Acetylcholinesterase: Structural Requirements for Blocking Deacetylation*

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ABSTRACT: The ability of various amines to block the deacetylation step in acetylcholine hydrolysis was tested by a kinetic method based on the competition between an inhibitory amine and a reference compound, trimethylammonium ion, for the anionic site at the active center. The results agreed with those from another method, related to the inhibitor's effect on the optimum velocity. When small quaternary and primary amines are bound to the acetyl enzyme, deacetylation continues, though at a somewhat reduced rate. On the other hand, deacetylation is largely blocked by secondary and tertiary amines and large quaternary and primary amines. Blocking by the larger ions is probably due to steric interference with the acetyl group, while inhibition by small secondary and tertiary amines may result from hydrogen bonding with an essential enzyme group. Experiments with a series of quaternary amines from tetramethyl- to tetra-*n*-butylammonium ions indicate that in the acetyl enzyme, as in the free enzyme, the anionic and esteratic sites are approximately 5 A apart.

Trimethylammonium ion inhibits by forming an inactive complex with acetylcholinesterase in the free form (E) and with the acetyl enzyme (EA), but not with the enzyme-substrate complex (ES) (Krupka, 1964). Inhibition by *cis*-2-dimethylaminocyclohexanol is similar, since the complex with EA does not form products; but in the case of tetramethylammonium and choline inhibition, the complex formed with EA is partially reactive (Krupka, 1963). The requirements for blocking deacetylation will now be explored further by using trimethylammonium ion as a reference, against which effects of other inhibitors are gauged. Briefly, if an inhibitor competes with trimethylammonium ion for EA but only partially blocks deacetylation, it should diminish the noncompetitive inhibition of trimethylammonium to an extent dependent upon its own effectiveness in blocking deacetylation. The kinetic theory leading to this idea will first be outlined, and then the results of the experiments will be given.

Kinetic Theory

In the reaction scheme shown in Figure 1, two cationic inhibitors, I_1 and I_2, compete for the anionic site in the enzyme. I_2, representing trimethylammonium ion, forms an inactive complex with EA (EAI_2), but I_1 may form an active complex, EAI_1, reacting a times as fast as EA. The reciprocal of the reaction velocity is then

$$\frac{1}{v} = \frac{1}{k_2[E_0]}\left\{1 + \frac{k_2}{k_3}\left(\frac{1 + K'_1[I_1] + K'_2[I_2]}{1 + aK'_1[I_1]}\right) + \frac{1 + K_1[I_1] + K_2[I_2]}{\bar{K}[S]}\right\} \quad (1)$$

where $\bar{K} = k_1/(k_{-1} + k_2)$. In analyzing the experiments we shall be concerned with the maximum velocities obtained under a number of conditions: in the absence of inhibitors (V_0), and in the presence of fixed concentrations of I_1 (V_1), I_2 (V_2), or both I_1 and I_2 at the same time (V_{12}). From (1), these are found by making $[S] = \infty$:

$$\frac{1}{V_0} = \frac{1}{k_2[E_0]}(1 + k_2/k_3) \quad (2)$$

$$\frac{1}{V_1} = \frac{1}{k_2[E_0]}\left\{1 + \frac{k_2}{k_3}\frac{1 + K'_1[I_1]}{1 + aK'_1[I_1]}\right\} \quad (3)$$

$$\frac{1}{V_2} = \frac{1}{k_2[E_0]}\left\{1 + \frac{k_2}{k_3}(1 + K'_2[I_2])\right\} \quad (4)$$

$$\frac{1}{V_{12}} = \frac{1}{k_2[E_0]}\left\{1 + \frac{k_2}{k_3}\frac{1 + K'_1[I_1] + K'_2[I_2]}{1 + aK'_1[I_1]}\right\} \quad (5)$$

Using these expressions, it can be shown that

$$\frac{a}{1 - a} = \frac{(1/V_1 + 1/V_2 - 1/V_0 - 1/V_{12})1/V_0}{(1/V_1 - 1/V_0)(1/V_2 - 1/V_0)} \quad (6)$$

If both inhibitors completely block deacetylation ($a = 0$), then from (6)

$$1/V_1 - 1/V_0 = 1/V_{12} - 1/V_2 \quad (7)$$

With equations (6) and (7) we may decide the extent to which an inhibitor blocks deacetylation, inde-

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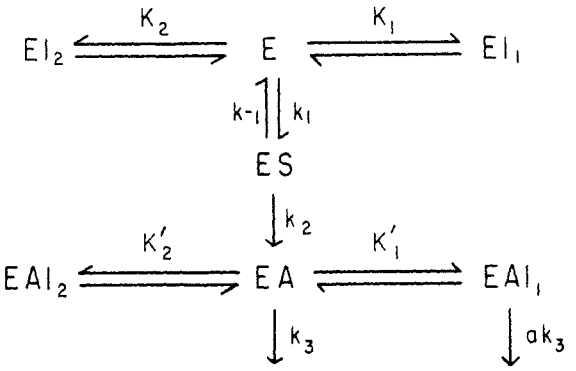

FIGURE 1: Reaction scheme for simultaneous inhibition by two amines, I_1 and I_2, either of which may add on to the free enzyme (E) or the acetyl enzyme (EA). I_2 represents trimethylammonium ion, whose complex with EA is unreactive. I_1 is the test compound and its effect on deacetylation, as reflected in the size of a, is to be determined.

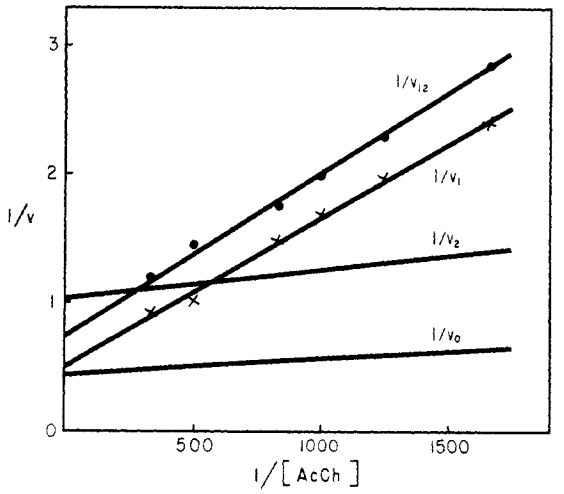

FIGURE 2: Inhibition of acetylcholine (AcCh) hydrolysis by 1.00×10^{-2} M tetraethylammonium bromide in the presence ($1/v_{12}$) or absence ($1/v_1$) of 5.03×10^{-3} M trimethylammonium chloride. The lines designated $1/v_0$ and $1/v_2$ refer to reactions in the absence of inhibitor and in the presence of 5.03×10^{-3} M trimethylammonium ion, respectively. Units of 1/[AcCh] are M^{-1}.

pendent of its ability to form a complex with the acetyl enzyme. In plots of $1/v$ against 1/[S] the intercepts on the $1/v$ axis represent the reciprocals of the maximum velocities ($1/V$). If a is larger than zero, the difference between the intercepts in the presence and absence of an inhibitor ($1/V_1 - 1/V_0$) is greater than the difference in intercepts for trimethylammonium ion alone, and for a mixture of trimethylammonium ion and the first inhibitor ($1/V_{12} - 1/V_2$). In this case a may be computed from equation (6).

The value of K_1 is obtained from the ratio of slopes

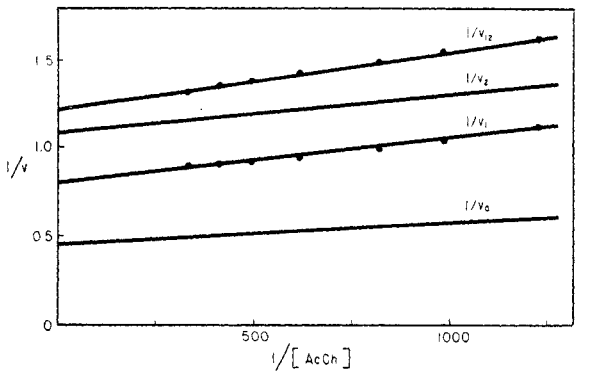

FIGURE 3: Inhibition by 5.10×10^{-3} M triethylammonium chloride together with 5.27×10^{-3} M trimethylammonium chloride. Otherwise as in Figure 2.

in $1/v \times 1/[S]$ plots in the presence and absence of inhibitor,

$$K_1 = \left(\frac{\text{slope } (+I)}{\text{slope } (-I)} - 1\right)\frac{1}{[I]} \quad (8)$$

and K'_1 (exptl) from the following relations, derived from equations (3) and (4):

$$K'_1(\text{exptl}) = V_0(1/V_1 - 1/V_0)/[I]$$
$$= K'_1(1 - a)/(1 + k_3/k_2)(1 + aK'_1[I_1]) \quad (9)$$

When $a = 0$, $K'_1(\text{exptl}) = K'_1/(1 + k_3/k_2)$, but when $a > 0$, $K'_1(\text{exptl}) < K'_1/(1 + k_3/k_2)$. To correct for a nonzero value of a, the following quantity may be computed:

$$K'_1(\text{corr}) = K'_1(\text{exptl}) \times \left(1 + \frac{a}{1 - a}K'_1(\text{exptl})[I]\right)/(1 - a) \quad (10)$$

If, as the evidence suggests, k_3/k_2 is small relative to unity in acetylcholine hydrolysis (Wilson and Cabib, 1956; Krupka, 1964), then K'_1 (corr) $\simeq K'_1$, and K'_1 (exptl) $\simeq K'_1$ when $a = 0$.

Methods

Details of the experimental procedure have been described before (Krupka, 1963, 1964). The acetic acid released in acetylcholine hydrolysis was automatically titrated against 0.01 N NaOH, and addition of base was recorded as a function of time. Such recordings gave initial rates directly. The reaction mixture contained 0.1 M NaCl and 0.04 M $MgCl_2$ at 26°. Purified acetylcholinesterase from bovine erythrocytes was supplied by Nutritional Biochemicals Corp. 3-Hydroxyphenyltrimethylammonium iodide was prepared by reacting 3-dimethylaminophenol and methyl iodide in ether at room temperature; the product was

TABLE I: Values of Inhibition Constants (Calculated from Equations 8, 9, and 10) and of a (Equation 6) for Various Amines.

Compound	a	K_1	K'_1(exptl)	K'_1(corr)
Tetramethylammonium Cl	0.42, 0.58 0.65	3.5×10^2	18	50
Tetraethylammonium Br	0.83, 0.83	8.3×10^2	13	1.3×10^2
Tetra-*n*-propylammonium Br	0.05, 0.13	8.6×10^3	4.2×10^3	4.9×10^3
Tetra-*n*-butylammonium I	0.04	1.5×10^4	6.7×10^3	7.2×10^3
Tetra-*n*-pentylammonium I		2.6×10^3	1.1×10^3	
Choline Cl	0.33, 0.40	7.7×10^2	77	1.6×10^2
Chlorocholine Cl	0.13, 0.19	5.4×10^2	2.3×10^2	2.6×10^2
Trimethylphenylammonium I	0.23, 0.28 0.28	1.3×10^4	1.2×10^3	1.9×10^3
3-Hydroxyphenyltrimethyl-ammonium I	0.00, 0.00	1.6×10^6	7.0×10^4	
3-Fluorophenyltrimethyl-ammonium I	0.22	7.1×10^3	1.4×10^3	2.0×10^3
1,1,1-Trimethylhydrazine	0.39, 0.56	1.7×10^2	17	52
Trimethylamine HCl	0.00	1.4×10^2	2.7×10^2	
Triethylamine HCl	0.29, 0.30	2.1×10^2	1.5×10^2	2.8×10^2
Tri-*n*-butylamine HCl	0.04	3.5×10^3	1.8×10^3	2.0×10^3
Dimethylaminoethanol HCl	0.13, 0.17	2.7×10^2	2.8×10^2	4.1×10^2
Dimethylaminocyclohexane HCl	0.39, 0.44	1.1×10^3	1.3×10^2	2.8×10^2
cis-2-Dimethylaminocyclo-hexanol HCl	0.10, 0.18	1.0×10^3	1.4×10^2	1.9×10^2
Dimethylamine HCl	0.06, 0.19	30	40	50
Methylamine HCl[a]	0.79	16	1.3	9
n-Propylamine HCl	0.48, 0.49 0.64	1.2×10^2	18	54
n-Hexylamine HCl	0.37, 0.37 0.43	1.5×10^2	17	36
Tryptamine HCl	0.09	1.0×10^3	2.1×10^2	2.4×10^2
Serotonin hydrogen oxalate	0.00	9.7×10^2	2.1×10^2	

[a] To avoid a large increase in ionic strength, 0.1 N NaCl was replaced in this experiment by 0.1 M methylamine HCl.

recrystallized from ethanol (mp 181°, lit. 179°, Oae and Price, 1958). 3-Fluorophenyltrimethylammonium iodide (decomp at 189°, lit. 180°) was prepared by the procedure of Bevan and Bye (1957). *cis*-2-Dimethylaminocyclohexanol was synthesized from 2-dimethylaminocyclohexanone according to the method of Baldridge *et al.* (1955) (mp 32–34°, lit. 35–37°). The other inhibitors were of reagent grade; the liquid primary, secondary, and tertiary amines were redistilled before use.

Experimental Results

Application of the theory to acetylcholinesterase is illustrated by the data in Figures 2, 3, and 4. The experiment reported in Figure 2 shows clearly that the maximum velocity is higher when tetraethylammonium and trimethylammonium ions are present together than when only trimethylammonium ion inhibits. Triethylammonium ion (Figure 3) does not increase the maximum velocity, but nevertheless gives a higher rate than it would if it completely blocked deacetylation; this is evident from the fact that $1/V_1 - 1/V_0 > 1/V_{12} - 1/V_2$. With tetra-*n*-propylammonium ion (Figure 4), $1/V_{12} - 1/V_2$ is almost as large as $1/V_1 - 1/V_0$, indicating that deacetylation is largely blocked. Data of this kind were used to calculate a (equation 6), K_1 (equation 8), K'_1(exptl) (equation 9) and K'_1(corr) (equation 10), and the results are listed in Table I. For duplicate or triplicate determinations, which were usually carried out at different inhibitor concentrations, the average deviation of the experimental value of a from the mean was 0.04. This degree of reproducibility serves to distinguish ions capable of largely blocking

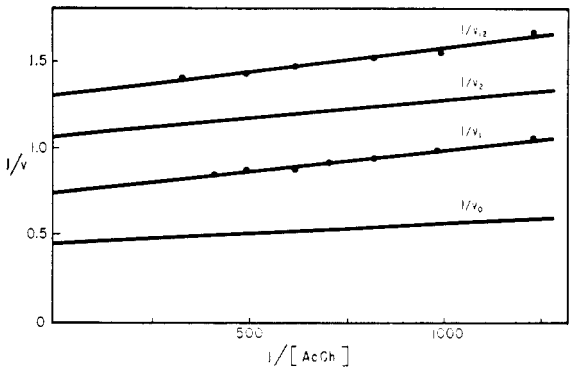

FIGURE 4: Inhibition by 1.54×10^{-4} tetra-*n*-propylammonium bromide with 5.27×10^{-3} M trimethylammonium chloride. Otherwise as in Figure 2.

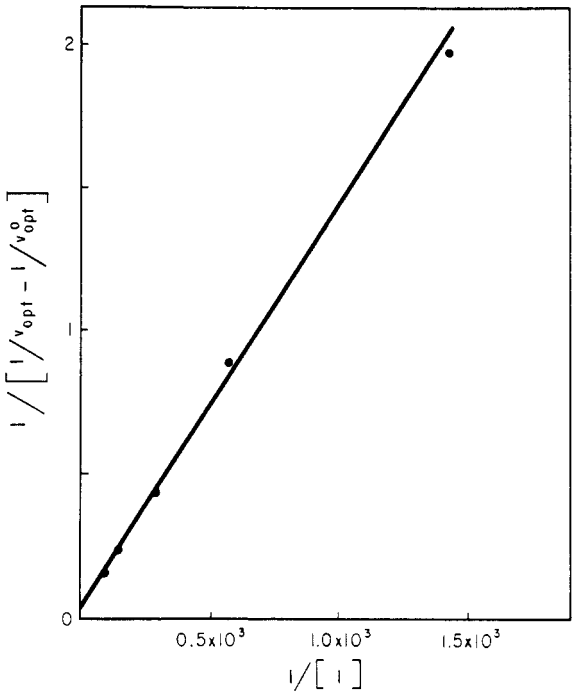

FIGURE 5: Effect of *cis*-2-dimethylaminocyclohexanol (I) on the optimum velocity (v_{opt}). Units of 1/[I] are M^{-1}.

deacetylation from those having a moderate or slight effect.

A second type of experiment was performed to determine the extent to which *cis*-2-dimethylaminocyclohexanol blocks deacetylation; the optimum velocity (v_{opt}) was determined as a function of the inhibitor concentration and $1/(1/v_{opt} - 1/v^0_{opt})$ was plotted against 1/[I] (Figure 5). As shown before (Krupka, 1963), a zero intercept indicates that $a = 0$, a positive intercept on the $1/(1/v_{opt} - 1/v^0_{opt})$ axis that $a > 0$.

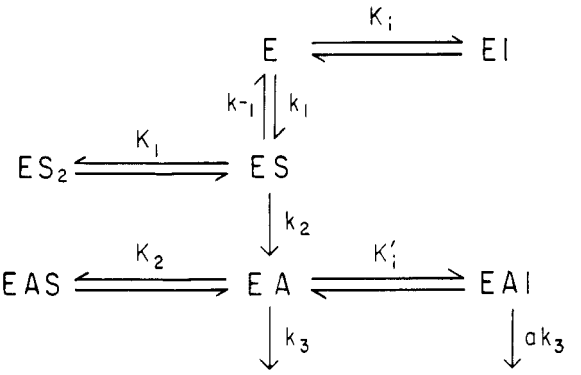

FIGURE 6: General reaction scheme for inhibition by substrate, S, and inhibitor, I. The substrate adds on to the enzyme-substrate complex (ES), and to the acetyl enzyme (EA), the inhibitor to the free enzyme (E), and to EA.

Discussion

To check the appropriateness of the scheme in Figure 1 to the actual enzymic mechanism, the slopes in $1/v \times 1/[S]$ plots for rates in the presence of two inhibitors (m_{12}) were estimated from the theory and compared with the experimental values. From equation (2) the slope should be equal to

$$(1 + K_1[I_1] + K_2[I_2])/\bar{K} = m_{12} = m_1 + m_2 - m_0 \quad (11)$$

where m_1, m_2, and m_0 are the slopes in the presence of I_1, I_2, and no inhibitor, respectively. In all cases, the predicted values of the slopes agreed well with the experimental results.

Another observation supporting the model is that with several of the inhibitors a has a small value, in some cases zero. The low value indicates that both the inhibitor in question and trimethylammonium ion must almost completely block deacetylation, which confirms the previous conclusion (Krupka, 1964) concerning the mechanism of trimethylammonium ion inhibition.

Comparison of Two Procedures for Determining a. The extent to which an inhibitor blocks deacetylation is reflected in its effect on the optimum velocity (Krupka, 1963); if EAI is reactive, $1/v_{opt}$ approaches an upper limit as the inhibitor concentration rises, but if it is unreactive the relationship between $1/v_{opt}$ and [I] is linear. The following equations show that measurements of v_{opt} may be used to find the approximate value of a. For the general reaction scheme in Figure 6, in which the substrate may form an inactive complex with either ES or EA, the predicted reaction rate is

$$v = \frac{k_2[E_0]\bar{K}[S]}{1 + K_i[I] + \bar{K}[S]\left\{1 + K_1[S] + \frac{k_2}{k_3}\frac{(1 + K_2[S] + K'_i[I])}{(1 + aK'_i[I])}\right\}} \quad (12)$$

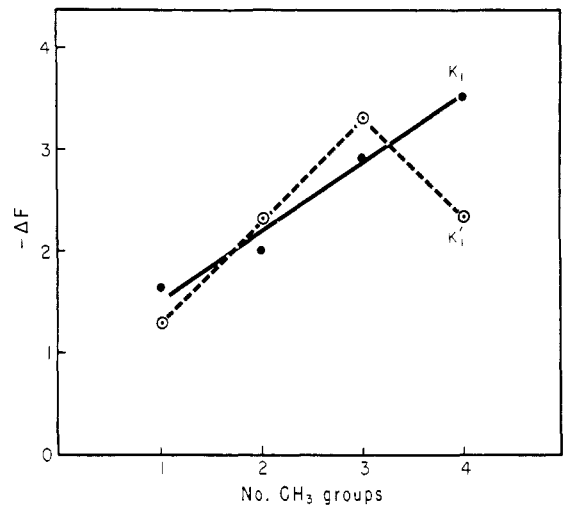

FIGURE 7: Free energies of binding to the free enzyme (K_i) and the acetyl enzyme (K'_i) as functions of the number of methyl groups in tetramethyl-, trimethyl-, dimethyl-, and methylammonium ions.

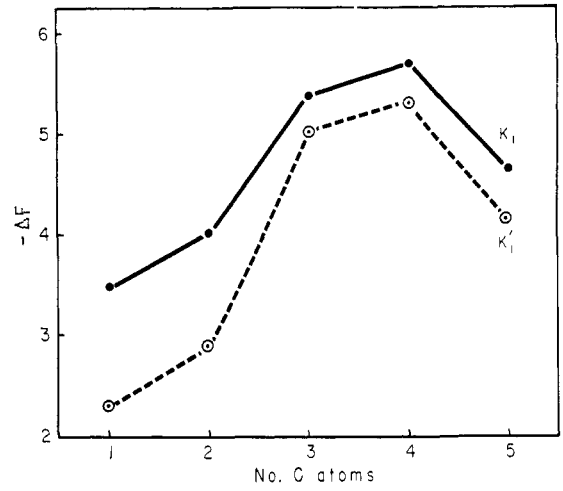

FIGURE 8: Free energies of binding to E (K_i) and EA (K'_i) as functions of the number of carbon atoms in the alkyl groups of tetramethyl-, tetraethyl-, tetra-*n*-propyl-, tetra-*n*-butyl-, and tetra-*n*-pentylammonium ions.

where $\bar{K} = k_1/(k_{-1} + k_2)$. The reciprocal of the optimum velocity is

$$\frac{1}{v_{opt}} = 1 + \frac{k_2}{k_3}\left(\frac{1 + K'_i[I]}{1 + aK'_i[I]}\right) + 2\left(\frac{1 + K_i[I]}{\bar{K}}\right)^{1/2} \times \left(K_1 + \frac{k_2}{k_3}\frac{K_2}{1 + aK'_i[I]}\right)^{1/2} \quad (13)$$

Since the association constant for substrate inhibition by acetylcholine is much smaller than that for binding to the free enzyme, the last term in (13) should be small compared with the second, especially as it involves the square root, rather than the first power, of [I]. As a good approximation the term may be neglected, so that

$$1/(1/v_{opt} - 1/v^0_{opt}) = k_3[E_0]\{a/(1 - a) + 1/(1 - a) \times K'_i[I]\} \quad (14)$$

Since the rate of acetylcholine hydrolysis is probably limited by the rate of deacetylation (Wilson and Cabib, 1956; Krupka, 1964) the maximum velocity, V_0, may be used to estimate $k_3[E_0]$. Hence the ratio of V_0 to the intercept in a plot according to equation (14) is approximately $1/a - 1$. From the data in Figure 5 for *cis*-2-dimethylaminocyclohexanol, a is not larger than 0.1, in fair agreement with the value of 0.18 from equation (6). Plots according to equation (14) were available for choline and tetramethylammonium ion inhibition (Krupka, 1963) and from these data the calculated values of a are 0.3 and 0.4, respectively, in agreement with the figures in Table I for these inhibitors. In the above calculations V_0 was taken to be $1.07 \times v_{opt}$ as may be shown from plots of $1/v \times 1/[S]$.

Binding of Inhibitors to the Free and Acetyl Enzymes. From the values of K_1 and K'_1(corr) in Table I, the free energies of binding, ΔF and $\Delta F'$, were calculated for tetramethyl-, trimethyl-, dimethyl-, and methylammonium ions, and these values were plotted as functions of the number of methyl groups attached to the positively charged nitrogen atom (Figure 7). Accompanying the removal of one methyl group from tetramethylammonium ion to give trimethylammonium ion, there is a large increase in binding to EA (1.0 kcal), but a decrease in binding to E (0.7 kcal). This suggests a restricted spatial arrangement in EA, such that a fourth methyl group prevents the close approach of the inhibitor to the anionic site. Trimethylammonium ion probably approaches the anionic site in EA more closely than that in E, since it is bound more strongly to the former than to the latter. Further removals of methyl groups from trimethylammonium lower the binding to both EA and E.

Values of ΔF, for binding to E, and $\Delta F'$, for binding to EA, are plotted in Figure 8 as functions of the number of carbon atoms in the alkyl substituents of tetramethyl-, tetraethyl-, tetra-*n*-propyl-, tetra-*n*-butyl- and tetra-*n*-pentylammonium ions. In all cases, binding to EA is weaker than to E, suggesting that the inhibitors cannot approach the anionic site in EA as closely as that in E. The difference in binding is greater with the methyl- and ethyl-substituted ions (1.1 kcal) than with the larger analogs (0.4 kcal), the explanation being, perhaps, that the latter are bound by Van der Waals forces to nonpolar amino acid side chains available in both E and EA.

Tetraethylammonium ion is bound to E and EA more strongly than the tetramethyl derivative by about 0.5 kcal mole^{-1}, but the increase in binding energy in going to the next higher derivative, tetra-*n*-propylammonium ion, is much larger than this, 1.4 kcal and

2.0 kcal for binding to E and EA, respectively. On the other hand, tetra-*n*-butylammonium ion is not bound much more strongly than the *n*-propyl derivative (0.3 kcal), and tetra-*n*-pentylammonium is more weakly bound. The large increase in inhibitory power of the tetra-*n*-propyl over the tetraethyl derivative may, as suggested in the preceding paragraph, be due to the involvement of nonpolar amino acid side chains in binding the former ion. The relatively low binding of the two largest ions is understandable if the ion–ion attraction is reduced, partly because of greater separation between the anionic site and the positive charge in the inhibitor, and partly because the major electrostatic attraction may, as Thomas (1961) suggested, involve the fractional positive charge on the α-carbon atoms rather than the charge on the nitrogen atom. In the larger ions, interactions between the enzyme and the α-carbon atoms would be restricted by the carbon chains.

Inhibition of Deacetylation by Cations. The experimental procedure gives a value for *a*, the ratio of rates at which EAI and EA react to form acetic acid and the free enzyme. When I is an alcohol, an ester could be formed from EAI, and this process would not be detected. Blocking of deacetylation by such cations may therefore have two causes, interference with hydrolysis of the acetyl enzyme and reaction of the inhibitor with the acetyl group to form an ester.

Observations on the blocking of deacetylation are summarized as follows: (a) Quaternary amines do not block when the substituents are small (tetramethyl- and tetraethylammonium ions) but do when they are as large as a propyl group (tetra-*n*-propyl- and tetra-*n*-butylammonium ions). Replacement of one methyl group in tetramethylammonium sometimes increases inhibition slightly (e.g., choline, and trimethylphenyl- and trimethyl-3-fluorophenylammonium ions) and sometimes greatly (e.g., 3-hydroxyphenyltrimethylammonium ion). (b) Tertiary amines in which the substituents are of equal size usually block deacetylation, and the same may apply to secondary amines (e.g., trimethyl- and tri-*n*-butylamines, dimethylaminoethanol, and dimethylamine). Tertiary amines with two small substituents and one large may not block deacetylation effectively, as in the case of dimethylaminocyclohexane. Addition of a hydroxyl group to the latter, forming *cis*-2-dimethylaminocyclohexanol, increases blockage. (c) Primary amines do not block deacetylation if the substituent is small, as in the case of methylamine and *n*-propylamine, but the degree of blocking increases when the substituent is enlarged, e.g., hexylamine, tryptamine, and serotonin. (d) Ethyl substituents have the peculiar property of blocking less than methyl groups, e.g., tetraethyl- and triethylammonium compared with tetramethyl- and trimethylammonium ions.

Several conclusions may be drawn from these observations. If a quaternary amine is symmetrical and large it may overlap the acyl group in the enzyme and interfere with its reaction with water. In the series tetramethyl-, tetraethyl-, tetra-*n*-propyl-, and tetra-*n*-butylammonium ions, the first and second members block only slightly, the third and fourth greatly. On the basis of Stuart-Briegleb models, the radii of unhydrated tetraethyl- and tetra-*n*-propylammonium ions in their fully compressed and extended forms are approximately 3.5 and 4.5 A, respectively, for the first ion and 3.9 and 5.9 A for the second. The acyl group may therefore be 4–5 A from the anionic site, which is consistent with other evidence on the relative positions of functional groups in the enzyme (Wilson, 1960; Krupka and Laidler, 1961).

If only one substituent in the quaternary amine is large, it may be directed away from the acyl residue and fail to block deacetylation (e.g., trimethylphenylammonium ion). On the other hand, a particular substituent, such as the hydroxyl group in 3-hydroxyphenyltrimethylammonium ion, may hold the larger group in the vicinity of the acyl residue, perhaps by bonding with an essential enzyme group, and block deacetylation. Alternatively, the inhibitor may react with the acetyl group, forming an ester.

Many tertiary and secondary amines block deacetylation. Inhibition is not simply dependent upon the small size of the ions, for dimethylaminoethanol largely blocks deacetylation while tetramethylammonium ion, of roughly the same size, does not, nor does the smallest ion tested, methylammonium. A possible explanation is that tertiary and secondary amines form a hydrogen bond with an essential enzyme group. The separation between the anionic site and this particular group would then be about 2.5 A.

A large substituent in tertiary amines, as in dimethylaminocyclohexane, may prevent complete blocking of deacetylation by hindering the ion's penetration into the spatially restricted region where it can inhibit the enzyme reaction. Alternatively, the large substituent may prevent the required hydrogen bond formation by affecting the orientation of the bound ion. 1,1,1-Trimethylhydrazonium ion may be similarly hindered from blocking deacetylation, either because of size or because the second nitrogen atom is unsuitably oriented in EA. The failure of primary amines to block deacetylation may also be because of an unsuitable orientation.

Relative Positions of the Anionic and Esteratic Sites in E and EA. From studies with substrates and inhibitors, Wilson (1960) concluded that in acetylcholinesterase from the electric organ of *Electrophorus electricus* the anionic site is about 5 A from the basic group in the esteratic site. This conclusion was based partly on experiments with hydroxy-substituted phenyltrimethylammonium ions. A key finding was that 3-hydroxyphenyltrimethylammonium ion was bound to the enzyme 120 times more strongly than phenyltrimethylammonium ion, probably because of a hydrogen bond formed between the phenolic hydroxyl of the inhibitor and the basic group at the active center (Wilson and Quan, 1958). Presumably hydrogen bonding could occur because the distance between the quaternary ammonium and hydroxyl groups in the inhibitor corresponded to that between the anionic site and basic

group in the enzyme. The same ratio of binding strengths for these inhibitors is found with bovine erythrocyte acetylcholinesterase (see Table I), indicating that the enzymes from these sources are very similar. It is also found that only the hydroxy-substituted ion completely blocks deacetylation, suggesting that the basic group which attracts the inhibitor does function catalytically, as Wilson and Quan thought.

The basic group, then, is probably about 5 A from the anionic site. It may well be involved, perhaps indirectly, in a nucleophilic attack upon the carbonyl carbon atom of the substrate, resulting in transfer of an acyl group from the ester substrate to the enzyme, and the acyl residue in EA may therefore occupy roughly the same region of the enzyme surface as the basic group. As seen before, the experiments with tetramethyl-, tetraethyl-, tetra-*n*-propyl-, and tetra-*n*-butylammonium ions suggest that the acyl residue in EA is approximately 5 A from the anionic site, in agreement with this idea. A second line of evidence comes from the experiments with phenyltrimethyl- and 3-hydroxyphenyltrimethylammonium ions. The latter is bound to EA approximately 37 times more strongly than phenyltrimethylammonium ion and, as noted, 120 times more strongly to E. The increased binding to EA indicates that the hydroxyl group is involved in binding, as in E, so that the two sites which attract the inhibitor probably occupy roughly the same positions in E and in EA. The smaller increase in binding to EA than to E may result from spatial restrictions about the basic group in EA, as expected if the acyl residue is nearby. The available evidence therefore suggests that the orientation of functional groups is not greatly changed during acetylation of the enzyme.

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