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The Effect of Fluoride on the Reaction of Acetylcholinesterase with Carbamates

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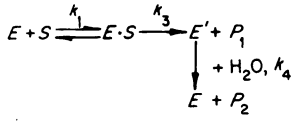
SUMMARY

The effect of fluoride on the rates of carbamylation and decarbamylation of acetylcholinesterase was studied using dimethylcarbamyl fluoride, dimethylcarbamylcholine, and monomethylcarbamylcholine as carbamylating agents. In all cases concentrations of fluoride between 10^{-4} M and 10^{-3} M, which markedly decreased the rate of hydrolysis of acetylcholine, also markedly decreased the rate of carbamylation. On the other hand, concentrations of fluoride as high as 0.1 M failed to affect the rate of decarbamylation of the dimethylcarbamyl-enzyme and the monomethylcarbamyl-enzyme. Fluoride was also without effect on the nucleophilic decarbamylation or reactivation caused by hydroxylamine and choline.

INTRODUCTION

This study of the effect of fluoride on the reaction of acetylcholinesterase with carbamates 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 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 (1-5).

Fluoride inhibition is best discussed with the aid of the kinetic scheme for enzymatic hydrolysis of substrates (Scheme 1), where S is acetylcholine, P_1 is choline, P_2 is acetic acid, E is enzyme, and E' is the acetyl-enzyme intermediate. It is apparent that there are three enzyme forms with which fluoride might combine, and three reaction steps that might be inhibited. In order to explain his results, Krupka (5) invoked all these possibilities.



(SCHEME 1)

The N-methyl- and N,N-dimethylcarbamates (of choline, for example) that we have used react with acetylcholinesterase in accord with the same scheme that applies for the reaction of substrates with the enzyme. The mechanism is thought to be the same, or at least very similar.

These substances react with the enzyme

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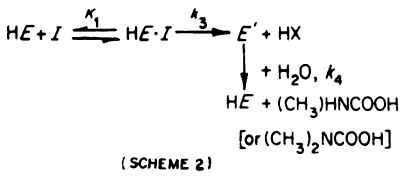

(SCHEME 2)

where I is $(CH_3)HNCOX$ or $(CH_3)_2NCOX$,

to form methyl- and dimethylcarbamyl-enzyme intermediates, which in turn slowly react with water to complete the hydrolysis of the carbamate. Thus, these substances are extremely poor substrates (6–10), but historically they have been regarded as inhibitors of this enzyme and we shall refer to them as inhibitors. The reaction is depicted in Scheme 2, where I is $(CH_3)HNCOX$ or $(CH_3)_2NCOX$, E' is the covalent *N*-methyl- or *N,N*-dimethylcarbamyl-enzyme, and X is the leaving group. HX is either choline or hydrogen fluoride in this study. All K are dissociation constants.

The symbol HE, in which the H indicates the hydrogen atom of the hydroxyl group of the active serine residue, is used to represent free enzyme rather than E, so that a balanced equation can be written. However, we do not wish to imply any mechanistic role here to H, although one may exist, or to imply that it is the same H as in HX. In the later mathematical development we shall use E as the symbol and not be concerned with balanced equations. HE and I are essentially in equilibrium with H$E \cdot I$. Because decarbamylation is very slow (half-time about 30 min), it is possible to study the effect of fluoride separately on carbamylation and decarbamylation. From these observations we may be able to infer what effect fluoride has on acetylation and deacetylation.

MATERIALS AND METHODS

Enzyme. Three preparations of acetylcholinesterase from *Electrophorus electricus* were used in this work. Two, LK 1 and LK 2, were gifts of Dr. Leon Kremzner, and the third was lot 6FA from Worthington Biochemical Corporation. The respective specific activities were 140, 100, and 60 mmoles of acetylcholine hydrolyzed per milligram of protein per hour at 25° and pH 7.0. (The purification procedure is described in ref. 11.)

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 (12). The buffer was 0.02 M Tris-HCl, 0.15 M NaCl, 10^{-5} M EDTA, 0.01% gelatin, and 1×10^{-2} M $CaCl_2$ (or 5×10^{-2} 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.

Carbamylation. When a carbamate is incubated with enzyme, a steady state is approached in which the rate of carbamylation equals the rate of decarbamylation, in accord with Scheme 2. The extent of carbamylation in the steady state depends upon the concentration of carbamate inhibitor. Inhibitor (dimethylcarbamyl fluoride, dimethylcarbamylcholine, monomethylcarbamylcholine) and dilute enzyme (approximately 2×10^{-8} N) were incubated in buffer with and without fluoride for at least six half-times for the approach to the steady state. Aliquots were assayed for enzyme activity at various times until constant values corresponding to the steady state were obtained. The decrease in enzyme activity in the steady state as compared to controls without carbamate inhibition gave the concentration of carbamyl-enzyme. The controls did not change with time. Small corrections were made for the slight decarbamylation that occurs during the time required for the assay.

Decarbamylation. Carbamyl-enzyme solutions in which about 80% of the enzyme was carbamylated were prepared as above, but with concentrated enzyme (10^{-5} N), using dimethylcarbamylcholine and monomethylcarbamylcholine as the carbamates. The carbamyl-enzyme solution was diluted 500 times into buffer with and without fluoride and other desired substances (choline, hydroxylamine), and after suitable times aliquots were withdrawn and assayed for enzyme activity. The rate of increase of

enzyme activity gives the rate of decarbamylation of the enzyme.

Buffer. The buffer used in carbamylation and decarbamylation was the same as for enzyme assay, except that the concentration of Tris-HCl was 0.001 M and the pH was 7.0.

RESULTS

Scheme 2 indicates that the reaction between a carbamate inhibitor and enzyme approaches a steady state in which the rate of carbamylation equals the rate of decarbamylation. In this way the carbamate is hydrolyzed, but if the concentration of carbamate is much greater than the concentration of enzyme there will be little change in the concentration of the carbamate, and the concentration of carbamyl-enzyme (E') will remain constant for many hours. If such a solution is extensively diluted, the rate of carbamylation becomes sensibly zero, but decarbamylation is unaffected so that the new steady state which will be approached corresponds to zero concentration of carbamyl-enzyme. The rate of decarbamylation is easily followed as the system moves toward this new steady state.

The rate of decarbamylation of the monomethylcarbamyl-enzyme in the presence and absence of fluoride is shown in Fig. 1. Even 10^{-2} M F^- does not affect the rate of decarbamylation. Similar results were obtained with the dimethylcarbamyl-enzyme (Table 1). Even 10^{-1} M F^- in one experiment did not inhibit decarbamylation (Table 2). These results must be contrasted with the effect of fluoride on the hydrolysis of acetylcholine, in which strong inhibition occurs with fluoride concentrations that are considerably less than 10^{-3} M.

The carbamyl-enzyme derivatives can be decarbamylated or reactivated by nucleophiles. Fluoride has no effect on the decarbamylation of the monomethylcarbamyl-enzyme by hydroxylamine (Table 2). Decarbamylation by choline at high concentration was not affected by fluoride, although at lower choline concentrations (data not given) fluoride may have enhanced the rate of decarbamylation; however, the effect was too small to be certain. It is clear, however, that

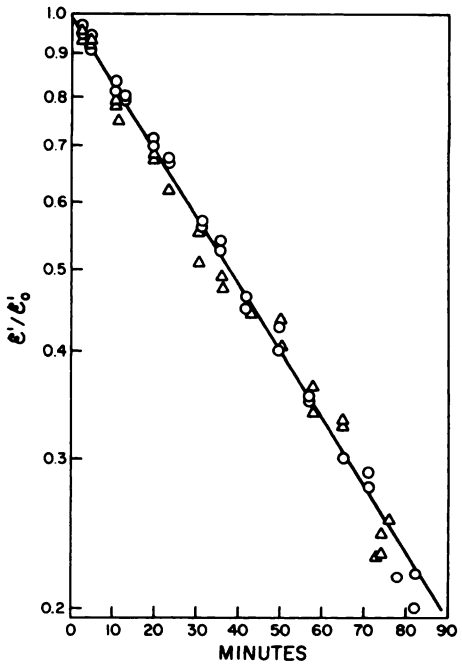

FIG. 1. *Rate of hydrolysis of monomethylcarbamyl-enzyme with and without NaF*

Acetylcholinesterase, Worthington lot 6FA, was inhibited with 3 × I_{50} monomethylcarbamylcholine (4.2×10^{-5} M), diluted 500-fold into buffer ± NaF, and incubated at 25°. ○, no NaF; △, 10^{-2} M NaF. $k_4 = 1.8 \times 10^{-2}$ min^{-1}. Data on the ordinate are plotted on a logarithmic scale.

TABLE I

Effect of fluoride on rate of decarbamylation of dimethylcarbamylacetylcholinesterase

Acetylcholinesterase, LK 1, was used in these experiments. $k_4 = 2.5$ min^{-1}. Phosphate experiments were done in duplicate, and those with Tris, in quadruplicate. The enzyme was inhibited with 3×10^{-4} M dimethylcarbamylcholine, diluted 500-fold into buffer ± NaF, and incubated at 25°. Samples were withdrawn at various times and assayed for active enzyme. Data were plotted separately for each entry and in a manner similar to that shown in Fig. 1.

Buffer	NaF	$t_{1/2}$
	M	*min*
Sodium phosphate	0	28
Sodium phosphate	1×10^{-3}	28.5
Sodium phosphate	4×10^{-3}	27.5
Tris-HCl	0	28
Tris-HCl	1×10^{-2}	28

TABLE 2

Effect of fluoride on rate of decarbamylation of monomethylcarbamylacetylcholinesterase

Acetylcholinesterase, Worthington lot 6FA, was used in these experiments.

NaF	Other addition	$t_{1/2}$
M		*min*
0	None	38.7
10^{-2}	None	38.5
10^{-1}	None	36.5
0	1 M NH_2OH	5.5
10^{-2}	1 M NH_2OH	5.3
0	10^{-2} M choline	21.0
10^{-2}	10^{-2} M choline	22.0
5×10^{-2}	10^{-2} M choline	22.5

fluoride does not inhibit decarbamylation by choline.

In the presence of fluoride the scheme for decarbamylation is

$$E' + H_2O \xrightarrow{k_4} E + (CH_3)NHCOOH$$

$$E' + F \underset{}{\overset{K_F''}{\rightleftharpoons}} E'\cdot F \xrightarrow{\beta k_4} E\cdot F + (CH_3)NHCOOH$$

yielding

$$\log \frac{\varepsilon'}{\varepsilon_0'} = \frac{-k_{4\,(app)}\, t}{2.3} \qquad (1)$$

where ε' is the concentration of carbamyl-enzyme in all forms ($E' + E' \cdot F$) at time t, and ε_0' is the initial concentration of carbamyl-enzyme.

$$k_{4\,(app)} = k_4 \frac{1 + \beta[(F)/K_F'']}{1 + [(F)/K_F'']} \qquad (2)$$

While it was possible *a priori* for $\beta > 1$, i.e., for fluoride to act either as a nucleophile or as an activator for decarbamylation, the expectation was that $\beta \leqq 1$; i.e., the question under investigation was whether or not fluoride inhibits decarbamylation. Our experimental results show that $k_{4(app)} = k_4$. This condition can be met in two ways. Either $K''_F = \infty$ or $\beta = 1$; either fluoride does not form a complex with the carbamyl-enzyme or, if it does, the complex is decarbamylated at the same rate as the carbamyl-enzyme. It is also clear that our experimental results show that fluoride is not a nucleophile toward the carbamyl-enzyme.

The effect of fluoride on the rate of carbamylation was studied by observing the effect of fluoride on the steady-state extent of carbamylation. Since fluoride does not affect decarbamylation, any diminution of the extent of carbamylation must be caused by inhibition of carbamylation.

The scheme for the steady state of the reaction of a carbamate, I, with the enzyme E in the presence of fluoride is

$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} E\cdot I \xrightarrow{k_3} E' \xrightarrow{k_4} E$$

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

$$E\cdot I + F \underset{}{\overset{K'_F}{\rightleftharpoons}} E\cdot I\cdot F \xrightarrow{\alpha k_3} E$$

$$E' + F \underset{}{\overset{K''_F}{\rightleftharpoons}} E'\cdot F \xrightarrow{\beta k_4} E$$

(The last reaction can be omitted, since either $K''_F = \infty$ or $\beta = 1$.)

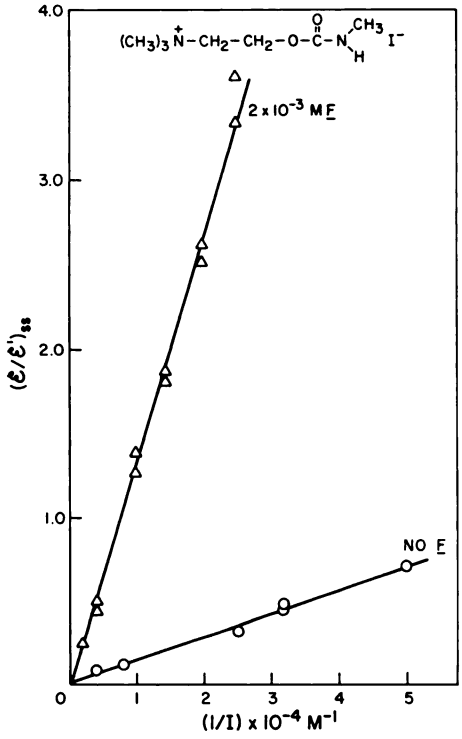

FIG. 2. *Effect of fluoride on steady-state ratios* $(\varepsilon/\varepsilon')_{ss}$ *plotted as a function of reciprocal of monomethylcarbamylcholine concentration*

Enzyme (LK2) was incubated at 25° and pH 7.0 with the carbamylating agent ± NaF until more than 98% of the steady-state extent of carbamylation was reached.

The steady-state solution,

$$\frac{\mathcal{E}}{\mathcal{E}'} = \frac{k_4}{k_3\{1 + \alpha[(F)/K'_F]\}} \cdot \left\{\left[1 + \frac{(F)}{K'_F}\right] + \left[1 + \frac{(F)}{K_F}\right]\frac{K_I}{(I)}\right\} \quad (3)$$

pertains when

$$\frac{d\mathcal{E}'}{dt} = k_3(E \cdot I) + \alpha k_3(E \cdot I \cdot F) - k_4(E' + E' \cdot F) = 0$$

where $\mathcal{E}'$ is the sum of the concentrations of the carbamyl-enzyme, including all its reversible complexes; namely,

$$\mathcal{E}' = (E') + (E' \cdot F)$$

and $\mathcal{E}$ is the sum of the concentrations of the noncarbamylated enzyme, including all its reversible complexes; namely,

$$\mathcal{E} = (E) + (E \cdot I) + (E \cdot I \cdot F) + (E \cdot F)$$

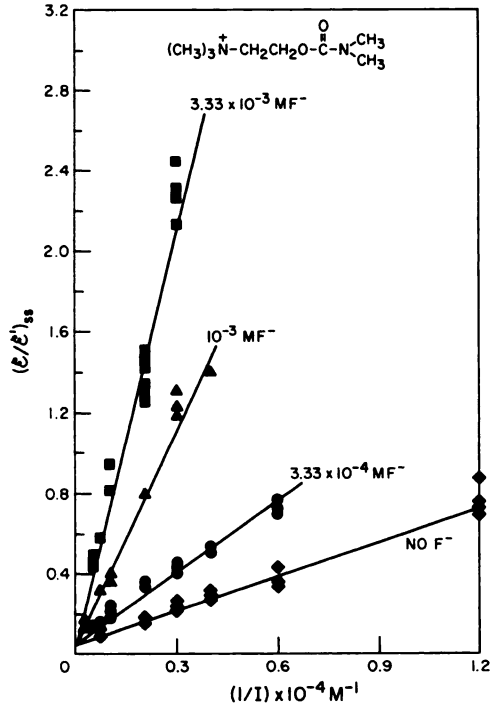

FIG. 3. *Effect of fluoride on steady-state ratios* $(\mathcal{E}/\mathcal{E}')_{ss}$ *plotted as a function of reciprocal of dimethylcarbamylcholine concentration*

Enzyme (LK2) was incubated at 25° and pH 7.0 with dimethylcarbamylcholine ± NaF until more than 98% of the steady-state extent of carbamylation was reached.

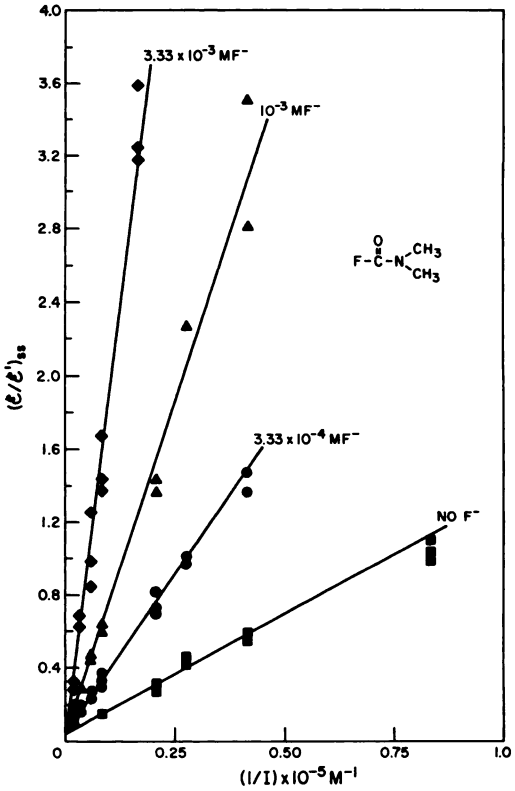

FIG. 4. *Effect of fluoride on steady-state ratios* $(\mathcal{E}/\mathcal{E}')_{ss}$ *plotted as a function of reciprocal of dimethylcarbamyl fluoride concentration*

Enzyme (LK2) was incubated at 25° and pH 7.0 with the carbamylating agent ± NaF until more than 98% of the steady-state extent of carbamylation was reached.

The results are put in this form because $\mathcal{E}$ and $\mathcal{E}'$ are the quantities that are evaluated. This is so because the system is diluted extensively in measuring the carbamyl-enzyme, and therefore the reversible complexes dissociate completely.

Our results show that fluoride markedly decreases the extent of carbamylation in the steady state and indicate that fluoride decreases the rate of carbamylation. This is true with all three carbamate inhibitors, as shown in Figs. 2–4, where the data are presented with respect to the reciprocal of the concentration of carbamate in accord with Eq. 3. In all cases the intercepts are close to the origin. This indicates that the concentrations of the carbamates are well be-

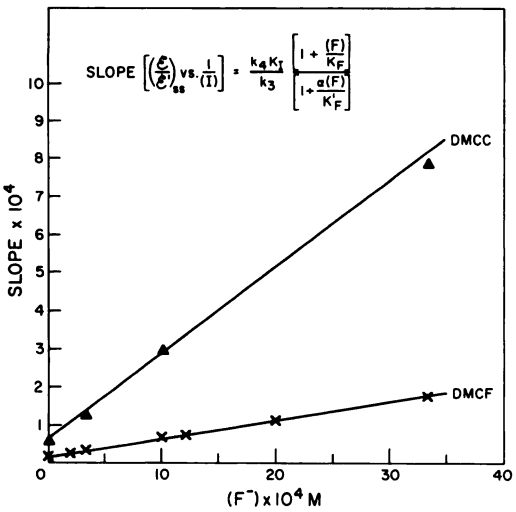

FIG. 5. *Slopes of the lines in Figs. 3 and 4 plotted as a function of fluoride concentration*

low the values of their reversible dissociation constants (K_I) with the enzyme, and that k_3 is very large compared to k_4. The effect of fluoride on the intercept could not therefore be observed. This is unfortunate, because the effect of fluoride on the intercept would have enabled us to evaluate α and K'_F.

The slopes of these lines are sensitive to the concentration of fluoride and increase linearly with fluoride concentration, as shown in Fig. 5. This indicates that α/K'_F is small compared to $1/K_F$; either α is small compared to 1 or K'_F is considerably larger than K_F, or some combination of both is true. If K'_F is larger than K_F, fluoride does not bind as readily to the reversible enzyme-carbamate complex as it does to the free enzyme, and this in turn implies that the carbamate does not bind as readily to the enzyme-fluoride complex as it does to the free enzyme. Thus we are left with two possibilities: either fluoride diminishes the formation of the reversible enzyme-carbamate complex or it diminishes the rate with which this complex reacts to form the carbamyl-enzyme.

We can of course evaluate K_F, the dissociation constant for the enzyme-fluoride complex. The values obtained range from 2×10^{-4} to 3×10^{-4}, with most values close to 2.4×10^{-4} M.

We have seen that the linearity of the plots in Fig. 5 indicate that $\{1 + \alpha[(F)/K'_F]\}$ is approximately 1. Therefore the ratio of the slopes from Figs. 2–4 in the presence and absence of fluoride gives $\{1 + [(F)/K_F]\}$, from which K_F may be calculated.

DISCUSSION

Our results clearly show that carbamylation of acetylcholinesterase as effected by monomethylcarbamylcholine, dimethylcarbamylcholine, and dimethylcarbamyl fluoride is inhibited by fluoride. These observations confirm the inhibition by fluoride of the acetylation step in the hydrolysis of acetylcholine.

Our results also clearly show that decarbamylation of neither the monomethylcarbamyl-enzyme nor the dimethylcarbamyl-enzyme is affected by fluoride. This finding does not confirm that fluoride inhibits the deacetylation step in the hydrolysis of acetylcholine. What can we infer from this result? Had we found fluoride to inhibit decarbamylation, it would surely have indicated that fluoride also inhibits deacetylation. However, we cannot so easily draw the conclusion that fluoride does not inhibit deacetylation from our actual result that it does not inhibit decarbamylation, because there is a very great difference in the rates of the two processes: decarbamylation is 20 million times slower. It is conceivable that the catalytic processes are not identical, that deacetylation involves an extra facet of catalysis, and it is possible that this facet is affected by fluoride.

The interpretation made by Krupka and others that fluoride inhibits deacetylation, while eminently reasonable, was necessarily indirect. Our observation that fluoride does not affect decarbamylation would seem to suggest that a definitive position on the effect of fluoride on deacetylation be held in abeyance until further work can better establish whether or not fluoride does inhibit deacetylation.

The constant for the dissociation of the enzyme-fluoride complex appears to be rather lower for electric eel acetylcholinesterase (2.4×10^{-4} M) than for bovine erythrocyte enzyme [1.8×10^{-3} M (5)].

The value itself is rather interesting, in that its low level, less than the dissociation constant of HF (7.2×10^{-4} M), makes it difficult to envisage what kind of binding might be involved. A simple hydrogen bond between the protein and fluoride would not seem to be of sufficient strength unless the proton donor was in a nonaqueous environment and was not initially hydrogen-bonded to water or another base. Perhaps two hydrogen bonds are involved, in which fluoride bridges two proton donors.

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