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The Effect of Fluoride on the Reactions of Methanesulfonates with Acetylcholinesterase

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SUMMARY

Methanesulfonates [(3-hydroxyphenyl)trimethylammonium iodide methanesulfonate, 1,1′-pentamethylenebis(3-hydroxypyridinium iodide) methanesulfonate, and methanesulfonyl fluoride] react with acetylcholinesterase to form an inactive methanesulfonyl-enzyme derivative analogous to the acetyl-enzyme that is formed during the hydrolysis of esters. The methanesulfonyl-enzyme is desulfonylated by reaction with thiocholine. When the effect of fluoride on these two steps was studied, it was found that fluoride inhibits sulfonylation but does not affect desulfonylation. It was also found that fluoride and a quaternary ammonium ion can bind simultaneously to the enzyme. In one case the binding was independent, in many cases it was partially competitive, in one case it was completely competitive, and in two cases it was cooperative.

INTRODUCTION

This study of the effect of fluoride on the reaction of acetylcholinesterase with methanesulfonates was undertaken for its own interest and also in the hope of clarifying the kinetic mechanism whereby fluoride inhibits acetylcholinesterase. Several studies of the inhibition by fluoride of the acetylcholinesterase-catalyzed (1–5) hydrolysis of acetylcholine have shown the phenomenon to be rather complicated, although the effect of fluoride is rapid and reversible and no metal ions are involved.

Fluoride inhibition is best discussed with the aid of the kinetic scheme for enzymatic hydrolysis (Eq. 1). In this equation, S denotes acetylcholine; P_1, choline; P_2, acetic acid; E, enzyme; and E', the acetyl-enzyme intermediate. It is apparent that there are three enzyme forms with which fluoride might combine, and two reaction

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_3} E' + P_1 \qquad E' \xrightarrow{+H_2O,\ k_4} E + P_2 \qquad (1)$$

steps that might be inhibited. In order to explain his results, Krupka (5) invoked all these possibilities.

The methanesulfonates that were used in this study, (3-hydroxyphenyl)trimethylammonium iodide methanesulfonate (methanesulfonate I, Fig. 3), 1,1′-pentamethylenebis(3-hydroxypyridinium iodide) methanesulfonate (methanesulfonate II, Fig. 4), and methanesulfonyl fluoride, react with acetylcholinesterase in a manner analogous

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to the reaction of acetylcholine with the enzyme and, it is thought, by a similar mechanism (6). These substances react with the enzyme to form a methanesulfonyl-enzyme derivative analogous to the acetyl-enzyme, but, unlike the latter, the methanesulfonyl-enzyme reacts with water at an almost negligible rate. The substances therefore are inhibitors of the enzyme. The methanesulfonyl-enzyme, however, does react with suitable nucleophiles such as thiocholine and pyridine oximes, which desulfonylate the enzyme and restore its catalytic activity (7). The scheme for the sulfonylation reaction is

$$E + I \overset{K_I}{\rightleftharpoons} E \cdot I \xrightarrow{k_i} E' + X$$

where I is CH_3SO_2X, E' is the covalent methanesulfonyl-enzyme, and X is the leaving group (e.g., X is fluoride when I is methanesulfonyl fluoride). Since the enzyme can be reactivated by thiocholine,

$$E' + \text{thiocholine} \rightarrow E + \text{thiocholinemethanesulfonate}$$

It is possible to measure separately the effect of fluoride on the rate of sulfonylation of the enzyme and on the rate of reactivation by thiocholine. The reaction of methanesulfonyl fluoride with acetylcholinesterase is greatly accelerated in the presence of certain ammonium ions, such as tetramethylammonium ion (8, 9), and we have also studied the effect of fluoride on this phenomenon.

METHODS

Enzyme. Acetylcholinesterase was obtained as lots 6FA and 6JA from Worthington Biochemical Corporation, and as lot LK2 prepared from *Electrophorus electricus* (for purification procedure, see ref. 10). The specific activity was 60 mmoles of acetylcholine hydrolyzed per milligram of protein per hour at 25° and pH 7.0 for lots 6FA and 6JA, and 100 for lot LK2.

Enzyme assay. Small aliquots of the enzyme solutions were added to 0.5 ml of buffer containing 2.9×10^{-3} M acetylcholine. Enzyme activity was measured at 25° and pH 8.0 by the decrease in acetylcholine concentration after 1–6 min, as determined by the colorimetric hydroxamic acid method (11). The buffer consisted of 0.02 M Tris-hydrochloride, 0.15 M NaCl, 10^{-5} M EDTA, 0.01% gelatin, and 0.01 M $CaCl_2$ (or 0.05 M). Calcium was included to remove fluoride that was present in high concentration in some of the enzyme solutions. The presence of calcium precluded the use of phosphate buffers; therefore Tris was used and the pH was set at 8.0.

Sulfonylation. The extent of sulfonylation was inferred from the extent of inhibition of the enzyme. Inhibitor and enzyme were incubated in buffer with and without fluoride and substituted ammonium ions. At suitable time intervals, aliquots were assayed for enzyme activity. Each aliquot was diluted 500-fold in the assay buffer.

Desulfonylation. Sulfonyl-enzyme solutions in which nearly all the enzyme was sulfonylated (nearly complete loss of enzyme activity) were diluted 500-fold with buffer containing 3×10^{-3} M thiocholine iodide. After suitable periods of 1 or more hours, aliquots were assayed for enzyme activity.

Buffer. The buffers used in sulfonylation and desulfonylation were the same as for enzyme assay, except that the concentration of Tris-chloride was 0.001 M and the pH was 7.0.

RESULTS

Desulfonylation. Desulfonylation by thiocholine (reactivation) may be represented as follows.

$$E' + R \overset{K'_R}{\rightleftharpoons} E' \cdot R \xrightarrow{k_R} E$$

$$E' + F \overset{K'_F}{\rightleftharpoons} E' \cdot F$$

$$E' \cdot F + R \overset{K''_R}{\rightleftharpoons} E' \cdot R \cdot F \xrightarrow{\alpha k_R} E$$

$$E' \cdot R + F \overset{K''_F}{\rightleftharpoons} E' \cdot R \cdot F$$

where E' is the methanesulfonyl-enzyme, R is thiocholine, F is fluoride, and E is the normal enzyme.

The last equation can be omitted, since the four equilibria are not independent, and

$$K''_F = K'_F \frac{K''_R}{K'_R}$$

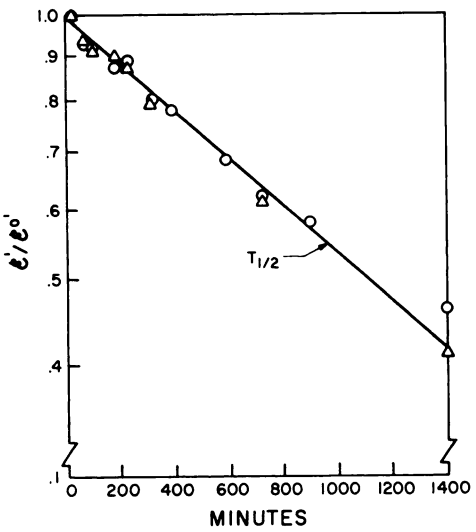


FIG. 1. *Rate of desulfonylation of methanesulfonyl-enzyme in thiocholine with and without NaF*

Methanesulfonyl-enzyme was diluted 500-fold into Tris incubation buffer, pH 7.0, at 25°. The buffer contained: ○, 3×10^{-3} M thiocholine; △, 3×10^{-3} M thiocholine plus 10^{-2} M NaF. The data are plotted on a semilogarithmic scale. ϵ'° is the initial concentration of sulfonyl-enzyme.

$$\epsilon' = E' + E'\cdot\mathrm{R} + E'\cdot\mathrm{R}\cdot F + E'\cdot F$$

The rate of desulfonylation (rate of appearance of active enzyme) is

$$\frac{d\epsilon'}{dt} = k_{\mathrm{R}}\,\epsilon' \frac{1 + \alpha(F/K''_F)}{[1 + (F/K''_F)] + [1 + (F/K'_F)][K'_{\mathrm{R}}/\mathrm{R}]}$$

Our results show that fluoride, even at 0.01 M, does not affect the rate of desulfonylation (Fig. 1). The concentration of fluoride is very high, since 2×10^{-4} M is sufficient to inhibit the hydrolysis of acetylcholine by about 50%. The lack of effect of fluoride may arise in different ways. Fluoride may not be able to bind to the sulfonyl-enzyme; i.e., K'_F, $K''_F \rightarrow \infty$ (K's are dissociation constants). Then adding fluoride could have no effect. A second possibility is that $\alpha = 1$ and $K'_F = K''_F$; i.e., fluoride binds equally well with the sulfonyl-enzyme and the sulfonyl-enzyme–thiocholine complex and in addition does not change the rate constant. (The equality of K_F and K''_F is equivalent to the equality of K'_{R} and K''_{R}.) Thus, either fluoride does not interact with the sulfonyl-enzyme or its interaction leaves the binding and kinetics unchanged. While we cannot distinguish between these two cases, it is nonetheless clear that fluoride does not affect desulfonylation by thiocholine. It is also clear that fluoride is not itself a reactivator of the methanesulfonyl-enzyme, in contrast to its ability to dephosphorylate dialkylphosphoryl-enzyme derivatives. Desulfonylation occurs only very slowly in water, less than 10% in 4 days, and this was not affected by 0.01 M NaF.

Sulfonylation. The sulfonylation of the enzyme by all three methanesulfonates was inhibited by fluoride. Some results with (3-hydroxyphenyl)trimethylammonium iodide methanesulfonate (methanesulfonate

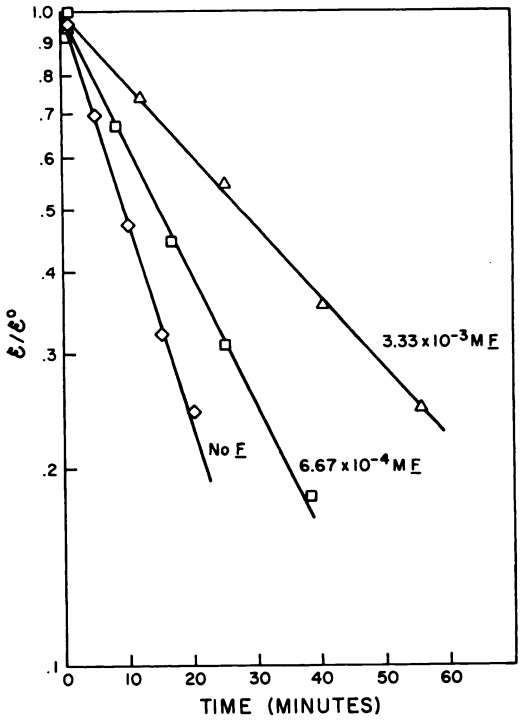


FIG. 2. *Effect of fluoride on rate of sulfonylation by (3-hydroxyphenyl)trimethylammonium iodide methanesulfonate*

The concentration of (3-hydroxyphenyl)trimethylammonium iodide methanesulfonate was 8×10^{-4} M. The enzyme was lot 6JA. ◇, no NaF; □, 6.67×10^{-4} M NaF; △, 3.33×10^{-3} M NaF. ϵ° is the initial concentration of active enzyme.

I) at high concentrations are shown in Fig. 2. The decrease in active enzyme indicates its progressive sulfonylation, and it is apparent that sulfonylation proceeds more slowly in the presence of fluoride. Relatively high fluoride concentrations are required, because, as will be seen, there is a considerable degree of competition between this sulfonylating agent and fluoride. Considerably less fluoride is required to inhibit sulfonylation by the other two methanesulfonates.

The scheme for sulfonylation in the presence of fluoride, F, allowing for the formation of reversible complexes between the methanesulfonates, I, and the enzyme, E, is

$$E + I \overset{K_I}{\rightleftharpoons} E\cdot I \xrightarrow{k_i} E'$$

$$E + F \overset{K_F}{\rightleftharpoons} E\cdot F$$

$$E\cdot I + F \overset{K'''_F}{\rightleftharpoons} E\cdot I\cdot F \xrightarrow{\beta k_i} E'$$

$$E\cdot F + I \overset{K'''_I}{\rightleftharpoons} E\cdot I\cdot F$$

(Single primes and double primes were used up for dissociation constants involving the sulfonyl-enzyme, E'.)

The last equation is redundant, since

$$K'''_I = K_I \frac{K'''_F}{K_F}$$

$$\epsilon = E + E\cdot I + E\cdot F + E\cdot I\cdot F$$

$$\frac{d\epsilon}{dE} = \frac{-k_i[1 + \beta(F/K'''_F)]\epsilon}{1 + (F/K'''_F) + [1 + (F/K_F)](K_I/I)} \quad (2)$$

and the observed first-order rate constants (plots as in Fig. 2) are

$$k_{obs} = \frac{k_i[1 + \beta(F/K'''_F)]}{1 + (F/K'''_F) + [1 + (F/K_F)](K_I/I)} \quad (3)$$

When no fluoride is present,

$$k^{\circ}_{obs} = \frac{k_i}{1 + (K_I/I)} \quad (4)$$

The plots in Figs. 3 and 4 correspond to

$$\frac{1}{k_{obs}} = \frac{1 + (F/K'''_F) + [1 + (F/K_F)](K_I/I)}{k_i[1 + \beta(F/K'''_F)]} \quad (5)$$

When no fluoride is present, the intercept is $1/k_i$ and the slope is K_I/k_i. With methanesulfonyl fluoride, the intercept is at the origin (not shown) (8), indicating that the range of concentrations is much below K_I; i.e., $[I] \ll K_I$. With methanesulfonate I and especially methanesulfonate II, the intercept is distinctly different from the origin, indicating that the concentration range is such that a good fraction of the enzyme exists as the complex $E\cdot I$. It is clear in both Figs. 3 and 4 that fluoride changes the slope. In Fig. 3, the intercept appears to be changed by fluoride, but this effect is not certain because the lines could be drawn so that there would be no change in intercept, although the latter seems less likely. In Fig. 4, however, there is no doubt whatsoever that fluoride affects the intercept. From Eq. 5 we see, therefore, that β must be less than 1. The maximum shift in the intercept is $1/\beta$. Therefore this figure alone shows that β must be less than $\frac{1}{3}$. Actually β is very small

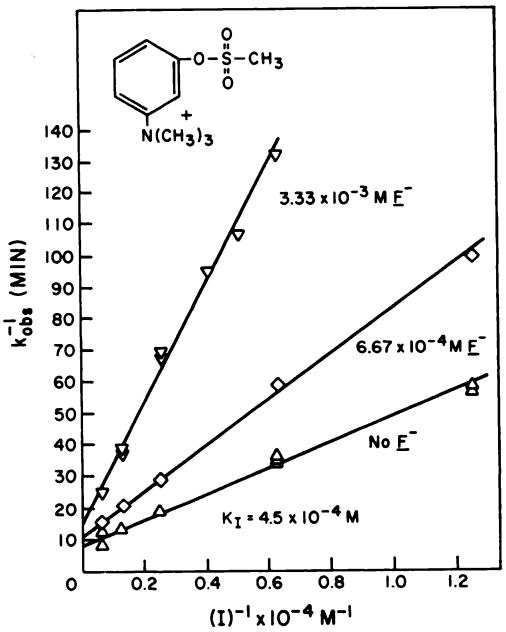


FIG. 3. *Dependence of k_{obs} on concentration of (3-hydroxyphenyl)trimethylammonium iodide methanesulfonate with and without NaF, plotted as reciprocals in accordance with Eq. 5*

The enzyme preparation was 6JA. △, no NaF; ◇, 6.67×10^{-4} M NaF; ▽, 3.33×10^{-3} M NaF.

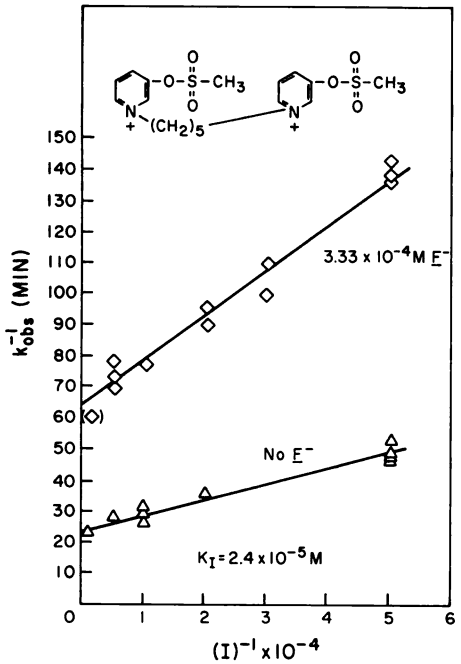


FIG. 4. *Dependence of k_{obs} on concentration of sulfonylating agent, 1,1-pentamethylenebis(3-hydroxypyridinium iodide) methanesulfonate, with and without NaF, plotted as reciprocals in accordance with Eq. 6*

Enzyme preparation LK2 was used. △, no NaF; ◇, 3.33 × 10^{-4} M NaF.

and may be zero. This is shown in the following way. The ratio of the slope to the intercept is independent of β and relates K_F and K'''_F, so that if K_F is known, K'''_F can be calculated. In a previous study with this enzyme preparation (LK2), and also in the present study, K_F was found to be 2.4 × 10^{-4} M and therefore K'''_F for methanesulfonate II is 2.1 × 10^{-4} M. Using this value for K_F, the value of β calculated from the intercept is −0.05. A negative value is not permissible and only reflects the errors in the measurements, but it is evident that β is quite small. If we assume $\beta = 0$, $K'''_F = 1.8 \times 10^{-4}$ M from the intercepts. Further evidence will be given that β is quite small.

The reaction of methanesulfonyl fluoride with the enzyme (as already mentioned) is second-order (pseudo-first-order) and can be represented as

$$E + I \xrightarrow{k_I} E'$$

$$E + F \underset{}{\overset{K_F}{\rightleftharpoons}} E \cdot F$$

$$E \cdot F + I \xrightarrow{\gamma k_I} E'$$

The solution for the pseudo-first-order rate constant for sulfonylation is

$$k_{obs} = \frac{k_I I[1 + \gamma(F/K_F)]}{1 + (F/K_F)} \qquad (6)$$

and

$$\frac{k^\circ_{obs}}{k_{obs}} = \frac{1 + (F/K_F)}{1 + \gamma(F/K_F)} \qquad (7)$$

where k°_{obs} is the pseudo-first-order rate constant for sulfonylation in the absence of fluoride.

Our results for the effect of fluoride on the rate of reaction of methanesulfonyl fluoride and acetylcholinesterase (lot 6JA) are shown in Fig. 5 (labeled F^- control), plotted in accordance with Eq. 7. The linearity of the plot over a 15-fold change in rate indicates that γ is quite small—less than 0.03—and may be zero. The slope then allows the calculation of K_F. The value obtained with the preparation shown (6JA) is 4.1 × 10^{-4} M. This value varies somewhat with the preparation. For 6FA it is 2.6 × 10^{-4} M, and for LK2 it is 2.4 × 10^{-4} M. Since γ is analogous to β/K_F, we have further evidence that β is quite small.

As already mentioned, the rate of reaction of methanesulfonyl fluoride with acetylcholinesterase is affected by the presence of substituted ammonium ions, some of which inhibit the reaction, some of which accelerate the reaction, often by quite large factors, and some of which have little effect although they are bound by the enzyme (8, 9). We have examined the effect of fluoride on the rate of reaction of methanesulfonyl fluoride, I, with the enzyme in the presence of ammonium ions, N. The scheme for the reaction is (remember that $\gamma \cong 0$)

$$E + I \xrightarrow{k_I} E'$$

$$E + N \underset{}{\overset{K_N}{\rightleftharpoons}} E \cdot N$$

$$E \cdot N + I \xrightarrow{\alpha k_I} E'$$

$$E + F \underset{}{\overset{K_F}{\rightleftharpoons}} E \cdot F$$

$$E \cdot N + F \rightleftharpoons E \cdot N \cdot F \qquad K'''_F$$

$$E \cdot F + N \rightleftharpoons E \cdot N \cdot F \qquad K'''_N$$

Again the last equation is redundant, since

$$K'''_N = K_N \frac{K'''_F}{K_F}$$

The solution for the observed pseudo-first-order rate constant for sulfonylation is

$$k_{obs} = \frac{k_I I[1 + \alpha(N/K_N)]}{1 + (N/K_N) + F/K_F[1 + (N/K'''_N)]} \quad (8)$$

For $F = 0$,

$$k^\circ_{obs} = \frac{k_I I[1 + \alpha(N/K_N)]}{1 + (N/K_N)} \quad (9)$$

and

$$\frac{k^\circ_{obs}}{k_{obs}} = \frac{1 + (F/K_F)[1 + (N/K'''_N)]}{1 + (N/K_N)} \quad (10)$$

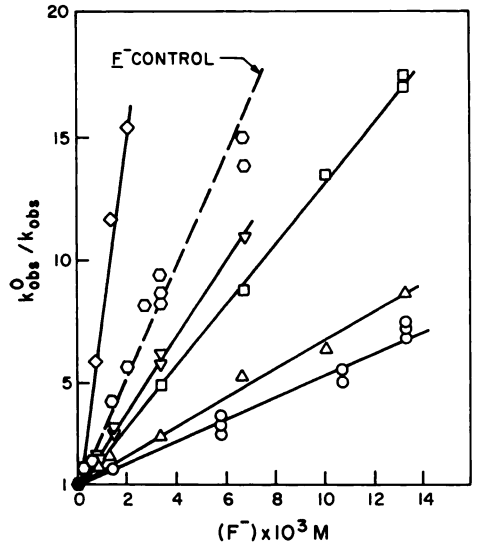


FIG. 5. *Effect of substituted ammonium ions on rate of sulfonylation by methanesulfonyl fluoride in the presence of sodium fluoride, plotted as* k_{obs}/k_{obs} *with respect to fluoride concentration in accordance with Eq. 7*

The enzyme was lot 6JA. Hexagons, no substituted ammonium ion; ○, 3.33×10^{-2} M choline; △, 6.67×10^{-3} M choline; □, 6.67×10^{-4} M 1,1′-pentamethylenebis(pyridinium); ▽, 2×10^{-5} M 1,1′-pentamethylenebis(pyridinium); ◇, 1.33×10^{-2} M (3-methylphenyl)trimethylammonium.

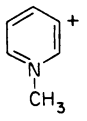
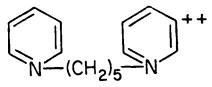
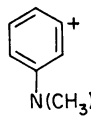
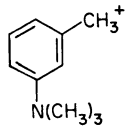
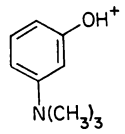
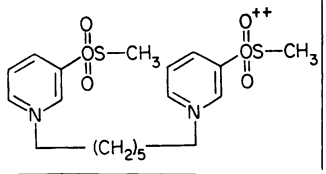
We have studied eight quaternary ammonium ions. The results were typically as shown in Fig. 5 for three amines plotted in accord with Eq. 10. The data for all are given in Table 1. In all cases the addition of F^- decreases the rate of methanesulfonylation of the enzyme. The value of k°_{obs}/k_{obs} was linear with fluoride concentration, as expected from Eq. 10. It will be noted that if the curve lies to the right of the "control" curve, i.e., the curve for $N = 0$, $K'''_N > K_N$ (the amine binds less readily to the enzyme-fluoride complex than to the free enzyme), and if it lies to the left, $K'''_N < K_N$ (the amine binds more readily to the enzyme-fluoride complex than to the free enzyme).

Because of the redundancy relationship, the former conditions also indicate that fluoride binds less readily with the enzyme-amine complex than with the free enzyme, and the latter conditions indicate that fluoride binds more strongly with the enzyme-amine complex than with the free enzyme. Thus the statement that (3-methylphenyl)trimethylammonium ion binds better to the enzyme-fluoride complex than to the free enzyme (Fig. 5) is equivalent to saying that fluoride binds better to the enzyme–(3-methylphenyl)trimethylammonium complex than to the free enzyme. It will be noted that a knowledge of K_N is not required in order to ascertain qualitatively whether the binding of fluoride and an amine is competitive, independent, or cooperative. The value of K_N can be determined from two lines corresponding to different values of N, provided that they are not so large that the slope is nearly independent of N. In the latter event, K_N cannot be determined but the ratio K_N/K'''_N is determined. Our concentrations of amine were too large in those cases when we do not report a value of K_N. In the three cases when K_N can be compared with values given in the literature as determined from the inhibition of the hydrolysis of acetylcholine, the agreement is good.

Our results indicate that the simultaneous binding of fluoride and a quaternary ammonium ion with the enzyme may be approximately independent, as in the case of methanesulfonate II; completely com-

TABLE 1

Sulfonylation of acetylcholinesterase by methanesulfonyl fluoride with substituted ammonium ions ± fluoride

Cation	Structure	Enzyme lot[a]	Cation properties				$K''_F/K'''_F = K_N/K_N$[c]
			Acceleration[b]		K_N		
			Observed	Reported[a]	Observed	Reported[a]	
					M	*M*	
Tetraethylammonium	$(C_2H_5)_4N^+$	6FA	33	33		$2.5\text{–}5\times10^{-4}$	11.2
Tetramethylammonium	$(CH_3)_4N^+$	6JA	7	6	1.3×10^{-3}	1.2×10^{-3}	8.7
Choline	$(CH_3)_3NCH_2CH_2OH^+$	6JA	5.2		7×10^{-4}		5.4
Pyridine methiodide		6JA	2.8	2.1	1.4×10^{-4}	1.1×10^{-4}	2.0
1,1′-Pentamethylenebis(pyridinium)		6JA	5.0		6×10^{-6}		1.9
Phenyltrimethylammonium		6FA	1.0	1.2		3.8×10^{-5}	0.5
(3-Methylphenyl)trimethylammonium		6JA	2.8			1.2×10^{-5}[d]	0.3
(3-Hydroxyphenyl)trimethylammonium		6JA	0	0	2×10^{-7}	3×10^{-7}	∞
1,1′-Pentamethylenebis(3-hydroxypyridinium) methanesulfonate		LK2			2×10^{-4}	2×10^{-4}	1

[a] Reference 8.

[b] Rate of sulfonylation in the presence of saturating concentrations of amine, divided by the rate without amine.

[c] A number greater than 1.0 in this column indicates that the amine and fluoride are partially competitive (∞ means completely competitive); less than 1.0 indicates that the bindings are cooperative; and precisely 1.0 means that the bindings are independent.

[d] Reference 12.

petitive, as in the case of (3-hydroxyphenyl)-trimethylammonium ion; partially competitive, as in the case of choline and methanesulfonate I; and cooperative, as in the case of (3-methylphenyl)trimethylammonium ion. Most of our compounds were partially competitive (Table 1).

DISCUSSION

Our results show that fluoride, even in high concentration, does not affect desulfonylation. This indicates that fluoride does not serve as a nucleophile to desulfonylate the methanesulfonyl-enzyme. Fluoride similarly does not act as a nucleophile with the carbamyl-enzyme (13, 14). In contrast, fluoride does act as a nucleophile toward the phosphoryl-enzyme (15, 16). Fluoride does not affect the desulfonylation brought about by thiocholine acting as a nucleophile, and similarly fluoride does not affect the decarbamylation brought about by water, hydroxylamine, or choline serving as nucleophiles. Thus fluoride is without effect in both desulfonylation and decarbamylation, in contrast to the interpretation that fluoride inhibits deacetylation during the normal course of the hydrolysis of acetylcholine (5). Deacetylation is enormously faster than decarbamylation (which in turn is very much faster than desulfonylation), and it is quite possible that fluoride may have different effects on the two processes. However, our results do suggest that the effect of fluoride on deacetylation be re-examined.

Fluoride does inhibit sulfonylation, in agreement with its effect on carbamylation (13) and its less directly observed effect on acetylation (1–5).

Our results show that fluoride and a sulfonylating agent (methanesulfonate II) can combine simultaneously with the enzyme, in agreement with the interpretation that fluoride and acetylcholine can combine simultaneously with the enzyme (5). This observation is part of the general result that fluoride and many quaternary ammonium ions (and probably tertiary ions, etc.) can combine simultaneously with the enzyme. However, one of our quaternary ions was completely competitive, and several were partially competitive. Interestingly enough, some were cooperative. Our results also suggest that any combination of fluoride with enzyme, be it free enzyme or enzyme–ammonium ion complex, is completely or nearly completely lacking in catalytic properties. With regard to the sulfonyl-enzyme, either fluoride does not combine with it, or, if it does, the resulting complex is still equally reactive with thiocholine.

The completely competitive binding of fluoride and (3-hydroxyphenyl)trimethylammonium ion which had been reported from different observations (5) is unusual, and we might speculate on a possible explanation. The 3-hydroxyl group makes a large binding contribution, a factor of 120, and it is thought that this group may be involved in a hydrogen bond with a basic group in the protein (12). If the fluoride ion forms a hydrogen bond with the conjugate acid of this basic group, it would preempt the binding site of the hydroxyl group and therefore be competitive (to the extent of a factor of at least 120).

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