Fluoride Inhibition of Acetylcholinesterase

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SUMMARY

Inhibition of acetylcholinesterase by sodium fluoride is reversible and depends on binding of one fluoride ion per active center. The inhibitor adds to the free enzyme, the enzyme–substrate complex (blocking acetylation), and the acetyl enzyme (blocking deacetylation). The fluoride binding site is probably in the active center, since cationic inhibitors compete with fluoride for the acetyl enzyme. Small cationic inhibitors do not compete with fluoride for the free enzyme, and fluoride can add to the enzyme–substrate complex whether the substrate is a cation or a neutral molecule. The pH dependence of fluoride binding to the free enzyme and the enzyme–substrate complex is practically identical, but a different pH dependence is observed for the acetyl enzyme. These findings indicate that the site of fluoride attachment is altered during the course of the enzyme reaction, suggesting a conformational change accompanying acetylation.

INTRODUCTION

Fluoride inhibition of AChE[1] has been known for many years (1, 2), but its detailed mechanism is still not understood. Binding of fluoride to the free enzyme, enzyme–substrate complex, and acetyl enzyme have now been studied, with particular reference to interactions with bound cations and ionizing groups in the enzyme. Since completion of this work, related papers have appeared (3, 4) and there is general agreement about the basic kinetics of the inhibition but not about the fluoride binding site and inhibition mechanism. Cimasoni (4) concluded that the site was probably outside the active center, since inhibition was found to be uncompetitive. Heilbronn (3), on the other hand, considered the possibility of binding at the substrate site but had difficulty reconciling this with the types of inhibition observed for different substrates, either purely noncompetitive or mixed competitive and noncompetitive. There were other difficulties: despite the noncompetitive inhibition with cationic substrates, fluoride did compete with trimethylammonium ion, and also with substrate molecules responsible for substrate inhibition. The present experiments lead to more definite conclusions regarding the binding site and inhibition mechanism, and explain these apparent contradictions.

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

MATERIALS AND METHODS

Rates of substrate hydrolysis in reaction mixtures maintained at 26.0° were determined by automatic titration of acid released, using a radiometer TTT1 Titrator and Titrigraph, with Radiometer G202C glass and K401 calomel electrodes. With ionic substrates a stream of nitrogen was passed over the reaction solution, and with volatile substrates the reaction mixture was closed off from the atmosphere. Appropriate controls were run in all cases, and where necessary corrections were applied for buffering by acetate produced in the hydrolysis. Statistical analysis was based on plots of $[S]/v$ against $[S]$ (5, 6). The enzyme, a purified preparation of bovine erythrocyte acetylcholinesterase, was sup-

plied by Sigma Chemical Co. Dipropylmethylaminoethyl acetate iodide was kindly donated by Dr. W. C. Dauterman, Pesticide Residue Laboratories, North Carolina State College, Raleigh, North Carolina. Reagent isoamyl acetate was redistilled before use. Other chemicals were of reagent grade and were used without further purification.

RESULTS

Effects of Inorganic Ions on Inhibition by NaF

Relative inhibitions of AcCh hydrolysis (0.002 M) by NaF in the presence of either 0.10 M NaCl or 0.10 M NaCl together with 0.04 M $MgCl_2$ were measured at pH 7.5 (Table 1). $MgCl_2$ diminishes fluoride inhibition slightly, and subsequent experiments were carried out in 0.10 M NaCl solutions. Similar measurements were made in various other media: 0.33 M sodium oxalate, 0.02 M NaBr, 0.02 M NaI, 0.10 M NaCl + 2% methanol, and with no added salt (Table 1). Neither Cl^-, Br^-, I^- nor oxalate gave protection against fluoride, and methanol did not alter the inhibition constant.

TABLE 1

K_F values (Eq. 8, Appendix) for inhibition of the hydrolysis of 0.0020 M AcCh by 0.0040 M NaF in various reaction media at pH 7.5

	Medium	K_F
1	0.10 M NaCl	$3.67 \pm 0.07 \times 10^{-3}$ M
2	0.10 M NaCl + 0.04 M $MgCl_2$	$8.21 \pm 0.21 \times 10^{-3}$ M
3	0.09 M NaCl + 0.02 M NaBr	$3.69 \pm 0.04 \times 10^{-3}$ M
4	0.09 M NaCl + 0.02 M NaI	$3.90 \pm 0.07 \times 10^{-3}$ M
5	0.033 M NaC_2O_4	$4.18 \pm 0.07 \times 10^{-3}$ M
6	No added salt	$6.28 \pm 0.11 \times 10^{-3}$
7	0.10 M NaCl, dialyzed enzyme	$3.25 \pm 0.12 \times 10^{-3}$ M
8	0.10 M NaCl, 2% methanol	$3.74 \pm 0.35 \times 10^{-3}$ M

The commercial AChE preparation used in this study contains a small quantity of sodium phosphate, giving a final concentration of less than 5×10^{-5} M in experiments with AcCh. Fluoride inhibition of succinic oxidase is known to depend on the presence of phosphate (7), suggesting that the latter could be involved in AChE inhibition. This possibility was excluded in an experiment with dialyzed enzyme. Enzyme, dissolved in 25 ml of a solution of 0.10 M NaCl + 0.001 M EDTA, pH 7.0, was dialyzed against 250 ml of the same salt solution over a period of 72 hr, at 2°, with three changes. Inhibition of this preparation was at least as strong as with undialyzed enzyme (Table 1), showing that phosphate is nonessential.

Reversibility of NaF Inhibition

When hydrolysis of AcCh in the presence of AChE and NaF was followed continuously, no changes in rate were observed over a period of 15–20 min that could not be explained by substrate depletion, suggesting that no progressive increase in inhibition occurred. In a confirmatory experiment, enzyme solution was incubated with 1.57×10^{-2} M NaF at 0°. The rate at which an aliquot of this solution (giving a final NaF concentration of 1.57×10^{-3} M) catalyzed the hydrolysis of 0.002 M AcCh in a reaction mixture containing 0.10 M NaCl at pH 7.5 was determined immediately and then again after 4.5 hr. The final and initial enzyme activities were identical, their ratio being 1.002 ± 0.013, and K_F was $4.29 \pm 0.20 \times 10^{-3}$ M, compared with 3.7×10^{-3} M measured in the usual experimental procedure (Table 1). Consequently inhibition by the high NaF concentration in the incubation mixture must have been reversed immediately after dilution. If an irreversible complex is formed with the enzyme, inhibition should increase with time and should persist after dilution. It is therefore concluded that the inhibition is reversible.

Failure of Oxalate, Bromide, and Iodide to Inhibit

Rates of hydrolysis of 0.002 M AcCh at pH 7.5 were determined in the presence of 0.033 M and 0.12 M sodium oxalate or 0.10 M NaCl. Relative rates were 0.99, 1.01, and

1.00, respectively. In the presence of 0.02 M NaBr + 0.09 M NaCl, the relative rate was 1.00 ± 0.01, and in 0.02 M NaI + 0.09 M NaCl it was 0.96 ± 0.01.

Kinetics of Fluoride Ion Inhibition

Rates of substrate hydrolysis were measured in the presence of 4.0×10^{-3} M NaF, using AcCh (Fig. 1), dipropylmethylaminoethyl acetate (1.46×10^{-3} to 7.30×10^{-3} M), acetyl-β-methylcholine (2.06×10^{-3} to 2.06×10^{-2} M), and isoamyl acetate (2.5×10^{-3} to 1.0×10^{-2} M) as substrates. In the experiment with isoamyl acetate the reaction mixture contained 2% methanol. In no case was inhibition competitive; inhibition constants are listed in Table 2.

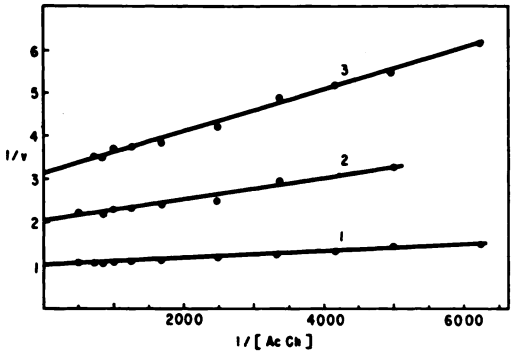

FIG. 1. *Lineweaver-Burk plot for inhibition of acetylcholine (AcCh) hydrolysis by NaF*

Curve *1*, No inhibitor; curve *2*, 4.00×10^{-3} M NaF; curve 3, 8.01×10^{-3} M NaF. Units of AcCh concentration are molar, and V_{max} in absence of inhibitor is arbitrarily set at unity.

The number of fluoride binding sites was determined from measurements, at pH 7.5, of the rates of hydrolysis of AcCh or isoamyl acetate with varying concentrations of NaF. Log $(v_0/v - 1)$ was plotted against log NaF, according to the following equation (see reference 8 and Appendix, Section iii):

$$\log (v_0/v - 1) = -\log K + n \log [F^-] \quad (1)$$

The plots (Fig. 2) are linear, and the slopes, equal to the number of binding sites, *n*, are 0.97 and 1.01 with AcCh and isoamyl acetate, respectively.

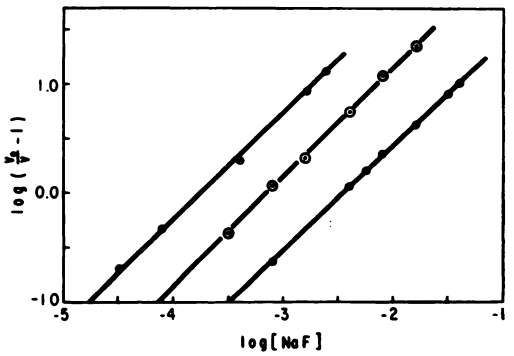

FIG. 2. *Relationship between inhibition and the molar concentration of NaF* (see Eq. 1)

The three lines (left to right) are for rates of hydrolysis of 0.0020 M acetylcholine bromide, pH 5.2; 0.010 M isoamyl acetate, pH 7.5; and 0.0020 M acetylcholine bromide, pH 7.5. The slopes, corresponding to the number of fluoride binding sites, are 0.98, 1.01, and 0.97 respectively.

pH Dependence of Fluoride Ion Inhibition

Relative inhibitions, v/v_0, were determined with several substrates (AcCh, isoamyl acetate, and acetyl-β-methylcholine) over the pH range 4.7–8.5. Inhibition constants, K_F, were then calculated from Eq. 8, and pK_F was plotted against pH (Fig. 3). If ionizations in the enzyme or inhibitor affect inhibitor binding, points in such a plot should lie along linear segments connected

TABLE 2

Inhibition constants for NaF with four substrates (pH 7.5)[a]

Substrate	K_i (M)	$K'_{i(app)}$ (M)
AcCh	$1.68 \pm 0.48 \times 10^{-3}$	$3.73 \pm 0.11 \times 10^{-3}$
Dipropylmethylaminoethyl acetate	—	$2.06 \pm 0.13 \times 10^{-3}$
Acetyl-β-methyl choline	—	$2.92 \pm 0.16 \times 10^{-4}$
Isoamyl acetate	$1.03 \pm 0.15 \times 10^{-3}$	$6.24 \pm 1.69 \times 10^{-4}$

[a] See Eqs. 6 and 7, Appendix.

by curved regions; the slope of a linear segment should equal unity if a single ionizing group affects binding, or zero if no ionization is involved (9, 10). The pH at the point of intersection of two linear segments corresponds to the pK value of an ionizing group in the enzyme or inhibitor, and at this pH the experimental points are expected to lie 0.3 pK units above or below the point of intersection. Application of these rules to the NaF data

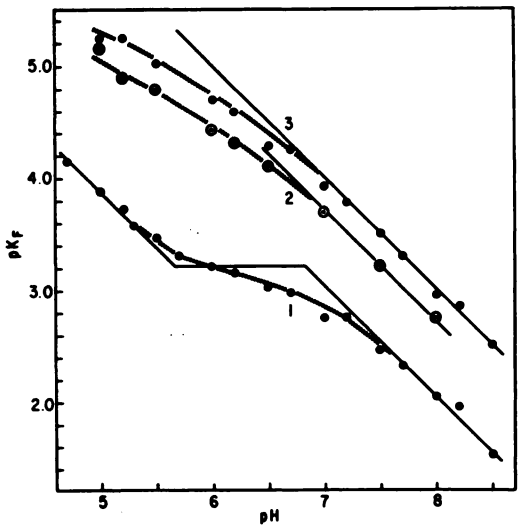

FIG. 3. *NaF inhibition of hydrolysis of three substrates as a function of pH*

Curve *1*, 0.0020 M acetylcholine bromide; curve *2*, 0.010 M isoamyl acetate; and curve *3*, 0.0103 M acetyl-β-methylcholine. pK_F is calculated from Eq. 8, Appendix. Guide lines drawn in the figure have slopes of 0 or −1 (see text).

indicates that two ionizations affect inhibition. With AcCh, fluoride appears to be bound only when a group of pK 6.8 becomes protonated. At low pH (<6) there is a secondary increase in inhibition, related to the ionization of an enzyme group of pK 5.7. With acetyl-β-methylcholine and isoamyl acetate the behavior is different, and pK_F rises continuously as the pH drops from 8.5 to 6.0. Below pH 6, the experimental constants fall progressively in relation to the guide line of unit slope, suggesting that inhibition depends on an ionization between pH 5 and 6.

K_F (Eq. 8) is a weighted mean of competitive and noncompetitive inhibition constants (K_i and $K'_{i(app)}$ respectively; Eqs. 6 and 7). The latter constants were determined separately for AcCh hydrolysis at pH 5.5 to 7.5 (Fig. 4). As expected, pK_F and $pK'_{i(app)}$ for AcCh exhibit a similar pH dependence (since 0.002 M AcCh, employed in determinations of K_F, saturates the enzyme). However, the pH dependence

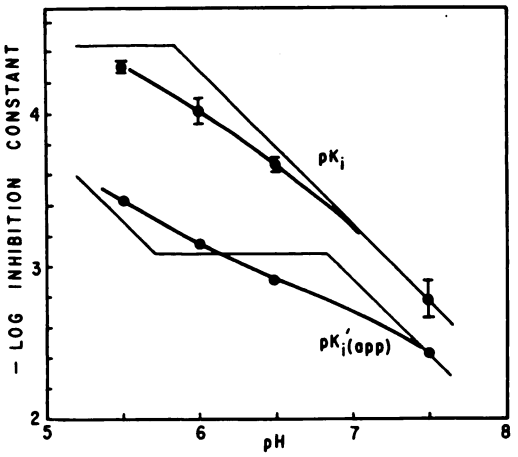

FIG. 4. *Constants for NaF inhibition of acetylcholine hydrolysis as a function of pH*

pK_i and $pK'_{i(app)}$ were calculated according to Eqs. 6 and 7. The average standard error of $pK'_{i(app)}$ was 0.015, and the standard errors of pK_i are indicated by bars in the figure. Guide lines have slopes of 0 or −1 (as in Fig. 3).

of pK_i is different, and resembles the behavior observed with acetyl-β-methylcholine and isoamyl acetate. That the difference is significant is shown by the values of $pK-pK'_{(app)}$, which are 0.872 ± 0.032, 0.870 ± 0.086, 0.724 ± 0.030, and 0.344 ± 0.125 at pH 5.5, 6.0, 6.5, and 7.5, respectively.

The secondary increase in inhibition of AcCh hydrolysis at low pH (Fig. 3) could result from binding of a second fluoride ion. An experiment similar to that described above (Eq. 1) was therefore carried out. Rates were now measured with AcCh at pH 5.2, and the results, included in Fig. 2, indicate that at low pH, as well as at high, only one fluoride ion is bound to the enzyme.

It was recently shown that two ionizing groups at the active center function catalytically in AChE; in 0.10 M NaCl + 0.04 M $MgCl_2$ the pK values of these groups are 5.5 and 6.3 (11). In view of the similarity between the pK values of the groups involved in catalysis and in fluoride inhibition, it was possible that the same groups determined both types of behavior. The ionization constants of the two catalytic groups were therefore redetermined in 0.10 M NaCl. Previous work (6, 11) showed that the lower ionization constant (pK 5.5) may be obtained from the pH dependence of isoamyl acetate hydrolysis, and the higher from AcCh hydrolysis, both at fixed substrate concentrations. From rate measurements with 0.010 M isoamyl acetate (pH 5.5–6.7) and 0.002 M AcCh (pH 6.5–8.2), the constants were found to be 5.49 ± 0.04 and 6.56 ± 0.03. Additional experiments were now performed to decide whether the fluoride binding site is near the active center.

Inhibition by NaF in the Presence of Substituted Ammonium Ions

Competition between two inhibitors may be detected in an experiment where both are allowed to act upon the enzyme at the same time, with analysis based on Eqs. 19–22 (Appendix, Section ii). Lineweaver-Burk plots for rates in the absence of inhibitor, or in the presence of NaF, of 3-hydroxyphenyltrimethylammonium iodide (12, 13), and of both inhibitors are shown in Fig. 5. Similar experiments with tetramethyl ammonium chloride, tetraethylammonium bromide, tetrapropylammonium bromide, choline chloride, and butyrylcholine iodide are reported in Table 3. An experiment was also carried out with trimethylammonium chloride, at a single AcCh concentration (0.002 M). The reciprocals of the relative rates were as follows: $1/v_0 = 1.00 \pm 0.013$; $1/v_F = 2.13 \pm 0.017$; $1/v_I = 4.26 \pm 0.028$; $1/v_{IF} = 5.04 \pm 0.072$, where the subscripts 0, F, I, and IF refer to rates in the absence of inhibitor, and in the presence of fluoride (F), ammonium ion (I), and both inhibitors, respectively.

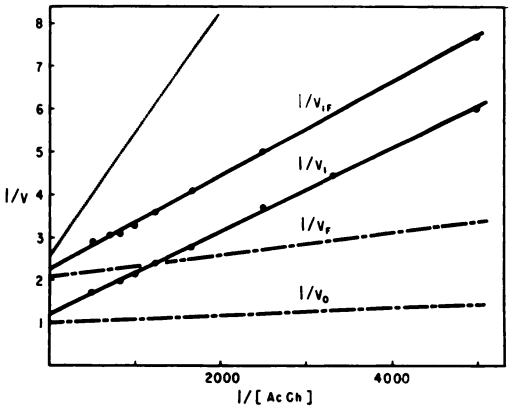

FIG. 5. *Lineweaver-Burk plots for rates of acetylcholine hydrolysis in the presence of* 2.66×10^{-6} M *3-hydroxyphenyltrimethylammonium iodide alone* ($1/v_I$) *or together with* 4.03×10^{-3} M *NaF* ($1/v_{IF}$)

Rates in the absence of inhibitor ($1/v_0$) and in the presence of 4.03×10^{-3} M NaF ($1/v_F$) are indicated by the two broken lines below. Dotted line in upper part of figure shows expected values of $1/v_{IF}$ if fluoride and the cationic inhibitor bind at separate sites without mutual interference. Slopes (m) and intercepts ($1/V$) of lines are given in Table 3, together with standard error estimates.

Effect of NaF on Acid Production during AcCh Hydrolysis

To decide whether inhibition of deacetylation is due to fluoroacetate production, the relation between substrate loss and acetic acid formation was examined. Reaction mixtures were prepared (10 ml total volume) containing 0.10 M NaCl, enzyme, 0.008 M NaF, and 4.0×10^{-4} M AcCh. Hydrolysis was started on addition of the latter. Other solutions were identical except for omission of NaF and use of a four times lower enzyme concentration. Acid released was titrated at pH 7.5 using a pH stat. After 22–30 min, when no further acid was produced, the relative volumes of added base were determined from the titrigraph tracing, and were found to be 75.6 ± 0.5 units in the presence of NaF, and 77.4 ± 0.6 in its absence. The relative initial rates of hydrolysis with and without NaF, corrected for differences in enzyme concentration, were 0.33 and 1.00.

TABLE 3

Values of 1/V (the reciprocal of the maximum velocity) and m (the slope in a 1/v × 1/[S] plot, or intercept in an [S]/v × [S] plot)

The subscripts 0, F, I, and IF refer, respectively, to rates in the absence of inhibitors, and in the presence of a fixed concentration of fluoride (F), of a cationic inhibitor (I), or of both. The concentration of NaF employed throughout was 4.03×10^{-3} M. Reaction mixture total volume 10 ml, pH 7.5, 26.0°, 0.10 M NaCl, 0.0020 M AcCh. Statistical analysis based on [S]/v × [S] plots. $1/V_0$ and m_0 are arbitrarily set at unity.

Inhibitor	Inhibitor concentration (M)	$1/V_0$	$1/V_F$	$1/V_I$	$1/V_{IF}$	m_0	m_F	m_I	m_{IF}
Tetramethylammonium chloride	9.01×10^{-3}	1.000 ± 0.018	2.107 ± 0.046	1.257 ± 0.030	1.831 ± 0.041	1.00 ± 0.20	2.92 ± 0.58	12.19 ± 0.52	30.35 ± 0.70
Tetraethylammonium bromide	7.14×10^{-3}	1.000 ± 0.018	2.107 ± 0.046	1.023 ± 0.031	1.301 ± 0.050	1.00 ± 0.20	2.92 ± 0.58	19.69 ± 0.53	48.70 ± 0.81
Tetrapropylammonium bromide	3.76×10^{-4}	1.000 ± 0.027	2.110 ± 0.065	2.095 ± 0.034	3.84 ± 0.41	1.00 ± 0.21	2.91 ± 0.58	16.71 ± 1.10	35.94 ± 1.47
Choline chloride	4.34×10^{-3}	1.000 ± 0.018	2.107 ± 0.050	1.350 ± 0.046	2.272 ± 0.029	1.00 ± 0.20	2.92 ± 0.58	8.62 ± 0.95	17.13 ± 0.53
Butyrylcholine iodide	6.01×10^{-4}	1.000 ± 0.047	2.110 ± 0.054	1.105 ± 0.009	2.440 ± 0.019	1.00 ± 0.21	2.91 ± 0.58	6.38 ± 0.19	12.29 ± 0.41
3-Hydroxyphenyltrimethylammonium iodide	2.66×10^{-6}	1.000 ± 0.026	2.107 ± 0.062	1.199 ± 0.019	2.241 ± 0.047	1.00 ± 0.20	2.92 ± 0.54	14.52 ± 0.38	15.96 ± 0.84

DISCUSSION

Inhibitions of AChE by fluoride ion and by chelators such as oxalate obviously have a different basis. Oxalate inhibits only impure enzyme preparations, whose activity is increased by addition of Mg^{++} (14, 15), while fluoride inhibits the purified enzyme. Other halides, NaBr, NaI, and NaCl, do not inhibit and give no protection against fluoride. A single mole of NaF is involved in inhibition at pH 7.5 and 5.2, and the binding is reversible [in agreement with the findings of Heilbronn (3, 16) and Cimasoni (4)]. There are several reasons for thinking that F^- rather than HF or HF_2^- is the main inhibitory species. First, as pointed out by Heilbronn (3), the concentrations of HF and HF_2^- are extremely low (for HF pK = 3.2), and K_i would be of the order of 10^{-7} M. Such strong inhibition is somewhat unlikely. Next, the concentration of HF_2^- is proportional to the square of the fluoride ion concentration, predicting that inhibition should be related to $[NaF]^2$, instead of to [NaF] as found. Another argument is that in certain pH regions the inhibition tends toward pH independence (see Figs. 2 and 3). If the two protonated species were responsible, inhibition should increase with at least the first power of $[H^+]$ over the entire pH range.

The observation of mixed competitive and noncompetitive inhibition with substrates whose hydrolysis is rate-limited at either the acetylation step (isoamylacetate, dipropylmethylaminoethyl acetate, and acetyl-β-methylcholine) or deacetylation step (acetylcholine) (11), indicates that fluoride binds to E, ES, and EA, blocking reaction of the latter two.

NaF reactivates AChE inhibited by organophosphorus compounds, presumably by attacking the phosphoryl enzyme to give a phosphoryl fluoride and the free enzyme (17). By analogy, reaction of fluoride with EA to form E and acetyl fluoride may account for the apparent interference with deacetylation. Since the half-life of this compound is 26 min at 25° and pH 7.5 (18, 19), little would be hydrolyzed during the 10-min period required for reaction rate measurement; furthermore the assay depends on release of acetic acid and would not detect acetyl fluoride. In the last experiment reported above, K'_i [NaF] = 2.2 = [EAI]/[EA]. Hence, approximately two-thirds of EA is combined with fluoride, and therefore two-thirds of the product could be acetyl fluoride and one-third acetic acid. During the roughly 26 min required for complete hydrolysis, some acetyl fluoride would be hydrolyzed spontaneously to acetic acid. If the average acetyl fluoride concentration is taken as half the final, and if half this is hydrolyzed while the experiment is in progress, then the final yield would be three-fourths of the total production, i.e. $(\frac{3}{4}) \times (\frac{2}{3}) = \frac{1}{2}$. Consequently the total acid formation in the presence of NaF should be about half the control. The quantities released are practically the same, and therefore inhibition of deacetylation cannot result from acetyl fluoride formation.

The experiments on competition between fluoride and cationic inhibitors strongly suggest that fluoride is bound at the active center. Analysis is based on Eqs. 19 and 20 (Appendix). For 3-hydroxyphenyltrimethyl ammonium, a highly specific competitive inhibitor of AChE (12, 13), $1/V_{IF} = 1/V_I + 1/V_F - 1/V_0$ and $m_{IF} = m_I + m_F - m_0$. The values found are 2.24 ± 0.047 and 2.30 ± 0.070 for the first equality, and 15.96 ± 0.84 and 16.46 ± 0.70 for the second (Fig. 5; Table 3). Hence $K_s = K''_s = 0$ (see Fig. 6), meaning that the free enzyme, E, and the acetyl enzyme, EA, can bind either fluoride or the cation, but not both. It may be noted that displacements in m (the slope in a Lineweaver-Burk plot) reflect binding to E, while displacements in $1/V$ (the intercept in this plot) reflect binding to EA. (The second rule holds because the rate-limiting step in hydrolysis at high AcCh concentrations is deacetylation—see Appendix, Section ii). The dotted line drawn in Fig. 5 shows the expected $1/v_{IF}$ values (for rates in the presence of both inhibitors) if the two inhibitors do not compete for either E or EA

(Eqs. 21 and 22). The experimental and predicted points obviously differ by more than experimental error.

If either tetramethylammonium or tetraethylammonium ion competes for EA with a second inhibitor that completely blocks deacetylation, then the maximum rate of AcCh hydrolysis, V, will be higher in the presence of both inhibitors than with the second alone. The explanation is as follows: only a partial blockade of deacetylation ensues when these ions are bound to EA (13), and consequently their substitution for the second inhibitor accelerates deacetylation, the rate-limiting step in AcCh hydrolysis (11, 20). Both ions are found to increase V when added to the system containing enzyme, AcCh and NaF (Table 3). The acetyl enzyme can therefore bind fluoride or the quaternary ammonium ion, but not both. The following rate equation describes this behavior (13), assuming that the complex of EA with F^- is inactive, as the experiments have indicated:

$$(1 + [I]/K''_1 + [F]/K''_2)/(1 + a[I]/K''_1)k_3[E_0] \quad (2)$$

where a is the rate of hydrolysis of EAI relative to that of EA. If I does not affect deacetylation, $a = 1$, while if it completely blocks the reaction, $a = 0$. Using the following expression (13), a may be evaluated:

$$\frac{a}{1-a} = \frac{(1/V_I + 1/V_F - 1/V_0 - 1/V_{IF})1/V_0}{(1/V_I - 1/V_0)(1/V_F - 1/V_0)} \quad (3)$$

It is seen that when both inhibitors block deacetylation ($a = 0$),

$$1/V_{IF} = 1/V_I + 1/V_F - 1/V_0 \quad (4)$$

This is the situation found with 3-hydroxyphenyltrimethylammonium iodide, in agreement with previous studies on this inhibitor (13). From the data in Table 3 values of a for tetramethyl and tetraethyl ammonium ions are approximately 0.65 and 0.97, respectively, which may be compared with previous values of 0.54 and 0.83 for reaction mixtures containing 0.10 M NaCl and 0.04 M $MgCl_2$ (13). The conclusion that fluoride competes with cations for EA is supported by the data for choline, butyrylcholine, and tetrapropylammonium, where the quantity $1/V_I + 1/V_F - 1/V_0$ is 2.46 ± 0.066, 2.22 ± 0.024, and 3.21 ± 0.078, respectively, and the corresponding experimental values of $1/V_{IF}$ 2.27 ± 0.029, 2.45 ± 0.020, and 3.84 ± 0.41. The experiment with trimethylammonium chloride at a single AcCh concentration (0.002 M) leads to the same conclusion. Since this ion, as well as NaF, is a strongly noncompetitive inhibitor, the ratio from $1/v$ and $1/V$ values should be equivalent (20), and $1/v$ may therefore be substituted for $1/V$ in Eq. 19: $1/v_F + 1/v_I - 1/v_0 = 5.39 \pm 0.04$ and $1/v_{IF} = 5.04 \pm 0.07$.

While there is competition for EA, both fluoride and small cationic inhibitors may add to the free enzyme at the same time, since m_{IF} is larger than the sum of $m_I + m_F - m_0$ (Table 3). With tetramethyl and tetraethyl ammonium ions, respectively, $m_I + m_F - m_0$ is 14.10 ± 0.80 and 21.61 ± 0.81, m_{IF} is 30.35 ± 0.70 and 48.70 ± 0.81, and the predicted value of m_{IF} when there is no interference between the ions is approximately 35 and 58 (Eq. 22). The latter are higher than the experimental values, suggesting slight interference between the ions. Choline, butyrylcholine, and tetrapropylammonium ions interfere with fluoride binding more than do tetramethyl and tetraethyl ammonium. With these ions $m_F + m_I - m_0$ is 10.54 ± 1.13, 8.29 ± 0.90 and 18.6 ± 1.26, m_{IF} is 17.13 ± 0.53, 12.29 ± 0.41 and 35.9 ± 1.47, and the predicted m_{IF} in the absence of competition is 25, 19, and 49. In sum, it appears that small cations interfere with fluoride binding to only a small extent, but that there is more interference when the cation is larger. With the bifunctional inhibitor, 3-hydroxylphenyltrimethylammonium, in which both the ammonium ion and hydroxyl group are involved in binding, interference is complete. These results suggest that in the free enzyme the fluoride binding site

is at the active center, but is more than 5 Å from the anionic site.

It will have become apparent that some of Heilbronn's (3) observations can now be explained. A noncompetitive type of inhibition is observed with cationic substrates because fluoride is capable of binding to ES and EA, as well as E. Competition is observed with trimethylammonium ion because its inhibition is caused mainly by addition to EA (13, 20), and as seen above cations compete with fluoride for this intermediate. Substrate inhibition is also due to binding to EA (21), and inhibitory substrate molecules, which are cations, therefore compete with fluoride. Neither the substrate nor trimethylammonium ion would compete with fluoride for the free enzyme. The uncompetitive inhibition reported by Cimasoni (4) with acetylthiocholine indicates that fluoride is bound much more strongly to EA than to E under the experimental conditions employed, namely 0.1 M phosphate buffer, pH 8. Heilbronn's experiments, and those reported here, were carried out in unbuffered solutions.

The pH dependence of fluoride inhibition cannot be explained with any certainty, but it is to be noted that all the experiments, including those with cationic inhibitors, are understandable if the catalytic basic groups are involved in fluoride binding, and specifically if fluoride is bound to a given enzyme intermediate only when the basic group which functions catalytically in that intermediate is in the protonated form. From this postulate and the known functions of the catalytic groups it follows that (a) the approximate pK for the pH dependence of fluoride binding to E and ES should be 5.5, while that for binding to EA should be 6.6; and (b) small ammonium ions should not compete with fluoride for binding to E or ES, but should compete for EA. The reasoning is as follows: First, the group of pK 6.6 is adjacent to the anionic site where substituted ammonium ions are bound (11), so that addition of a cation to the anionic site prevents addition of a proton to this ionizing group. If fluoride binding depends upon the latter process, it should be prevented by cation binding. On the other hand, if binding depends on the second ionizing group (pK 5.5), which is more distant from the anionic site and does not strongly interact with it, no interference from the cation should be experienced. The group of pK 6.6 performs an essential catalytic role in EA but not in ES, while the other ionizing group is essential in E and ES but not in EA (11).

The pK_F values reported in Fig. 3 for inhibition with AcCh should be a measure of binding to EA, since the substrate saturates the enzyme ($[S]/K_m = 24.2$), and since the rate-limiting step in hydrolysis is deacetylation. Binding appears to be influenced by two ionizations in EA, of pK roughly 6.8 and 5.7. In hydrolysis of isoamyl acetate and acetyl-β-methylcholine, the rate-limiting step is acetylation rather than deacetylation (11). With the former, $[S]/K_m$ is 0.61, and with the latter approximately 18. With isoamyl acetate, K_F should therefore be a measure of binding to E and, to a lesser extent, ES, but with acetyl-β-methylcholine it should measure binding to ES alone. A similar pH dependence is observed with both substrates, suggesting a similar mechanism of fluoride binding in E and ES. The linear segments drawn in the diagram, on which the experimental points fall, have unit slopes, indicating that a single ionizing group is involved, and its pK appears to be roughly 5.7. The values of pK_i and $pK'_{i(app)}$ for AcCh hydrolysis are in agreement with these ideas (Fig. 4). K_i, which measures binding to E exclusively, reflects the ionization of only one group (pK 5.8), while $K'_{i(app)}$, for binding to EA, is dependent on two groups, of pK 6.8 and 5.7. It may be noted that the difference in measured pK values for catalysis (6.6) and for fluoride binding (6.8) does not rule out participation of a single group in both processes. The latter pK could combine a proton dissociation constant with an equilibrium constant for a conformational change at the active center, as follows:

$$E \rightleftharpoons EH \rightleftharpoons EH' \rightleftharpoons EH'F$$

Addition of a proton to the ionizing group does not immediately create a fluoride binding site, but first EH must be converted to EH′.

The nature of the fluoride binding site cannot yet be determined, although one hypothesis at least can be ruled out, namely that fluoride binds directly to both basic groups in the active center, and inhibits by keeping them in their protonated, i.e. inactive, forms. Fluoride does not compete with tetramethyl and tetraethyl ammonium ions for E, but does for EA, whereas if it always binds to the group of pK 6.6 competition should always be observed. A possible interpretation is a single primary binding site in all three enzyme species, whose location varies. In E and ES it borders the group of pK 5.5 but is remote from the group of pK 6.6, while in EA it is within range of both, possibly located between them. This hypothesis would account for the pH dependence of fluoride binding to E, ES, and EA, as well as for competition between F^- and cations for EA and for lack of competition in ES and E. Fluoride inhibits by binding to, and stabilizing, an inactive form of the enzyme, i.e. one in which an essential basic group is protonated.

A seemingly unavoidable conclusion from these experiments is that the site of fluoride attachment in EA differs from that in ES and E. The evidence is (a) small cations compete with fluoride for binding to EA but not to ES or E, and (b) the pH dependence of fluoride binding to E and ES differs from that for binding to EA. A change in the configuration of chemical groups in the enzyme may therefore occur during acetylation (ES → EA). Such a conformational change was suggested previously by the discovery that different basic groups in the enzyme function in acetylation and deacetylation (11).

APPENDIX

(i) *Calculation of Inhibition Constants*

In analyzing inhibition experiments, constants for addition of F^- to E, ES, and EA were determined. For a reaction proceeding by the following mechanism,

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

in which inhibitor (I) binding to E, ES, and EA (forming EI, ESI, and EAI) is governed by dissociation constants K_i, K'_i and K''_i, respectively, the rate of substrate hydrolysis is

$$v = k_2[E_t]/\{1 + k_2/k_3 + [I]/K'_i + k_2[I]/k_3K''_i + (1 + [I]/K_i)K_m/[S]\} \quad (5)$$

where $K_m = (k_{-1} + k_2)/k_1$ and E_t is the total enzyme concentration. K_i may be calculated from the ratio of slopes of Lineweaver-Burk plots ($1/v \times 1/[S]$) with and without inhibitor:

$$K_i = [I]/(m_I/m_0 - 1) \quad (6)$$

where m_I and m_0 are slopes of the lines in the presence and absence of inhibitor, respectively.

A weighted mean of K'_i and K''_i is found from the intercepts:

$$K'_{i(app)} = (1 + k_2/k_3)/(1/K'_i + k_2/k_3K''_i) = [I]/V_0(1/V_I - 1/V_0) \quad (7)$$

where $1/V_I$ and $1/V_0$ are intercepts with and without inhibitor, respectively. $K'_{i(app)}$ reduces to K''_i when $k_2 \gg k_3$, and to K'_i when $k_3 \gg k_2$.

In some experiments an inhibition constant was determined at a fixed substrate concentration:

$$K_F = [I]/v_0(1/v_I - 1/v_0) = \frac{K_m/K_i[S] + 1/K'_i + k_2/k_3K''_i}{K_m/[S] + 1 + k_2/k_3} \quad (8)$$

where v_I and v_0 are rates in the presence and absence of inhibitor. At saturating substrate concentrations $K_F \simeq K'_{i(app)}$, and at concentrations much lower than K_m, $K_F \simeq K_i$.

(ii) *Addition of Two Different Inhibitors to the Enzyme*

The reaction scheme in Fig. 6 represents binding of a substituted ammonium ion, I, and a fluoride ion, F, to AChE in the presence of AcCh. I may become bound at the anionic site in E and EA

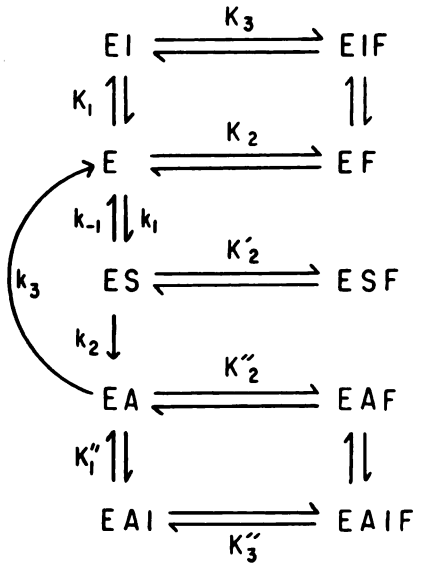

FIG. 6. *Reaction scheme for inhibition of acetylcholine hydrolysis by two inhibitors having different binding sites*

A cationic inhibitor (I) is bound to the free enzyme, E, and acetyl enzyme, EA, but not to the enzyme–substrate complex, ES; but fluoride (F) is bound to all three. Acetylation and deacetylation are governed by the rate constants k_2 and k_3, respectively. See Appendix, Section ii.

but not in ES (20). Fluoride may add to all three enzyme species. If both I and F become bound at the same time, EIF and EAIF are formed. The reciprocal of the reaction rate is found to be

$$\frac{1}{v} = \frac{1}{k_2[E_t]}\left\{1 + \frac{[F]}{K'_2} + \frac{k_2}{k_3} \times \left[1 + \frac{[I]}{K''_1} + \frac{[F]}{K''_2} + \frac{[F][I]}{K''_1K''_2}\right] + \frac{K_m}{[S]}\left[1 + \frac{[I]}{K_1} + \frac{[F]}{K_2} + \frac{[F][I]}{K_1K_3}\right]\right\}. \quad (9)$$

Deacetylation is probably the rate limiting step in AcCh hydrolysis (11, 20), so that (k_2/k_3) $(1 + [I]/K''_1 + [F][I]/K''_1K''_2)$ should be large compared to $1 + [F]/K'_2$. Equation 9 then reduces to

$$\frac{1}{v} = \frac{1}{k_3[E_t]}\left\{1 + \frac{[I]}{K''_1} + \frac{[F]}{K''_2} + \frac{[F][I]}{K''_1K''_2} + \frac{K'_m}{[S]}\left(1 + \frac{[I]}{K_1} + \frac{[F]}{K_2} + \frac{[F][I]}{K_1K_3}\right)\right\} \quad (10)$$

where $K'_m = K_mk_2/k_3$.

According to Eq. 10, Lineweaver-Burk plots ($1/v \times 1/[S]$) for rate measurements in the presence of one or the other inhibitor, or both, or neither, should give four straight lines with intercepts ($1/V$) given by

$$1/V_0 = 1/k_3[E_t] \quad (11)$$

$$1/V_I = (1 + [I]/K''_1)/k_3[E_t] \quad (12)$$

$$1/V_F = (1 + [F]/K''_2)/k_3[E_t] \quad (13)$$

$$1/V_{IF} = (1 + [I]/K''_1 + [F]/K''_2 + [I][F]/K''_1)/k_3[E_t] \quad (14)$$

where the subscripts 0, I, F, and IF refer, respectively, to rates in the absence of inhibitor, and in the presence of I, F, and I and F together. Similarly the corresponding slopes of the lines, m, are given by

$$m_0 = K'_m/k_3[E_t] \quad (15)$$

$$m_I = (1 + [I]/K_1)K'_m/k_3[E_t] \quad (16)$$

$$m_F = (1 + [F]/K_2)K'_m/k_3[E_t] \quad (17)$$

$$m_{IF} = (1 + [I]/K_1 + [F]/K_2 + [I][F]/K_1K_3)K'_m/k_3[E_t]. \quad (18)$$

Using Eqs. 11–18, the values of $1/V_{IF}$ and m_{IF} may be computed for two cases: (a) F and I compete for a single binding site:

$$1/V_{IF} = 1/V_I + 1/V_F - 1/V_0 \quad (19)$$

$$m_{IF} = m_I + m_F - m_0 \quad (20)$$

and (b) the binding of I does not interfere with the binding of F:

$$1/V_{IF} = 1/V_I + 1/V_F - 1/V_0 + (1/V_F - 1/V_0)(1/V_I - 1/V_0)V_0 \quad (21)$$

and

$$m_{IF} = m_I + m_F - m_0 + (m_I - m_0)(m_F - m_0)/m_0 \quad (22)$$

(iii) *Number of Fluoride Binding Sites*

Equation (1) is based on a simplified Michaelis-Menten reaction scheme (8), but an equation of similar form is obtained when EA, ESI, and EAI are taken into account. From Eq. 5 it follows that

$$\frac{v_0}{v} - 1 = \frac{[I]\,(K_m/K_i[S] + 1/K'_i + k_2/k_3K''_i)}{(K_m/[S] + 1 + k_2/k_3)} \quad (23)$$

If n_1, n_2, and n_3 inhibitor molecules are bound to E, ES, and EA, respectively the equation takes the form

$$v_0/v - 1 = C_1[I]^{n_1} + C_2[I]^{n_2} + C_3[I]^{n_3} \quad (24)$$

where C_1, C_2, and C_3 are constants for a given substrate concentration. The same arguments used in treating Eq. 8 above may now be applied to Eq. 24. At saturating substrate concentrations a plot of log $(v_0/v - 1)$ against log [I] should have a slope dependent on the values of n_2 and n_3; if deacetylation (k_3) is rate-limiting the slope equals n_3, the number of inhibitor molecules bound to EA, while if acetylation (k_2) is rate limiting the slope is n_2, which refers to complex formation with ES. When $[S] \ll K_m$ the slope gives n_1, for binding to E.

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REFERENCES

1. K. B. Augustinsson, *Acta Physiol. Scand.* **15**, Suppl. 52 (1948).
2. B. N. Smallman and L. S. Wolfe, *Enzymologia* **17**, 133 (1954).
3. E. Heilbronn, *Acta Chem. Scand.* **19**, 1333 (1965).
4. G. Cimasoni, *Biochem. J.* **99**, 133 (1966).
5. J. E. Dowd and D. S. Riggs, *J. Biol. Chem.* **240**, 863 (1965).
6. R. M. Krupka, *Biochemistry* **5**, 1983 (1966).
7. E. C. Slater and W. D. Bonner, *Biochem. J.* **52**, 185 (1952).
8. F. H. Johnson, H. Eyring and R. W. Williams, *J. Cellular Comp. Physiol.* **20**, 247 (1942).
9. M. Dixon, *Biochem. J.* **55**, 161 (1953).
10. J. L. Webb, "Enzyme and Metabolic Inhibitors," Vol. 1, p. 694. Academic Press, New York, 1963.
11. R. M. Krupka, *Biochemistry* **5**, 1988 (1966).
12. I. B. Wilson and C. Quan, *Arch. Biochem. Biophys.* **73**, 131 (1958).
13. R. M. Krupka, *Biochemistry* **4**, 429 (1965).
14. I. B. Wilson and E. Cabib, *J. Am. Chem. Soc.* **56**, 5154 (1954).
15. S. L. Friess, I. B. Wilson and E. Cabib, *J. Am. Chem. Soc.* **56**, 5156 (1954).
16. E. Heilbronn-Wikstrom, *Svensk Kem. Tidskr.* **77**, 11 (1965).
17. E. Heilbronn, *Biochem. Pharmacol.* **14**, 1363 (1965).
18. C. G. Swain and C. B. Scott, *J. Am. Chem. Soc.* **75**, 246 (1953).
19. D. P. N. Satchell, *J. Chem. Soc.* p. 555 (1963).
20. R. M. Krupka, *Biochemistry* **3**, 1749 (1964).
21. R. M. Krupka, *Can. J. Biochem.* **42**, 677 (1964).