i')}{1 + [\mathrm{H}]/K'' + [\mathrm{I}]/K_i'} \tag{6}$$

where a is the rate of reaction of EAI relative to that of EA. The maximum velocity in the absence of I is given by

$$V \simeq k_3/(1 + [\mathrm{H}]/K'') \tag{7}$$

The term in the denominator of (7) is responsible for the reduction in rate at low pH, provided that a single ionizing group governs the pH response. However, the rates at pH 5.0 and 5.5 are not governed by a single ionization, since the experimental points would then lie along the guide line of unit slope, instead of above it, as the data show (Krupka, 1966). Therefore, for purposes of insertion into eq 6 and 7, the term $1 + [\mathrm{H}]/K''$ must be replaced by the ratio of V at the maximum, pH 7.5, to that at low pH, 5.0 or 5.5; *i.e.*, the pH term is replaced by a direct experimental measure of the decline in rate resulting from high hydrogen ion concentration. From Figure 4 in the preceding paper (Krupka, 1966) the ratios of V at pH 7.5 and at pH 5.0 or 5.5 are 6.23 and 4.15, respectively.

Equations 6 and 7 may be combined to give the ratio of maximum velocities in the absence and presence of inhibitor

$$V_0/V \simeq \frac{1 + [\mathrm{H}]/K'' + [\mathrm{I}]/K_i'}{(1 + [\mathrm{H}]/K'')(1 + a[\mathrm{I}]/K_i')} \tag{8}$$

In previous experiments (Krupka, 1965a), a was found

to be 0.83 for tetraethylammonium bromide, and K_i', after correction for partial reactivity of EAI, was 7.7 $\times$ 10^{-3} M. Insertion of these values into eq 8 leads to predictions of V_0/V.

With 4.75 $\times$ 10^{-3}, 9.56 $\times$ 10^{-3}, 1.90 $\times$ 10^{-2}, and 4.78 $\times$ 10^{-2} M inhibitor, the predicted V_0/V ratios (pH 5.0) are 0.73, 0.59, 0.46, and 0.32, respectively, and the experimental ratios 0.789 $\pm$ 0.024, 0.559 $\pm$ 0.021, 0.461 $\pm$ 0.013, and 0.522 $\pm$ 0.030. At pH 5.5, [I] = 4.76 $\times$ 10^{-2} M and V_0/V is predicted to 0.41, and is found to be 0.47 $\pm$ 0.10. Agreement with theory is excellent, except at the highest inhibitor concentration. Secondary inhibitor effects may account for this failure to predict V_0/V accurately, since a nonlinear relation between $1/v$ and [I] has been demonstrated, which suggests binding of a second inhibitor molecule at high concentrations.

As expected, other substrates rate limited at deacetylation are also activated by tetraethylammonium ion at low pH. With 1.43 $\times$ 10^{-2} M inhibitor, V_0/V at pH 5.0 was 0.463 $\pm$ 0.064 and 0.542 $\pm$ 0.078 for phenyl acetate and acetylthiocholine, respectively, compared to a predicted ratio of 0.51. In the case of a substrate rate limited by the acetylation step no activation is observed with the same concentration of tetraethylammonium ion: V_0/V for diethylbutylaminoethyl acetate at pH 5.0 is 1.05 $\pm$ 0.25.

It is thus established that a group in the free enzyme of pK = *ca.* 6.3 is within 5 A of the anionic site and in the protonated form repels cations from the active center. A group near the anionic site with the same pK is present in the acetyl enzyme, is essential in deacetylation, and in the protonated form repels cations from the active center. Therefore, the same group probably functions catalytically in EA and is responsible for neutralizing the anionic site in E and EA. This essentially confirms the original suggestion made by Bergmann and co-workers (1956) that neutralization of the anionic site is due to protonation of an adjacent basic group having a catalyic function.

The Second Basic Group (pK = 5.5). This group appears to be at least 8–10 A from the anionic site, as the following evidence shows. First, the plot of log K_i *vs.* pH for tetramethylammonium ion, which reflects ionizations in the free enzyme, shows an inflection at pH 6.1, but none at lower pH. The plot of log K_i' *vs.* pH for trimethylammonium ion, which reflects ionizations in the acetyl enzyme, is similar and shows an inflection at pH 6.2. The increase in log K_i' at pH 5.0 above the expected value cannot be due to protonation of an enzyme group, which would diminish the strength of binding. In E and EA, therefore, the group of pK = 5.5 must be at least 6–7 A from the anionic site (Webb, 1963), since otherwise second downward inflections should have been observed in the plots of K_i and K_i'.

The measured values of pK' (Table III) show that there is at most a very weak interaction between the second basic group (pK = $\sim$5.5) and cations bound to the anionic site in the enzyme–substrate complex. With two of the substrates (methylaminoethyl acetate and dipropylmethylaminoethyl acetate) pK' is lower than with isoamyl acetate, but with the others there appears to be no change in pK' when the substrate is a cation.

Another line of evidence comes from the pH dependence of deacetylation. The maximum velocity for AcCh or phenyl acetate hydrolysis reflects an ionization at pH 6.3, but the values of V at pH 5.0 and 5.5, which as already noted are higher than expected (Krupka, 1966), suggest that a second ionization affects the rate. This ionization cannot involve a catalytic group, which would displace the points at low pH below the guide line of unit slope, but appears to maintain the group of pK'' = 6.3 in the active, *i.e.*, unprotonated form possibly as a result of electrostatic repulsion between the two groups. If the second ionizing group is that which functions in ES (pK = $\sim$5.5), it may be shown that ionization of one of the two catalytic groups shifts the pK of the other by 0.4–0.6 unit. According to Webb's calculations, this would correspond to a distance of *ca.* 8 A between the groups. We may conclude that there is little or no interaction between the second basic group (pK = 5.5) and cations bound at the anionic site in E, ES, or EA, but that there may be interaction between the two basic groups (pK = 5.5 and 6.3).

The Acidic Group (pK = 9.2). The functional acidic group (pK = 9.2), whose deprotonation is connected with reversible loss of activity (Wilson, 1960; Bergmann *et al.*, 1958; Krupka and Laidler, 1960b), probably functions in acetylation, and it may also function in deacetylation, though the evidence for the latter is weak. The experiments on tetrapropylammonium inhibition at pH 8.0–9.5 have given no indication of charge interactions between this group and the anionic site; *i.e.*, competitive and noncompetitive inhibition constants are altered little, if at all, as the pH is raised to 9.5. This confirms the finding of Wilson (1951) that prostigmine inhibition of AChE from the electric organ of the electric eel remains unchanged up to pH 11. Like the second basic group (pK = 5.5), the acidic group, therefore, appears to be at least 9 A from the anionic site.

Catalytic Functions of the Two Basic Groups. We should now consider the roles of the basic groups. As seen above, the pK value for ionization in the free enzyme depends on substrate charge: with neutral substrates pK = $\simeq$5.5, and with cationic substrates or inhibitors, pK = 6.0–6.3. Ionizations in ES (pK') are reflected in measurements of V with slowly hydrolyzed substrates, where acetylation (k_2) is rate limiting; pK' is found to be 5.2–5.5 with various substrates. Ionizations in the acetyl enzyme are reflected in the pH dependence of V for AcCh, acetylthiocholine, and phenyl acetate hydrolysis, where deacetylation (k_3) is rate limiting. As expected, the values of pK'' (6.3) with these substrates do not depend upon substrate charge.

It is clear that the group of pK = 6.3 plays no essential catalytic role in E or ES. When protonated it bears a positive charge which repels cations from the active center, explaining why the hydrolysis of cationic substrates depends upon its ionization in the free en-

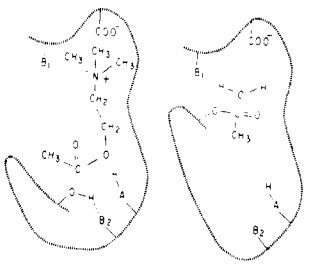

FIGURE 7: Diagrammatic representation of the active center of AChE. B_1 and B_2 are the basic groups of pK = 6.3 and 5.5, respectively, AH the acidic group of pK = 9.2, and OH and COO^- the serine hydroxyl and the anionic site. The left hand figure shows the enzyme–substrate complex for AcCh. While the substituted ammonium ion is held at the anionic site, B_2 and the acidic group catalyze transfer of the substrate acetyl group to the serine hydroxyl (acetylation). On the right is shown the product of this reaction, the acetyl enzyme. As the result of a conformational change, the acetyl residue has been brought near B_1, which catalyses hydrolysis of EA (deacetylation).

zyme. The essential catalytic group in E and ES has a pK = ~5.5, is distant from the anionic site, and has little effect on the binding of cations at the active center. The group adjacent to the active center, of pK = ~6.3, is essential in deacetylation. The group of pK = 5.5, however, is not, since its involvement would require a second downward inflection, at pH 5.5, in the values of log V for AcCh and phenyl acetate hydrolysis.

In summary, available evidence leads to the unexpected conclusion that two basic groups are present at the active center, one near the anionic site and interacting with it (pK = 6.3), the other more distant (pK = 5.5). The latter group, but not the former, functions in acetylation, while the former, but not the latter, functions in deacetylation. The group of pK = 6.3 is positively charged when protonated, suggesting that it may be an imidazole residue. If so, its pK value is normal (Tanford, 1962), despite its proximity to the negative charge in the anionic site, which should alter its pK; the shift should be of similar magnitude to that in log K_i for tetramethylammonium ion caused by protonation of the basic group, *i.e.*, at least 1.2 pK units. A possible explanation is a neighboring positive charge which counterbalances the effect of the anionic site.

The nature of the second basic group, pK = 5.6, is unknown, though it too may be an imidazole side chain. Sequence studies with chymotrypsin, trypsin, and elastase have shown two histidine residues to be held near one another by a disulfide bond, their positions relative to the bond being very similar in all three enzymes. The suggestion was therefore made that both histidines may be involved in catalysis (Walsh *et al.*, 1964; Smillie and Hartley, 1964). According to available evidence, the mechanisms of AChE, chymotrypsin, and trypsin are similar, and it is tempting to suggest further that the two histidine residues may act like the two basic groups of AChE, one catalyzing acylation, and the other, deacylation.

Catalytic Mechanism. Why should different ionizing groups function in acetylation and deacetylation? It is known that a substance must have unusual properties to be effective in covalent catalysis, where an intermediate is formed in which the substrate or part of the substrate is covalently bound to the catalyst. Here the catalyst must promote two reactions: (1) formation of a covalent bond with the substrate, and (2) rupture of this bond to re-form the catalyst. In general, structural modifications favoring one of these reactions disfavor the other (Jenks, 1963); *e.g.*, an electron-releasing group adjacent to a nucleophilic atom increases the latter's ability to attack an ester and form an acyl compound, but also increases the stability of the acyl compound to hydrolysis. A second example relates to the probability of having two ionizing groups in the states required for catalysis. Bender and Kézdy (1964) and Jenks (1963) have suggested that deacylation may be regarded as the reverse of acylation and that it is, therefore, reasonable to expect a group functioning as a base in one reaction to function as the conjugate acid in the other. Where two groups, A and B, function in catalysis, AH and B promoting an initial reaction and A and BH promoting a second, the probabilities of finding the combinations AH and B and A and BH will normally be different. If the ionization constant for A is K_1 and for B is K_2, it may be shown that the concentration of AH and B will be K_2/K_1 times that of A and BH. If pK_1 and pK_2 are 6 and 9, this factor is 10^3. Such difficulties may be overcome if a conformational change in the enzyme follows the first reaction, so that the environment of the covalently bound acyl group is different from that of the acyl group in the enzyme–substrate complex.

This mechanism is in accord with observations on AChE suggesting that the ester grouping in the enzyme–substrate complex may have a different location on the enzyme surface than the acetyl group in the acetyl enzyme. When bound at the anionic site, tetramethyl- and tetraethylammonium ions partially block deacetylation, and tetrapropyl- and tetrabutylammonium ions block almost completely (Krupka, 1964). These ions also reduce the rate of an analogous reaction, decarbamylation of a dimethylcarbamyl enzyme (Metzger and Wilson, 1963). However, carbamylation of AChE by dimethylcarbamyl fluoride, a reaction analogous to acetylation, is accelerated by tetraethyl- and tetrapropylammonium ions. It would appear from this that the acyl group in ES is farther from the anionic site than in EA.

A possible interpretation of the experiments is that in the free enzyme the "active" serine hydroxyl group is near the second basic group (pK = 5.5) and the acidic group (pK = 9.2), which are some 10 A from the anionic site. In ES all three of these may be involved in interaction with the acetyl group in the substrate, while

the trimethylammonium ion in AcCh is anchored to the anionic site. If so, AcCh would be in an extended conformation, since the distance from the trimethylammonium group to the carbonyl carbon atom is *ca.* 8 A in this form, according to measurements of Stuart-Briegleb molecular models. Possibly the ester bond is then strained and more labile, as in the "rack" mechanism discussed by Jenks (1963). In acetylation, catalyzed by the basic and acidic groups, the acetyl residue is transferred to the serine hydroxyl. Subsequently the acetylated serine may move nearer the anionic site, as the result of a conformational change in the protein, and the first basic group (pK = 6.3) would then be in a position to catalyze deacetylation. A diagrammatic representation of such a mechanism is shown in Figure 7.

These ideas resolve a problem encountered if the same catalytic groups are postulated to function in acylation and deacylation, as in the mechanism proposed by Bender and Kézdy (1964) for chymotrypsin. This mechanism rests on the assumption that acylation is essentially the microscopic reverse of deacylation. In the case of chymotrypsin reactions, Bender and his associates have shown that the rate of acylation declines above pH 9, while the rate of deacylation is constant from pH 8 to 12, so that participation of an acidic group in acylation but not deacylation must be explained. No difficulty is encountered if different sets of catalytic groups are assumed to function in the two reactions. In this connection it may be noted that the detailed mechanism of deacylation (k_3) is not necessarily, from the principle of microscopic reversibility, the reverse of acylation (k_2), since two separate reactions are involved; *e.g.*, in A $\rightleftharpoons$ B $\rightleftharpoons$ C, the intermediates formed in going from A to B cannot be the same as those from B to C, for A, which here represents E and S, is not identical with C, standing for E and products. Only the foreward and reverse directions of a single reaction (*e.g.*, A $\rightleftharpoons$ B) necessarily proceed through identical intermediates. It is possible, of course, for the intermediates between A and B to be completely analogous to those between B and C, but this must be proven rather than assumed. In the case of AChE the evidence indicates that a strict analogy is not maintained, since different enzyme groups participate in the two reactions.

The experiments described here support a number of conclusions on AChE reached before. (1) Cations may be bound to EA. (2) The complex between EA and I is unreactive in the case of some inhibitors, but in other cases reacts to form acetic acid and free enzyme. (3) Deacetylation limits the rate of AcCh, acetylthiocholine, and phenyl acetate hydrolysis, but acetylation limits the hydrolysis of acetyl esters that are relatively poor substrates. (4) A basic group located *ca.* 5 A from the anionic site functions in deacetylation. However, a revision is necessary in certain other suggestions. It was correctly concluded (Krupka and Laidler, 1960b) that the group of pK = 6.3 does not ionize in ES when S is a cation, but since ionization of this group in E was revealed by plots of log V/K_m *vs.* pH, the group was incorrectly assumed to play an essential role in either the formation or reaction of ES. It now appears that it is not involved in acetylation, and that its role in ES formation is accidental, since in the ionized state it simply repels cations from the active center. Its failure to ionize was explained by hydrogen bonding involving the substrate (Krupka, 1962) but is now known to be due to electrostatic repulsion from a cationic substrate. Finally, experiments of Friess and McCarville (1954) on bifunctional inhibitors such as *cis*-2-dimethylaminocyclohexanol, which contain a cationic nitrogen atom and an electronegative group, were interpreted on the basis of binding to two enzyme sites, the anionic site and the protonated acidic group (pK = 9.2) (Krupka and Laidler, 1961). It now appears that the acidic group is not adjacent to the anionic site and probably could not play a direct role in inhibitor binding, though some other group with acidic properties may do so.

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The Influence of Metal Ions on the Hydrolysis of Polyphosphates*

J. van Steveninck

ABSTRACT: The influence of some metal ions on the hydrolytic degradation of polyphosphates has been studied. Uranyl salts catalyze the hydrolysis of polyphosphates.

A number of metal ions (*e.g.*, uranyl and nickelous ions) change the rate of hydrolysis, presumably by changing the conformation of the polyphosphate chains. The influence of metal ions on the hydrolysis of pyrophosphate is quite different from the influence of these ions on the hydrolysis of larger polyphosphates. The possible relationship between these effects and the influence of some metal ions on enzymatic reactions involving polyphosphates is briefly discussed.

Polyphosphates occur in a wide variety of microorganisms (Lohmann, 1958; Kuhl, 1960). More recently polyphosphates have been detected in liver nuclei (Penniall and Griffin, 1964) and mitochondria (Lynn and Brown, 1963). Most investigators consider these condensed phosphates as phosphate and energy reserve of the cells.

Recently it has been proposed that polyphosphates play an important role in the process of glucose transport into yeast cells (van Steveninck, 1962; van Steveninck and Booij, 1964). Polyphosphates at the surface of yeast cells probably take part in an enzymatic phosphorylating process, preceding the uptake of glucose into the cells. The binding of certain metal ions (*e.g.*, Ni^{2+} and UO_2^{2+}) to these polyphosphates inhibits glucose transport. This inhibition was ascribed to changes in the spatial arrangement of the polyphosphate chains, induced by the metal ions.

It seems to be of interest to compare the influence of metal ions on this enzymatic scission of the P–O–P linkage with the influence of these ions on the hydrolytic scission of the P–O–P bond in acidic solutions. Some experiments on the influence of metal ions on the hydrolytic degradation of polyphosphates will be presented in this communication.

Methods

The sodium salts of the polyphosphates were dissolved in distilled water and converted into the corresponding acid over an ion-exchange column (Amberlite IR-120, H^+ form). In most experiments the solution contained finally 100 μg of P/ml. The pyrophosphate used in these experiments appeared to be chemically pure. The other polyphosphates were mixtures of molecules with an average chain length of n = 8, 13, 20, 120, and 200, respectively.

Metal ions were added as the corresponding nitrates. With the low polyphosphate concentrations used in these experiments, no precipitates were formed. The pH of the solution was adjusted with HCl or tetramethylammonium hydroxide, as it has been shown that tetramethylammonium ions do not influence the hydrolysis of polyphosphates (Van Wazer *et al.*, 1952). The pH was measured again at the end of the experiment. The pH shift, caused by the hydrolysis of the polyphosphate, was always <0.02 pH unit; therefore, the velocity of the hydrolysis was not appreciably influenced by it (see Results). In most experiments the pH was between 0.60 and 2.40. The reaction mixture was kept at a constant temperature in a water bath. Samples were withdrawn at intervals, cooled rapidly in melting ice, and analyzed for liberated orthophosphate, by the method of Fiske–Subbarow, as modified by Meyerhof and Oesper (1947). In preliminary experiments it was noted that under the described experimental conditions, at 40–50°, the liberation of orthophosphate proceeds linearly with time for *ca.* 1 hr, with all polyphosphates tested. So determination of the amount of ortho-phosphate, liberated in the course of time, is a good indication for the rate of hydrolysis.

The velocities of orthophosphate formation at a

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