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Action of Fluoride on Cholinesterase

I. On the Mechanism of Inhibition

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The effect of sodium fluoride upon acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) has been studied. The compound inhibits both enzymes reversibly, the type and degree of inhibition depending upon the substrate used. When the latter is acetylcholine, "mixed" inhibition of AChE is found, while the inhibition of AChE with N-methylaminoethyl acetate as substrate is noncompetitive. Some kinetic constants are calculated. The inhibition of crude plasma BuChE produced by sodium fluoride and that produced by excess of the substrate benzoylcholine interfere with each other, the total inhibition being less than that produced by fluoride alone. Inhibition at pH 7.4, due to the simultaneous presence of trimethylammonium bromide and fluoride, is less than additive. The sodium fluoride induced inhibition of both AChE and BuChE increases when the temperature is lowered and also when the pH is lowered. Some possible mechanisms of inhibition are discussed. It is suggested that both the fluoride ion, hydrogen fluoride and the hydrogen difluoride ion are inhibitors of cholinesterases and that acylation and possibly also deacylation of the enzymes may be blocked.

It was recently shown[1] that sodium fluoride is able to reactivate Sarin (methyl-isopropoxy-phosphoryl fluoride) inhibited human blood cholinesterases (ChE). An interpretation of this observation may be facilitated by information about the interaction of fluoride and ChE. It is known that fluoride inhibits ChE, but the papers describing this effect are confined to those dealing with effects of variations in the composition and the ionic strength of the medium on the ChE activity [2] or those reporting utilization of differences in the degree of ChE inhibition obtained with fluoride to distinguish between phenotypes of human plasma ChE's.[3–5] The present paper gives the results of a study on the mechanism of the ChE inhibition produced by sodium fluoride.

MATERIALS AND METHODS

Enzymes. Source of acetylcholinesterase (AChE) were human erythrocytes, treated as previously described.[6] In some experiments a partly purified preparation of bovine erythrocytes (Winthrop Laboratories, New York) was used, dissolved in 0.1 M potassium chloride.

Human plasma, serum fraction IV-6c or serum fraction IV-6-3 (obtained from human postpartum blood by AB Kabi, Stockholm) was used as source for butyrylcholinesterase (BuChE). When the latter ones were used, the lyophilized preparations were first dissolved in buffer or in 0.1 M potassium chloride solution. The BuChE preparations used may contain more than one ChE.[7-9] In that case it cannot be excluded that the enzymes present exhibit differences in their sensitivity towards sodium fluoride. However, such differences have so far only been observed in the presence of very low concentrations of benzoylcholine (3×10^{-5} M).[3,4] In the present investigation BuChE was studied mainly at substrate concentrations above 2×10^{-3} M, because, according to earlier experiments,[7] the enzyme preparations follow the Michaelis-Menten equation in this substrate range. Possibly this indicates that only one ChE acts upon the substrate.

Once it was established that enzyme, dialyzed for three days before addition of fluoride, was inhibited to the same degree as undialyzed enzyme, no further efforts were made to dialyse the enzyme preparations before use.

Substrates. Benzoylcholine chloride was obtained commercially. Acetylthiocholine iodide was synthesized by Hansen and the other choline esters by Enander. N-Methylaminoethyl acetate hydrochloride (MAEA) was synthesized according to Wilson.[18]

Inhibitors. Methyl-isopropoxy-phosphoryl fluoride (Sarin), methyl-isopropoxy-phosphoryl thiocholine iodide (37 S-N^+), diisopropoxy-phosphoryl fluoride (DFP) and dimethylamido-ethoxy-phosphoryl cyanide (Tabun) were synthesized by L. Fagerlind according to methods described in the literature.[11-14] Liquid compounds were freshly distilled before use. Sodium fluoride (NaF) was a commercial analytical grade preparation (Baker Chem. Co., Phillipsburg, N. J.), containing 0.04 % free acid (HF), 0.08 % sodium fluorosilicate (Na_2SiF_6) and 0.001 % heavy metals.

Measurement of enzyme activity. Enzyme activity was measured as described earlier,[6] using either an automatic recording constant pH titrator or an electrometric method. Either pH 7.0, 7.4, or 8.0 was used except in experiments on the pH dependence of inhibition, where the pH was varied between 5.5 and 9.5. The titrator was at each temperature standardized against either a borate buffer [15] or a phosphate buffer.[15] The temperature used was either 37°C or 25°C. In experiments on the influence of temperature on inhibition, temperatures of 38°C, 30°C, 22°C, and 15°C were used. All values given are the means of at least two determinations and are corrected for the spontaneous hydrolysis of the substrate used. When necessary the ionic strength of the medium was kept constant with added sodium chloride.

The effect of sodium fluoride on AChE in the presence of low concentrations of substrate (10^{-3} M and less) was studied by means of the double syringe method previously [7] described. With this method the substrate concentration is held constant throughout the experiments by the continuous addition from a second syringe of substrate solution of the same molarity as the sodium hydroxide used to keep the pH constant.

CALCULATIONS

The reaction to be studied [29] is outlined in scheme 1.

$$\mathrm{EH} + \mathrm{S} \underset{k_{+1}}{\overset{k_{-1}}{\rightleftharpoons}} \mathrm{EHS} \xrightarrow[k_2]{\text{alcohol}} \mathrm{EAc} \xrightarrow[k_3]{\mathrm{H_2O}} \mathrm{EH} + \text{acid} \qquad (1)$$

$$\mathrm{EHS} \xrightarrow[k_4]{} \mathrm{EH} + \text{acid}$$

where EH is the free enzyme (either AChE or BuChE), EHS the enzyme-substrate complex and EAc the acylated enzyme. Some results indicated mixed[16] inhibition. This type of inhibition is obtained when an inhibitor acts both on K_m, the substrate concentration giving half maximum velocity, and on V, the maximum velocity in excess of substrate. The correct interpretation of K_m in the case of AChE and BuChE is not known. It has been claimed[17] that K_m for AChE is a true Michaelis constant, *i.e.* equal to the dissociation constant K_s of the system described in scheme 1 above, thus that

$$K_m = K_s = k_{-1}/k_{+1} \tag{2}$$

It has also been claimed[18] that K_m for serum cholinesterase either approaches or is equal to the dissociation constant K_s. The uncertainty as to the significance of K_m makes it difficult to interpret the results. K_i, the dissociation constant for an enzyme-inhibitor complex, can be calculated by using eqns. 3 and 4, developed by Friedenwald *et al.*[19] and cited by Dixon and Webb,[16] provided the inhibition is truly mixed, meaning that both competitive and noncompetitive effects occur, and further that the enzyme-substrate-inhibitor complex does not decompose.

$$V_p = \frac{V}{1 + ([I]K_m/K_iK_m')} \tag{3}$$

$$K_p = \frac{K_m[1 + ([I]/K_i)]}{1 + ([I]K_m/K_iK_m')} \tag{4}$$

K_p is the substrate concentration giving half maximum velocity in the presence of inhibitor, K_m' is the value of K_m in the presence of excess of inhibitor and V_p is the maximum velocity in excess of substrate in the presence of inhibitor. The equations have been used for the calculation of some of the apparent values of K_i for AChE—ACh—NaF and BuChE—ACh—NaF, given in Table 4. V, V_p, K_m, and K_p were obtained from Lineweaver-Burk plots of $1/v$ *versus* $1/[S]$, where v is the observed initial velocity and [S] the substrate concentration.

In another series of experiments, where the concentration of the inhibitor ([I]) was varied at fixed substrate concentration, the apparent values of K_i were obtained graphically[20] from plots of $1/v$ against [I] (Dixon plot).

The apparent K_i for AChE—MAEA—NaF was obtained graphically as above or from the equation for noncompetitive inhibition[16]

$$V_p = \frac{V}{1 + ([I]/K_i)} \tag{5}$$

Due to a difference in the dissociation constants of the N-methylamino group of the ester MAEA (pk_a 8.7 according to own determinations) and of the alcohol N-methylaminoethanol (pk_a 9.77),[21] formed upon hydrolysis of the ester, corrections of 8—11 %, depending upon the pH of the solution, had to be added to the experimentally obtained rates.

It has previously been shown[22,23] that K_m for AChE—ACh is constant over the temperature range studied. If K_m further, as claimed,[17] is equal to

K_s, then at a fixed substrate concentration the velocity of the reaction described in scheme 1 varies with the temperature as V varies. This is seen from the Michaelis-Menten equation

$$v = \frac{V}{1 + (K_m/[S])} \tag{6}$$

No thermal inactivation of AChE was seen during the course of the experiments. If it is therefore assumed that the concentration of the active enzyme is constant over the temperature range studied, thus if the observed velocity of the reaction outlined in scheme 1 can be written as

$$v = k_4 \, [EHS] \tag{7}$$

where [EHS] does not change with the temperature, then the apparent energy of activation (E) for the breakdown of the enzyme—substrate complex for the system AChE—ACh to free enzyme and products can be obtained from an Arrhenius plot of log v *versus* $1/T$, using the equation [16]

$$\mathrm{d} \log v = \frac{E}{2.303 \; R} \times \frac{\mathrm{d}T}{T^2} \tag{8}$$

where v has been substituted for V. This has been done in Fig. 8.

RESULTS

Reversibility of inhibition. Plasma and erythrocytes were incubated with 1.5×10^{-2} M sodium fluoride for 30 min. After determination of the ChE activity of the samples and of the untreated controls the solutions were dialysed for 20—24 h against 0.9 % sodium chloride at 0°C. Table 1 shows the result obtained in a typical experiment. At least 98 % of the original enzyme activity was always restored after one day of dialysis. Enzyme preparations incubated with sodium fluoride and subsequently diluted before measurement of enzyme activity were inhibited proportionally to the amount of inhibitor present after dilution. From Table 2 it is seen that upon incubation of human blood ChE with sodium fluoride an equilibrium is rapidly reached; the time chosen for the addition of the substrate ACh making hardly any difference to the degree of inhibition.

Table 1. ChE activity of human plasma and erythrocytes inhibited with NaF, measured before and after 20 h of dialysis at 0°C against a solution of 0.9 % sodium chloride.

	Enzyme activity in % of control	
	Plasma	Erythrocytes
Before dialysis	11	5
After dialysis	98	102

Table 2. Influence of substrate (7.3×10^{-3} M ACh) and incubation time upon the degree of inhibition of plasma BuChE and erythrocyte AChE obtained with 1×10^{-3} M NaF at pH 7.4, 25°C.

Time for addition of sodium fluoride in relation to substrate	% inhibition obtained	
	BuChE	AChE
8 min after	48	31
together	44	—
5 min before	47	—
10 min »	—	30
20 » »	43	30
30 » »	—	30
40 » »	43	—
60 » »	45	31
sodium fluoride and substrate incubated together before addition of enzyme	46	—

Inhibition of cholinesterase by organophosphorus compounds and by trimethylammonium bromide in the presence of sodium fluoride. Human plasma BuChE and human erythrocyte AChE were incubated for 30 min at pH 8.0 and 25°C with varying concentrations of organophosphorus compounds and with or without sodium fluoride. The ChE activity was subsequently determined by the addition of ACh. The results, which are seen in Table 3, show a small protecting effect of sodium fluoride. This effect of sodium fluoride is not a general salt effect, because the ionic strength of the buffer solution itself is high (~ 0.7) and because addition of the corresponding amount of sodium chloride has no effect upon the inhibition produced by Tabun or Sarin. Possibly the observed effect is not only due to protection of ChE by fluoride. In the case of Tabun a chemical change of the organophosphorus compound may be caused by fluoride. It has been shown [24] that fluoride in water solution is able to displace the cyanide group from the phosphorus of Tabun, producing an anticholinesterase with a lower pI_{50} than Tabun.

At pH 7.4 inhibition of AChE by 5×10^{-3} M trimethylammonium bromide and 2×10^{-3} M sodium fluoride, added together, is lower (62 %) than the algebraic sum of the inhibition produced by either one alone (56 and 36 %, respectively).

Influence of substrate. Before describing the results it has to be pointed out that due to the biological variations in the sensitivity of human plasma BuChE to sodium fluoride [3-5] the results given may not be valid for every such enzyme preparation. The pI_{50} values at pH 8.0 and 25°C in barbital-phosphate buffer for the inhibition by sodium fluoride after addition of 7.3×10^{-3} M ACh to human plasma BuChE and erythrocyte AChE were found to be, respectively, 2.2 and 2.3. At pH 7.4, 25°C and in 0.1 M potassium chloride, sodium fluoride had only a very small influence upon the position of the pS-optimum of the bell-shaped pS-activity curve obtained with eryth-

3. Remaining activity of human plasma BuChE and human erythrocyte AChE with or without 10^{-3} M sodium fluoride after 30 min incubation with Sarin, 37 S-N$^+$, DFP and Tabun at 25°C and pH 8.

centration of ium fluoride (M)	Enzyme activity in % of control without sodium fluoride					
	BuChE Controls	AChE Controls	BuChE		AChE	
			Sarin			
			4×10^{-9} M	8×10^{-9} M	1.25×10^{-9} M	2.5×10^{-9} M
—	100	100	62	21	59	38
10^{-3}	76	83	70	60	74	57
			37 S-N$^+$			
			8×10^{-7} M	16×10^{-7} M	5×10^{-9} M	15×10^{-9} M
—	100	100	21	7	67	48
10^{-3}	75	82	32	20	74	57
			DFP			
			1.3×10^{-9} M	2.5×10^{-9} M	2.5×10^{-7} M	5×10^{-7} M
—	100*	100	61*	36*	66	44
10^{-3}	87*	86	62*	41*	68	51
			Tabun			
			4×10^{-9} M	8×10^{-9} M	8×10^{-9} M	16×10^{-9} M
—	100*	100	73*	55*	41	13
10^{-3}	88*	86	79*	70*	71	65

: Different batch of plasma used.

rocyte AChE and ACh (Fig. 1). As can be calculated from the left branch of the curve, at each substrate concentration the percentage of inhibition produced by excess of substrate and the percentage of inhibition produced

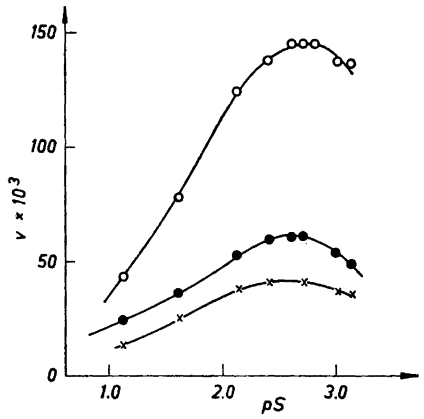

Fig. 1. pS-activity curve for the systems purified bovine erythrocyte AChE-ACh and AChE-NaF-ACh. The enzyme activity is expressed in μmoles of ester split per ml incubation solution and minute at pH 7.4 and 25°C. ×, 1×10^{-2} M NaF present; ●, 5×10^{-3} M NaF present; O, substrate only.

by fluoride is somewhat less than the additive value. The degree of inhibition of plasma BuChE produced by sodium fluoride was found to depend upon the type of the substrate used for the determination of the enzyme activity. Experiments in barbital-phosphate buffer with equimolar concentrations (9.1×10^{-3} M) of ACh, acetylthiocholine (AThCh), butyrylcholine and benzoylcholine (BzCh) as substrates and the same batch of plasma as enzyme source gave pI_{50}-values of, respectively, 2.1, 2.1, 3.0, and 3.4.

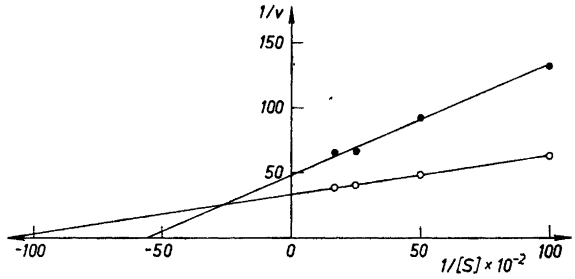

Fig. 2. Lineweaver-Burk plot for human erythrocyte AChE and ACh showing "mixed" inhibition. The enzyme activity is expressed as in Fig. 1 but measured at 37°C. ●, NaF (5×10^{-3} M) present; O, substrate only.

The nature of the inhibition was studied further in 0.1 M potassium chloride with the substrates ACh, BzCh, and MAEA. With ACh as substrate a mixed type [16] of inhibition is obtained with human erythrocyte AChE (Fig.2) and with purified bovine erythrocyte AChE (double syringe method), while the latter enzyme preparation with MAEA as substrate exhibits virtually noncompetitive [16] inhibition (Fig. 3). The inhibition of crude plasma BuChE and of partly purified human serum BuChE, measured with ACh as substrate,

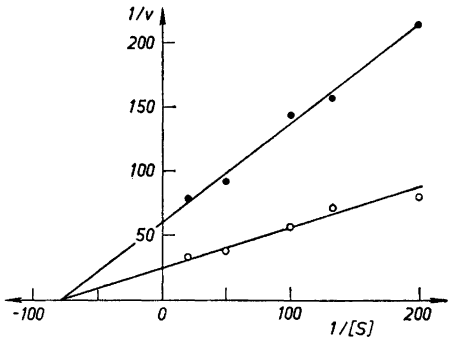

Fig. 3. Lineweaver-Burk plot for purified erythrocyte AChE and MAEA showing noncompetitive inhibition. The enzyme activity is expressed and measured as in Fig 1. ●, NaF (1×10^{-3} M) present; O, substrate only.

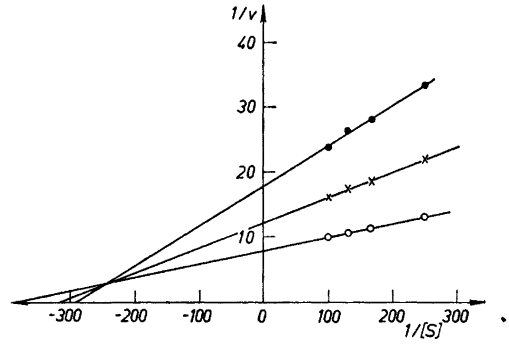

Fig. 4. Lineweaver-Burk plot for purified human serum BuChE (IV-6-3) and ACh showing "mixed" inhibition. The enzyme activity is measured and expressed as in Fig. 2. ●, 5×10^{-3} M and ×, 2×10^{-3} M NaF present; O, substrate only.

seems largely to be noncompetitive, but to some extent also K_m is changed by fluoride (Fig. 4), especially at higher substrate and inhibitor concentrations. Some kinetic data for AChE and BuChE, calculated from Lineweaver-Burk plots or from Dixon plots with the aid of the method of least squares, are seen in Table 4.

With BzCh (5×10^{-4} — 1×10^{-2} M) as substrate and plasma BuChE at pH 7.4 and 37°C the wellknown decrease of enzyme activity [25] with increasing substrate concentration was observed. However, the inhibition of BuChE produced by 1×10^{-4} M sodium fluoride in the presence of the lower BzCh concentrations was not further increased due to additional inhibition produced by an increasing substrate concentration. On the contrary, in the presence of sodium fluoride the enzyme activity increased with increasing substrate concentration (Fig. 5) though the activity was lower over the whole range of substrate than in the absence of fluoride.

Influence of pH. The experiments were performed in a 0.1 M solution of potassium chloride at 37°C (plasma) or 25°C (erythrocytes and serum fraction IV-6-3). At each pH, enzyme activity was found to be linear during the course of the experiment, which excludes loss of activity due to falling substrate concentration or due to enzyme denaturation. Fig. 6 shows that the

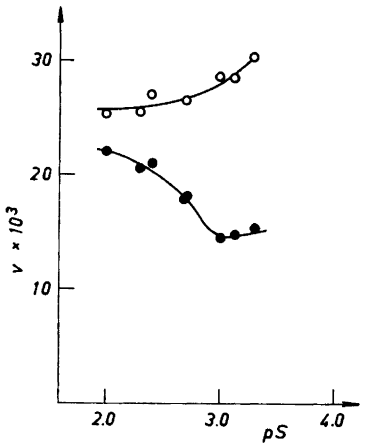

Fig. 5. pS-activity curve for the system human plasma BuChE-BzCh. The enzyme activity is measured and expressed as in Fig. 2. O, substrate only; ●, substrate and 1×10^{-4} M NaF.

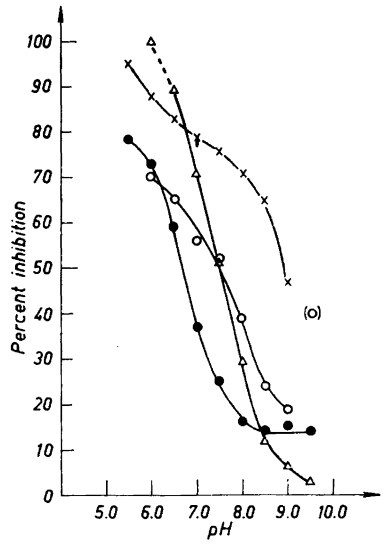

Fig. 6. Change of inhibition of ChE activity with pH as produced by fluoride. ●, human erythrocyte AChE, 25°C, 5×10^{-4} M NaF and 4×10^{-4} M ACh; △, human erythrocyte AChE, 25°C, 1×10^{-3} M NaF and 4×10^{-4} M AThCh; ×, purified human serum BuChE, 25°C, 5×10^{-3} M NaF and 1×10^{-2} M ACh; O, human plasma BuChE, 37°C, 5×10^{-3} M NaF and 1×10^{-2} M ACh.

inhibition of plasma, serum and erythrocyte ChE, produced by sodium fluoride and measured with ACh or AThCh as substrate, increases with decreasing pH. The inflexion point of the curve obtained with erythrocyte AChE and ACh is at 6.8. The shape of the curves obtained with crude or purified BuChE allows no such calculations. With all enzyme preparations a displacement of the acid side of the pH-activity curve towards a higher pH-value was obtained in the presence of fluoride. The pH optimum for AChE-ACh was not changed in the presence of fluoride. As expected,[26] the pH optimum for purified BuChE was not reached in the pH range studied (Fig.7.).

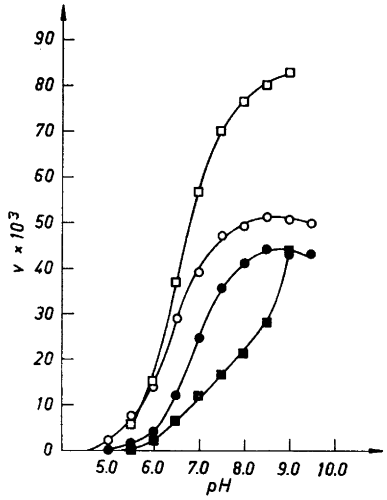

Fig. 7. Influence of NaF on pH-activity curves for ChE at 25°C. v expressed as in Fig. 1: O, human erythrocyte AChE—ACh (4×10^{-4} M); ●, AChE-ACh-NaF (5×10^{-4} M); □, purified human serum BuChE-ACh (1×10^{-2} M); ■, BuChE-ACh-NaF (5×10^{-3} M).

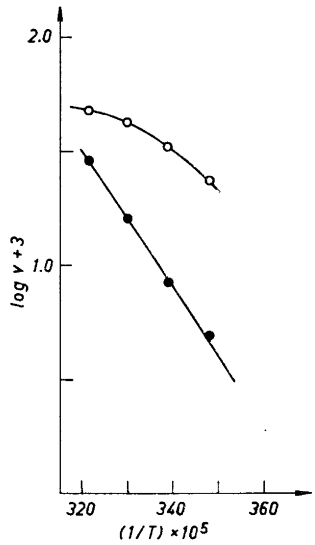

Fig. 8. Arrhenius plot of log v *versus* $1/T$. pH 7.4. v expressed as in Fig. 1: O, human erythrocyte AChE-ACh (4×10^{-4} M); ●, AChE-ACh-NaF (5×10^{-3} M). Double syringe method to avoid the range where excess of inhibitor inhibits the enzyme.

Influence of temperature. It was found that in the presence of ACh sodium fluoride inhibition of erythrocyte and plasma ChE increases as the temperature is lowered. As described in "Calculations", for erythrocyte AChE log v was plotted against $1/T$ both for the uninhibited and the inhibited reaction (Fig. 8). In the latter case this is possible because, as shown above in this paper, no time factor could be demonstrated in the inhibition produced by fluoride. Wilson and Cabib [23] found that the Arrhenius plot for the uninhibited reaction of AChE and ACh was not a straight line. The curves seen in Fig. 8 confirm this finding, but show also that in the presence of fluoride the plot of log v against $1/T$ results in a straight line.

As seen from Table 4, the apparent K_i for BuChE decreases with temperature.

Table 4. Some experimentally obtained Michaelis-Menten constants (K_m) and inhibitor constants (K_i) for the binding of the substrate or the inhibitor to the enzymes AChE or BuChE at various temperatures and pH.

Enzyme	Substrate Type	Substrate M × 10^4	Inhibitor M × 10^3	Temp. °C	pH	K_m M × 10^3	K_i M × 10^3
AChE							
Human erythrocytes	AChI	1–6	5	37	7.4	0.092	2.77
Purified bovine erythrocytes	AChI	6–20	1	25	7.4	0.131	0.62
Purified bovine erythrocytes	MAEA	50–500	1	25	7.4	13	0.74
Purified bovine erythrocytes	MAEA	75–500	0.2–1	25	7.0	10.2 ± 0.2	0.42 ± 0.02
BuChE							
Human plasma	AChI	30–150	5	37	7.4	1.42	2.2
Purified human serum (IV-6c)	AChI	60–100	0.8–4	25	7.4	2.5	0.44
Purified human serum (IV-6c)	AChI	50–75	0.8–4	30	7.4	2.1	0.60
Purified human serum (IV-6c)	AChI	25–75	0.8–4	37	7.4	0.99 ± 0.06	1.42 ± 0.25

DISCUSSION

The experimentally observed increase in ChE inhibition with decreasing pH, produced by sodium fluoride, could be explained if hydrogen fluoride (HF) and hydrogen difluoride ion (HF_2^-) were the active species of the inhibitor, as the pK values for the dissociation of these compounds are 3.17 and 0.59, respectively,[27] at 25°C. However, from these dissociation constants it is found that sodium fluoride in an aqueous solution of pH 7.4 occurs mainly as F^-, very small amounts of HF (3×10^{-8} M in a 5×10^{-4} M solution of NaF) and HF_2^- (6×10^{-11} M) being present at 25°C. The corresponding K_i values would be 10^{-8} M or less. Such values are usually obtained only with inhibitors fitting an enzyme particularly well. Nothing is known about the active centre of ChE that justifies the thought that HF (or HF_2^-) should be a particularly powerfull anticholinesterase. Therefore the results reported in this paper suggest that the fluoride ion itself is an inhibitor of ChE.

Inhibition was observed to decrease with increasing pH up to pH 8.0. Between this pH and pH 9.5 (highest pH used in the experiments) the percentage of fluoride inactivated enzyme is roughly constant for AChE–ACh,

while it continues to decrease for BuChE—ACh and AChE—AThCh. At least the latter observation could indicate that the inhibition is due to a reaction where F^- and OH^- can compete as nucleophilic agents. Such a reaction would be a nucleophilic attack of F^- at the carbonyl carbon of the acyl group of the substrate, attached to the enzyme when the latter is acylated. If ChE catalyzes transacylation from the substrate to F^-, as it does from *e.g.* ethyl acetate to hydroxylamine,[29] a new substrate might be created. Formation of *e.g.* acetyl fluoride would result in an apparent reduced rate of enzyme activity, because the amount of acid available for titration would be reduced. However, the following makes the suggested reaction less probable. Acetyl fluoride is rather labile in water solution and subject both to solvolysis and OH^- as well as H^+ catalyzed hydrolysis.[30,31] Further, available data [23] indicate that the deacylation of acylated AChE is the rate determining step in the catalyzed hydrolysis of ACh at 25°C and higher temperature. This may also be true for AThCh,[32] a compound that is an about equally good substrate of AChE.[10] Therefore, in the presence of these two substrates AChE catalyzed acylation of fluoride should appear as the same degree of inhibition, as the same acylated enzyme is obtained with both substrates. However, different degrees of inhibition were found in the presence of ACh and AThCh. This is not in agreement with the mechanism of fluoride inhibition outlined above.

It seems that an understanding of the mechanism of action of sodium fluoride requires a consideration of the mechanism of action of ChE. The current picture of the active centre of these enzymes [29] includes two sites with four groups interacting with substrates and inhibitors, the anionic site (questioned by some authors [33] in the case of BuChE) and the esteratic site, including a basic (imidazole?) and an acidic group and a reactive serine. Krupka [32] has recently summarized the evidence accumulated from the work of several authors and proposed a mechanism for the action of AChE involving the anionic site, the basic and the acidic group in the acylation and deacylation of serine. Various other mechanisms have been proposed,[29] among them one by Brestkin and Rozengart [34] suggesting the formation of a hydrogen bond between the carbonyl oxygen of the substrate and the hydrogen of the imino group of imidazole as a first step in the splitting of the substrate.

The acid branch of the pH-activity curve of ChE is considered to be the titration curve of the basic group, which is supposed to be inactive in its protonated form.[29] Possibly also the anionic site contributes to the shape of this branch ($pk_a \sim 6.3$).[29] The pH-activity curve for AChE-ACh, obtained in the presence of fluoride, was steeper than the one obtained in the absence of this compound. The pH optimum is hardly changed, but the presence of fluoride apparently alters the degree of dissociation of the group(s) determining the enzyme activity on the acid side of the pH optimum, resulting in a change of the apparent pk_a of the titrated group(s) from 6.4 to 6.8 in the presence of 5×10^{-4} M fluoride. Hence, the proportion of enzyme being in the inactive form increases as the pH decreases, when inhibited and uninhibited reaction are compared. This observation may indicate that fluoride either reacts with the titrated group(s) itself, *e.g.* by addition to the protonized form, or with a group influencing the pk_a of the titrated group(s).

Under the conditions used the inhibition by fluoride was found to be completely noncompetitive with MAEA as substrate, but a competitive element was observed when ACh was the substrate. It is assumed that the competitive element is real and not due to K_m not being equal to K_s, but including *e.g.* k_2 in scheme 1 in the case of ACh. Competition with ACh suggests reaction of the inhibitor with the free enzyme at a substrate binding site, *e.g.* the anionic site. Such an interpretation is also suggested by the reduced fluoride inhibition in the presence of excess of substrate, especially noted with BuChE—BzCh, and by the experimentally observed less than additive inhibition in the presence of the two inhibitors trimethylammonium bromide and sodium fluoride. However, at pH 7.4 inhibition with MAEA as substrate should in such a case also contain a competitive element. This is not so, and consequently a reaction between fluoride and the dissociated anionic site is improbable and is also unlikely, as both carry a negative charge. Inhibition of any other currently discussed substrate binding enzyme group should also result in the same type of inhibition in the presence of either ACh or MAEA. Therefore, explanation is probably found elsewhere. Possibly fluoride inhibition of AChE-ACh results in an impaired fit between enzyme and substrate. It has been shown that a second methyl group at the nitrogen atom of MAEA contributes substantially to the binding of the substrate [17] and that the third methyl group drastically lowers the entropy change accompanying the binding of the substrate to the enzyme.[23] Obviously ACh and AChE can arrange themselves in a very suitable position towards each other and the methyl groups at the quaternary nitrogen of the substrate play then an important role. The presence of fluoride in the reaction medium could result in interference with the formation of van der Waals forces, or with the formation of a hydrophobic enzyme surface,[28] suitable for substrate binding. This would perhaps explain the observed higher degree of BuChE inhibition obtained in the presence of substrates carrying long (BuCh) or bulky (BzCh) acyl groups. However, from the experimental results it can be seen that one mole of fluoride is bound by one mole of active site, an observation suggesting specific binding of fluoride to the enzyme. This binding seems somehow to reduce the space available for the substrate.

Fluoride thus seems to inhibit ChE in a specific way. Among the proposed catalytically active enzyme groups the acidic group is supposed to have electrophilic properties. In AChE this group is supposed to form a hydrogen bond with the ether oxygen of the substrate, [32] a bond that probably cannot be formed when sulphur in the substrate is substituted for oxygen.[35] Fluoride bound to the acidic group should prevent the formation of such a hydrogen bond. Inhibition should then be noticeable when ACh is the substrate but not when AThCh is the substrate. However, inhibition was observed in the presence of both substrates. Thus either fluoride is not bound to the acidic group or the mechanism of action proposed for this group is incorrect. The other catalytically active groups could form a hydrogen bond with fluoride, known to form such bonds. Some of them may even bind fluoride in their protonated form or else a hitherto unknown group binds fluoride. Binding to the catalytic centre of ChE should result in inhibition of either acylation or deacylation of the enzyme or in inhibition of both steps. The observed, largely noncom-

petitive inhibition of ACh splitting by AChE indicates interference with the deacylation of the enzyme.[36] On the basis of the nonlinear Arrhenius plot for AChE—ACh Wilson and Cabib [23] have suggested that at temperatures above approximately 20°C deacylation (k_3 in scheme 1) is the rate determining step for the splitting of ACh by this enzyme, while at lower temperature the acylation of the enzyme (k_2 in scheme 1) is rate determining. However, as shown in this paper plots of log v *versus* $1/T$ in the presence of fluoride result in a straight line. Thus the apparent energy of activation for this system seems to be independent of temperature, suggesting that in the presence of fluoride the same step in the reaction sequence is rate determining throughout the temperature range studied. Judging from the slope of the line, the step could be the acylation of the enzyme. In the reaction between AChE and MAEA, k_2 in scheme 1, thus the acylation of the enzyme, is supposed to be rate determining.[23] The noncompetitive inhibition by fluoride observed with this substrate may therefore indicate that acylation is inhibited due to a reaction of the inhibitor with the enzyme-substrate complex.[36]

Summarizing, the inhibition produced by sodium fluoride is reversible and largely noncompetitive. Quaternary ammonium compounds seem to compete with fluoride. Lowering of pH and temperature promotes inhibition. Interaction with a group in the active site, necessary for the acylation of the enzyme, seems indicated. Possibly deacylation is also disturbed.

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