The Acceleration of Acetylcholinesterase Activity at Low Ionic Strength by Organic and Inorganic Cations

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SUMMARY

The effect of three tetraalkylammonium compounds on the hydrolysis of phenylacetate by acetylcholinesterase at low ionic strength has been examined. Tetraethylammonium iodide is shown to increase noncompetitively the maximum velocity of hydrolysis, probably by accelerating the rate-limiting deacetylation step of the hydrolysis. Tetramethylammonium iodide acts as a purely competitive inhibitor, while tetra-*n*-propylammonium iodide decreases the maximum velocity, probably by blocking deacetylation. The effects of $MgCl_2$ and NaCl on phenylacetate hydrolysis have been studied, and a competition between organic and inorganic cations for the anionic site of acetylated acetylcholinesterase proposed.

INTRODUCTION

Roufogalis and Thomas (1) reported that some quaternary ammonium compounds potentiated the rate of hydrolysis of acetylcholine by acetylcholinesterase, and that this occurred above optimum substrate concentrations. When the medium contained NaCl (0.1 M) and $MgCl_2$ (0.04 M), this accelerated rate of hydrolysis was less than that of the optimum rate in the absence of the quaternary ammonium compounds. However, at ionic strengths of 0.01 or less the optimum rate of hydrolysis was greater than that of the optimum rate of the control. A suggestion which arose from these results was that at low ionic strength of the medium, compounds such as tetraethylammonium (TEA) potentiated the acetylcholinesterase by accelerating the deacetylation step in the hydrolysis as well as by competing with excess acetylcholine for the acetylated enzyme. In order to obtain more direct evidence that the deacetylation step can be accelerated by tetraethylammonium and other organic and inorganic cations, the effect of a number of these cations on the hydrolysis of phenylacetate by acetylcholinesterase has now been studied. The choice of substrate was governed by the following considerations: (a) the rate-determining step in the hydrolysis of phenylacetate is, like acetylcholine, the deacetylation step (2); (b) phenylacetate is not subject to inhibition by excess substrate and consequently, unlike acetylcholine, its kinetics can be conveniently studied at low ionic strength over a range of relatively high substrate concentrations; (c) phenylacetate, being neutral, does not enter into coulombic interaction with the anionic site of acetylcholinesterase and consequently does not compete with cations for this site; and (d) phenylacetate does not contribute to the ionic strength of the medium.

MATERIALS AND METHODS

An automatic titrimetric method of analysis was used, essentially as previously described (3). The required quantities of phenylacetate (a solution of 0.5 M freshly prepared in redistilled methanol) methanol (to a final concentration of 1% by volume) and compound being studied were made up to 19.0 ml with glass-distilled water in a jacketed glass vessel maintained at 37°. Nitrogen was flushed through this solution for 2.5 min. A portion of a solution of purified bovine erythrocyte acetylcholines-

terase (Nutritional Biochemicals Corporation; prepared in glass-distilled water as a 0.3 mg/ml solution and kept at 4°) was kept at 37° for 5 min. This solution was added to the reaction vessel via a 1-ml pipette, and the titrator was started. The reactions were carried out at either pH 7.4 $\pm$ 0.1 or pH 8.4 $\pm$ 0.1. At low ionic strength the value of the pH of a solution as indicated by a meter was found to be influenced by stirring of the solution. The observed value of a stirred solution was lower than the value of the same solution when the stirrer was switched off. Since the reaction solutions were stirred during the course of the experiments, the pH stat was set at a pH value that was found to be equivalent to pH 7.4 or pH 8.4 in the unstirred solution and the actual pH of the reaction mixture was read at the end of each run on the cessation of stirring. Nonenzymatic rates of hydrolysis were determined at various ionic strengths and at the two pH values, and the necessary corrections were made to the results.

Phenylacetate (British Drug Houses) was redistilled before use. The following compounds were used as received: tetramethylammonium iodide and tetra-*n*-propylammonium iodide (British Drug Houses), and tetraethylammonium iodide (Hopkins and Williams). Sodium chloride (British Drug Houses) and magnesium chloride (By-Products and Chemicals Pty. Ltd.) were both of analytical reagent grade.

Estimation of kinetic parameters. Values of $K_{m(app)}$, V, V_0 and the standard errors of these were estimated by the weighted statistical analysis described by Wilkinson (4). A KDF 9 computer was used for the analysis of the results.

The probable error in the ratio of V/V_0 was estimated from the formula (ref. 5)

$$p = \pm \frac{1}{V_0}\sqrt{b^2 + \frac{V^2a^2}{V_0^2}}$$

where a and b are the standard errors of V_0 and V respectively.

RESULTS AND DISCUSSION

The hydrolysis of acetyl esters by acetylcholinesterase is considered to proceed in three stages (ref. 6):

$$E + \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E - S \xrightarrow{k_2} EA \xrightarrow{k_3} E + \text{acid}$$

where E is the free enzyme, S is the substrate, E — S is the Michaelis complex, and EA the acetylated enzyme. It has been shown that such a three-step mechanism follows Michaelis-Menten kinetics (7) as shown in Eq. 1:

$$v = \frac{k_{(cat)}[E_0][S]}{[S] + K_{m(app)}} \quad (1)$$

where v is initial velocity of product formation, $k_{(cat)}$ the overall catalysis rate is equal to $(k_2k_3)/(k_2 + k_3)$ and $K_{m(app)} = (K_Sk_3)/(k_2 + k_3)$ (K_S is the equilibrium constant for Michaelis complex formation). Equation 1 can be rearranged into the form of Eq. 2

$$v = V_0 - K_{m(app)}\left(\frac{v}{[S]}\right) \quad (2)$$

where $V_0 = k_{(cat)}\,[E_0]$ and is the maximum velocity in the absence of inhibitors or accelerators. From Eq. 2 it can be seen that a plot of v against $v/[S]$ gives a straight line which has a slope of $-K_{m(app)}$ and the y intercept is V_0. This is the plot due to Eadie (8). In the presence of accelerators or inhibitors the y intercept gives V, the maximum velocity in the presence of these compounds. When V is greater than V_0 there is noncompetitive acceleration, and when V is less than V_0 there is noncompetitive inhibition. When $V = V_0$ the kinetics are purely competitive. Since the hydrolysis of phenylacetate is rate-limited by deacetylation (i.e., $k_2 > k_3$) and since a cationic compound can react with both the free enzyme, E, and the acetylated enzyme, EA (9), an increase or decrease of V with respect to V_0 will probably be related to an effect on the deacetylation step of the hydrolysis sequence (2).

In Fig. 1 the effect of three tetraalkylammonium iodides is shown in media of ionic strength less than 0.005 and pH 8.4. In the presence of tetraethylammonium iodide (TEA) the hydrolysis of phenylacetate proceeds with a maximum velocity

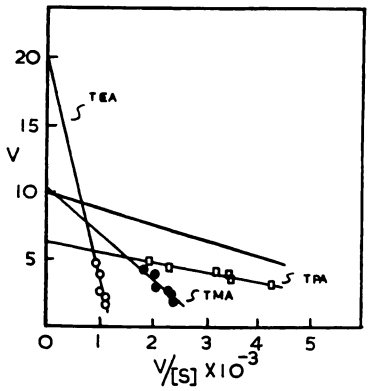

FIG. 1. *Effect of TMA, TEA, and TPA on phenylacetate hydrolysis at pH 8.4 ± 0.1 and ionic strength less than 0.005*

The control curve is the composite of five separate determinations. The values of $V/[S]$ for TMA and TPA have been doubled to avoid crowding of the diagram. V_0 for the control curve has been arbitrarily set at 10.00, and represents a maximum hydrolysis rate of approximately 40 μM/min. Batch 1 acetylcholinesterase was used (see text). ●——● TMA, 1.12×10^{-3} M; ○——○ TEA, 1.12×10^{-3} M; □——□ TPA, 2.21×10^{-4} M.

approximately twice that of the control. Under these conditions TEA inhibits phenylacetate hydrolysis but does so with a noncompetitive component of acceleration. In the presence of tetramethylammonium iodide (TMA) the maximum velocity does not differ significantly from that of the control. Under these conditions TMA is thus a purely competitive inhibitor of phenylacetate hydrolysis. In the presence of tetra-*n*-propylammonium iodide (TPA) the maximum velocity is lower than that of the control. TPA is thus a partially noncompetitive inhibitor of phenylacetate hydrolysis. The tetraalkylammonium compounds have been studied at a number of different concentrations. These results are summarized in Table 1.

It has been shown (10, 11) that acetylcholinesterase is polydisperse at ionic strengths of less than 0.003, and as the ionic strength is increased it becomes more homogeneous. The acceleration now observed with TEA cannot be due to a nonspecific ionic strength effect because both TMA and TPA at similar ionic strengths to that used with TEA show completely

TABLE 1

The effect of TMA, TEA, and TPA on the maximum velocity of hydrolysis of phenylacetate at pH 7.4 and 8.4 and ionic strength less than 0.005

V/V_0 gives the ratio of the maximum velocity in the presence of compound (V) to that in its absence (V_0) and the probable error associated with this ratio.

Compound	Concentration × 10^3 M	V/V_0 pH 7.4	V/V_0 pH 8.4
TMA	1.12	1.30 ± 0.08	1.02 ± 0.15
	2.24	1.24 ± 0.06	1.05 ± 0.29
	4.48	1.26 ± 0.11	—
TEA	0.51	1.94 ± 0.34	—
	1.12	2.00 ± 0.19	2.21 ± 0.48
	4.48	1.50 ± 0.41	1.68 ± 0.37
TPA	0.00221	—	0.62 ± 0.03
	0.0111	—	0.43 ± 0.02

different effects. An even more striking structural specificity among spiran quaternary ammonium compounds has been reported in a previous communication (1).

Krupka (12) has shown that at pH 5.0 hydrolysis of acetylcholine is accelerated by TEA in a manner similar to the effect of TEA on phenylacetate hydrolysis now reported. This was explained in terms of a protection by TEA against protonation of an essential group of $pK \sim 6.2$ in the vicinity of the anionic site.

Kinetic studies on the hydrolysis of phenylacetate at low ionic strength have now been made at pH 8.4 and 7.4. A group of $pK \sim 6.2$ will be protonated to the extent of only 0.6% at pH 8.4 and 6% at pH 7.4, and consequently it is unlikely that protection of such a group against protonation could be the mechanism of acceleration by TEA at these higher pH values. Table 1 shows that the effects of tetraalkylammonium compounds are essentially the same at pH 7.4 and pH 8.4.

The results obtained here at low ionic strength differ quite markedly from the results obtained by other workers in media containing relatively high concentrations of inorganic ions. The effect of tetraalkylammonium compounds on acetylcholine hy-

drolysis at pH 7.4 and 26° has been studied by Krupka (13) in a medium containing NaCl (0.1 M) and $MgCl_2$ (0.04 M). The results obtained under these conditions indicated that TMA inhibits the deacetylation step of the hydrolysis quite strongly, TEA inhibits the deacetylation, but only weakly, and TPA inhibits the deacetylation almost completely. In order to obtain some insight into the reasons for the differences obtained between the results of Krupka at high ionic strength and those reported here at low ionic strength the effects of NaCl and $MgCl_2$ on acetylcholinesterase hydrolysis of phenylacetate have been examined. The results are shown as Eadie plots in Fig. 2. It can be seen from these plots that in the presence of Na^+ and Mg^{2+} the hydrolysis of phenylacetate proceeds with a maximum velocity (V) that is greater than that of the control hydrolysis in their absence. Furthermore, this acceleration is not simply dependent on ionic strength, but to some extent on the nature of the cation. $MgCl_2$ at an ionic strength of 0.12 accelerates the hydrolysis to a greater degree than does NaCl at a similar ionic strength (0.1). During the course of this work two batches of acetylcholinesterase were used. The two batches had different apparent Michaelis constants and were accelerated differently by the inorganic ions. The comparative order of acceleration by NaCl and $MgCl_2$ was, however, the same. The acceleration by TEA, using the two batches, was quantitatively the same. These results are summarized in Table 2.

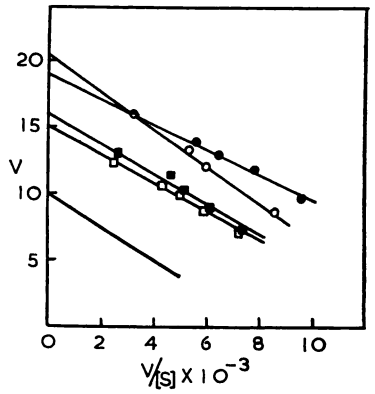

FIG. 2. *Effect of NaCl and $MgCl_2$ on phenylacetate hydrolysis at pH 7.4 ± 0.1*

The control curve was obtained at an ionic strength of less than 0.001 and is a composite of seven separate determinations. V_0 for the control curve has been arbitrarily set at 10.00 and represents a maximum hydrolysis rate of approximately 25 μM/min. Batch 1 acetylcholinesterase was used (see text). ○——○ $MgCl_2$, 0.04 M; ●——● $MgCl_2$, 0.10 M; □——□ NaCl, 0.04 M; ■——■ NaCl, 0.10 M.

Both organic and inorganic cations are likely to compete for the same sites on

TABLE 2

The effect of NaCl and $MgCl_2$ on the maximum velocity of phenylacetate hydrolysis at pH 7.4 and 8.4

V/V_0 gives the ratio of the maximum velocity in the presence of the inorganic ions (V) to that of a control at ionic strength less than 0.001 (V_0), and the probable error associated with this ratio. The apparent Michaelis constants ($K_{m(app)}$) and their standard errors are given. Two batches (1 and 2) of acetylcholinesterase have been used (see text).

Compound	Concentration M	pH	$K_{m(app)} \times 10^3$ M		V/V_0	
			Batch 1	Batch 2	Batch 1	Batch 2
Control	—	7.4	1.24 ± 0.24[a]	0.73 ± 0.12[c]	—	—
	—	8.4	1.18 ± 0.11[b]	—	—	—
NaCl	0.04	7.4	1.06 ± 0.05	1.41 ± 0.09	1.51 ± 0.04	2.53 ± 0.06
	0.10	7.4	1.13 ± 0.11	—	1.60 ± 0.06	—
	0.10	8.4	1.61 ± 0.26	—	1.37 ± 0.12	—
$MgCl_2$	0.04	7.4	1.39 ± 0.08	1.45 ± 0.08	2.04 ± 0.13	3.31 ± 0.11
	0.10	7.4	0.95 ± 0.04	—	1.90 ± 0.12	—
	0.04	8.4	1.84 ± 0.31	—	1.73 ± 0.15	—

[a–c] Standard deviations obtained from (a) 7, (b) 5, (c) 9 determinations.

acetylcholinesterase, presumably for the anionic site. There is evidence that the addition of inorganic cations to the medium decreases the affinity of acetylcholinesterase for acetylcholine (14, 15) and for cationic inhibitors (16, 17), and this is presumably due to competition between the organic and inorganic cations for the anionic site in the free enzyme (18). By analogy, it is likely that there will be competition between organic and inorganic cations for the free anionic site in the acetylated enzyme. This can be illustrated in the following scheme:

$$\begin{array}{ccccc} EAI & \rightleftharpoons & EA & \rightleftharpoons & EAM \\ \downarrow ak_3 & & \downarrow k_3 & & \downarrow bk_3 \\ E + A + I & & E + A & & E + A + M \end{array}$$

where the hydrolysis of EA, the acetylated enzyme, is rate-limiting [as is the case in phenylacetate hydrolysis (2)], EAI is the organic cation-acetylated enzyme complex, and EAM is the metal-acetylated enzyme complex. EAI and EAM undergo deacetylation at a and b times that of EA, respectively. If there is competition between I and M for EA then the effect of M on the maximum velocity of hydrolysis will be influenced by the presence and nature of I. This point is illustrated in Figs. 3 and 4. As shown in Fig. 3, the maximum velocity of hydrolysis in the presence of NaCl (0.04 M) and TMA is lower than that of the maximum velocity in the presence of NaCl alone. Presumably TMA, which does not accelerate deacetylation ($V/V_0 \approx 1$) competes with the Na^+ which does accelerate deacetylation ($V/V_0 \approx 2.5$) and so reduces its effect. The maximum velocity of hydrolysis in the presence of NaCl (0.04 M) and TEA is greater than that of the maximum velocity in the presence of NaCl alone. TEA accelerates deacetylation to approximately the same extent as Na^+. As shown in Fig. 4, the maximum velocity of hydrolysis in the presence of $MgCl_2$ (0.04 M) and TEA is lower than the maximum velocity in the presence of $MgCl_2$ alone. Presumably TEA, which is a weaker accelerator than $MgCl_2$ ($V/V_0 = 2$ and 3.3, respectively) competes with Mg^{2+} for the anionic site of EA and so reduces its ac-

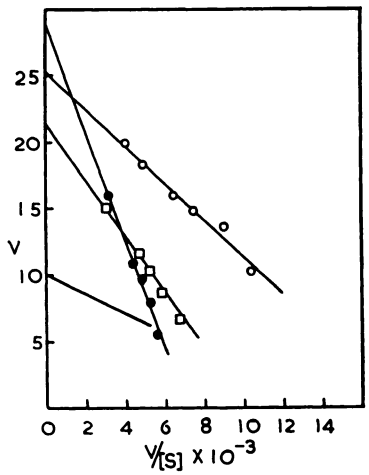

FIG. 3. *Effect of TMA and TEA on phenylacetate hydrolysis in the presence of NaCl 0.04 M, pH 7.4 ± 0.1*

The control curve was obtained at ionic strength less than 0.001 and is a composite of nine separate determinations. V_0 for control curve has been arbitrarily set at 10.00 and represents a maximum hydrolysis rate of approximately 20 μM/min. Batch 2 acetylcholinesterase was used (see text). ○—○ NaCl, 0.04 M; ●—● NaCl 0.04 M, + TEA 1.12×10^{-3} M. □—□ NaCl 0.04 M + TMA 1.12×10^{-3} M.

celerating effect. These results are similar to results obtained by Krupka (13), who has shown that in the presence of a mixture of NaCl (0.1 M) and $MgCl_2$ (0.04 M) TEA slightly decreased the maximum velocity of acetylcholine hydrolysis and TMA decreased the maximum velocity quite strongly.

The conclusion to be drawn from these results is that the observable effect of tetraalkylammonium ions on hydrolysis of phenylacetate by acetylcholinesterase depends on the medium used for the study. The true effect of these compounds is probably observed when the reaction medium contains no added inorganic ions. Under these conditions TMA inhibits the hydrolysis of phenylacetate in a purely competitive manner, TEA inhibits the hydrolysis with a noncompetitive acceleration component, and TPA inhibits the hydrolysis with a noncompetitive inhibition component. The effect of these compounds in a medium containing added inorganic ions is

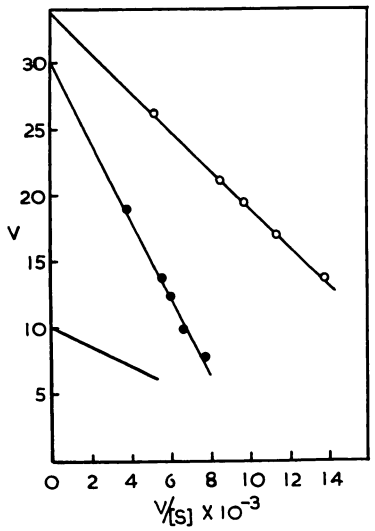

FIG. 4. *Effect of TEA on phenylacetate hydrolysis in the presence of $MgCl_2$ (0.04 M), pH 7.4 ± 0.1*

The control curve was obtained at an ionic strength of less than 0.001 and is a composite of nine separate determinations. V_0 for the control curve has been arbitrarily set at 10.00, and represents a maximum hydrolysis rate of approximately 20 μM/min. Batch 2 acetylcholinesterase was used (see text). ○—○ $MgCl_2$, 0.04 M; ●—● $MgCl_2$ 0.04 M, + TEA 3.36×10^{-3} M.

only a relative one and is influenced by the nature of the inorganic cation present. An equilibrium is established, and the organic and inorganic cations compete for the anionic site of the acetylated enzyme.

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