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ACTION OF FLUORIDE ON CHOLINESTERASE—II.
IN VITRO REACTIVATION OF CHOLINESTERASES INHIBITED BY ORGANOPHOSPHOROUS COMPOUNDS

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Abstract—*In vitro* reactivation of phosphorylated human blood cholinesterases by sodium fluoride was studied. The initial rate of reactivation of Sarin inhibited enzyme was found to be proportional to the concentration of sodium fluoride. The reactivation of Sarin inhibited blood cholinesterases increases with temperature and also when the pH is lowered. Reactivation of Sarin inhibited purified human serum butyrylcholinesterase occurs in two steps with different rates of reactivation. Sarin inhibited crude plasma butyrylcholinesterase is reactivated faster than enzyme inhibited by the thiocholine analogue of Sarin, methyl-isopropoxy-phosphoryl thiocholine iodide. Purified human serum butyrylcholinesterase and human erythrocyte acetylcholinesterase did not show this difference in the rate of reactivation.

On a molar basis at pH 7·4 and 37° sodium fluoride was found to be less effective than P2S as a reactivator of Sarin inhibited blood cholinesterases and less effective than Toxogonin or TMB-4 as a reactivator of Tabun inhibited blood cholinesterases. Soman inhibited enzyme could not be reactivated. The mechanism of the reactivation is discussed.

SARIN inhibited blood cholinesterases are, as previously communicated,[1] reactivated by sodium fluoride. The present paper reports results from a further study of this observation. Besides methyl-isopropoxy-phosphoryl fluoride (Sarin) the anticholinesterases methyl-isopropoxy-phosphoryl thiocholine iodide (^{37}S-N^+), dimethylamido-ethoxy-phosphoryl cyanide (Tabun) and methyl-pinacolyloxy-phosphoryl fluoride (Soman) have been included in the experiments.

MATERIALS AND METHODS

Enzymes, substrates, inhibitors and buffer solution

Acetylcholinesterase (AChE) from human erythrocytes and butyrylcholinesterase (BuChE) from human plasma or serum were studied with $7{\cdot}3 \times 10^{-3}$ M acetylcholine iodide (ACh) as substrate. Inhibitors and buffers have been described in previous papers.[2, 3]

Reactivators

Sodium fluoride (NaF) was of p.a. quality as described previously.[3] A preparation of potassium fluoride (Mallinckrodt Chem. Works, St Louis) containing 0·05% free acid (HF), 0·003% heavy metals, 0·05% potassium fluosilicate (K_2SiF_6), was used. N-methylpyridinium-2-aldoxime methane sulphonate (P2S) and N,N′-trimethylene bis (pyridinium-4-aldoxime) dibromide (TMB-4) were synthesized in this laboratory by I. Enander according to Creasy and Green[4] and Poziomek *et al.*[5] Bis (4-hydroxy-iminomethyl-pyridinium (1) methyl)-ether dichloride (Toxogonin) was commercial. Fresh solutions of the compounds were prepared each day.

Inhibition of enzyme, test for free inhibitor, determination of enzyme activities, reactivation and ageing

The methods have been described earlier.[2] In the present paper some variations in the concentration of the inhibitors and the time of inhibition have been introduced. As previously[2] either the automatic recording pH-titrator or the electrometric method was used. Reactivation in unbuffered solutions was studied with the aid of an extra incubation vessel connected with the titrator. Before the enzyme activity of each step was determined, the enzyme-reactivator incubation solution was diluted 1:20. When not otherwise stated, measurements of enzyme activities carried out with the titrator were undertaken at pH 7·4 and 25° or 37°. Temperature dependence of reactivation was studied at pH 7·4. Calculations of rates of reactivation or of percentage of reactivations have been described earlier.[2] When necessary corrections for the spontaneous hydrolysis of the substrate were made. When reactivation is given in percent, $E\infty$, as previously the total inhibited enzyme concentration available for reactivation, is equal to 100 and E_t, the concentration of the reactivated enzyme after time t, is expressed in percent of $E\infty$. Also as previously[2]

$$k' = \frac{k_{obs}}{[A]}$$

where $[A]$ is the total concentration of fluoride.

RESULTS

Rate of reactivation and influence of concentration of fluoride

In these experiments Sarin inhibited human plasma or Sarin inhibited human serum fraction IV-6-3, free from excess of inhibitor after two days of dialysis against saline at +4°, or Sarin inhibited human erythrocytes, free from excess of inhibitor after 5 washings with saline, were incubated either with sodium fluoride or with potassium fluoride at pH 7·4 and 37° or 25°. ChE activities were measured with the constant pH titrator. A plot of log (100 − % reactivation) against time (Fig. 1) shows that under

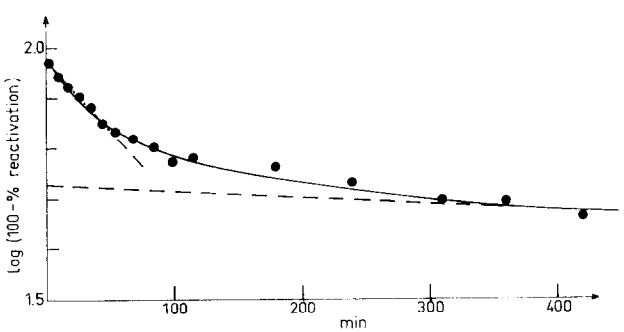

FIG. 1. Reactivation at pH 7·4, 25° of Sarin inhibited BuChE in human serum fraction IV-6-3 with 5×10^{-3}M NaF.

the conditions used reactivation of Sarin inhibited BuChE from serum fraction IV-6-3 begins as a first order reaction with respect to the concentration of the inhibited enzyme. This stage of reactivation is followed by slower reactivation with time. Both reactivation of Sarin inhibited human plasma BuChE and human erythrocyte AChE

increased with increasing fluoride concentration (Figs. 2 and 3). From the curves rate constants (k_{obs}) for the first stage of the reactivation of Sarin inhibited human plasma BuChE were evaluated. When k' was calculated it was seen that this value was constant. k' for AChE was found to vary more, but is still fairly constant. In this case the lines drawn through the experimentally obtained values meet the ordinate at log 94 instead of log 100 which means that the reactivation of the first 6 per cent of the enzyme is not accounted for. All rate constants are seen in Table 1.

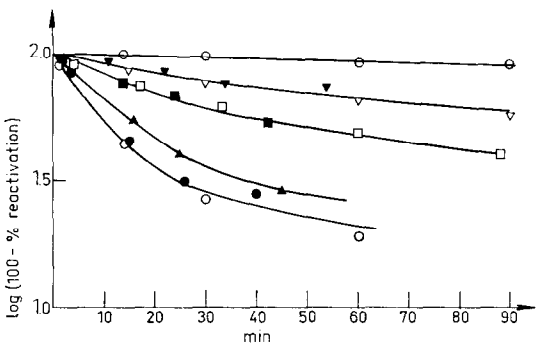

FIG. 2. Reactivation at pH 7·4, 37° of Sarin inhibited BuChE in human plasma. ●(○) = 3×10^{-3}M NaF (KF), ▲ = 2×10^{-3}M NaF, ■ (□) = 1×10^{-3}M NaF (KF), ▼ (▽) = 5×10^{-4}M NaF (KF), ⊖ = 1×10^{-4}M KF.

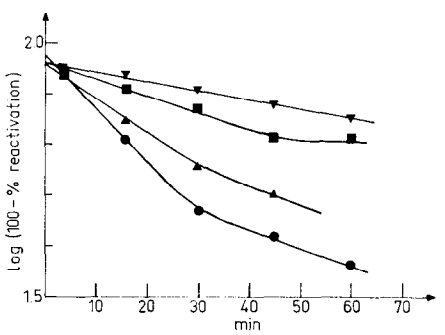

FIG. 3. Reactivation at pH 7·4, 25° of Sarin inhibited AchE in human erythrocytes. ● = 3×10^{-3}M NaF, ▲ = 2×10^{-3}M NaF, ■ = 1×10^{-3}M NaF, ▼ = 5×10^{-4}M NaF.

TABLE 1. RATE CONSTANTS FOR THE REACTIVATION WITH VARYING CONCENTRATIONS OF SODIUM FLUORIDE OF SARIN INHIBITED HUMAN PLASMA BuChE AT 37° AND pH 7·4 AND OF SARIN INHIBITED HUMAN ERYTHROCYTE AChE AT 25° AND pH 7·4

Conc. of NaF (M)	Plasma		Erythrocytes	
	k_{obs} (min^{-1})	k' (l mol^{-1} min^{-1})	k_{obs} (min^{-1})	k' (l mol^{-1} min^{-1})
3×10^{-3}	0·0568	18·9	0·0245	8·17
2×10^{-3}	0·0368	18·4	0·0157	7·85
1×10^{-3}	0·0184	18·4	0·00767	7·67
$0{\cdot}5 \times 10^{-3}$	0·0092	18·4	0·00422	8·44

The rate of reactivation of Sarin inhibited human erythrocyte AChE at pH 7·4 and 37°, obtained with either 5×10^{-4} M NaF or 5×10^{-5} M P2S, was compared. k_{obs} for the NaF induced reactivation was found to be $1{\cdot}53 \times 10^{-2}$ min^{-1} and for the P2S induced reactivation $6{\cdot}90 \times 10^{-2}$ min^{-1}. Assuming that k' for the initial reactivation produced by NaF, as found above at 25° reasonably constant down to a concentration of 5×10^{-4} M, is a constant also at 37° and down to a concentration of 5×10^{-5} M NaF, k' would be 30·6 for NaF and 1380 for P2S. Thus initial reactivation of Sarin inhibited human erythrocyte AChE at pH 7·4 and 37° occurs 45 times faster with P2S than with NaF.

Further experiments made it clear that reactivation of crude plasma BuChE inhibited with Sarin differs from that of purified human serum fraction IV-6-3 BuChE. ^{37}S-N$^+$ is the thiocholine analogue of Sarin. This compound and Sarin ought to give the same type of inhibited enzyme, methyl-isopropoxy-phosphorylated cholinesterase, splitting off respectively thiocholine and fluoride during inhibition. It has been shown by Tammelin[6] that incubation at 25° of human erythrocyte AChE with a 10^{-6} M solution of the inhibitors results in inhibited enzymes, which are reactivated at the same rate when treated with $2{\cdot}5 \times 10^{-4}$ M 2-PAM. This experiment was confirmed and repeated with 5×10^{-3} M sodium fluoride as reactivator. As seen in Fig. 4

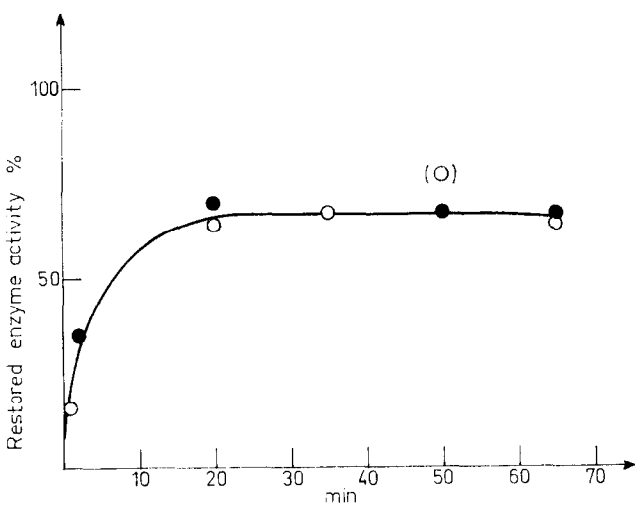

FIG. 4. Reactivation at pH 7·4, 37° of Sarin (●) or ^{37}S—N$^+$ (○) inhibited AChE in human erythrocytes with 5×10^{-3}M NaF.

the same rate of reactivation after inhibition with either Sarin or ^{37}S-N$^+$ was obtained also with this reactivator. A different result is obtained after inhibition of human plasma BuChE with either Sarin or ^{37}S-N$^+$. At pH 7·4 and 37° the rate of reactivation both with P2S and with NaF is dependent upon the time of contact between inhibitor and plasma and the concentration of the inhibitor during incubation in spite of absence of free inhibitor after dialysis of the inhibited plasma according to our test method and in spite of good reactivatability (96 per cent for ^{37}S-N$^+$ and 100 per cent for Sarin in ~18 hr with 0·01 M P2S), taken as criterium for the absence of aged enzyme. Plasma inhibited with Sarin was reactivated more rapidly than plasma

inhibited with ^{37}S-N^+ (Fig. 5). In some further experiments, using human serum fraction IV-6-3 BuChE, it was shown that in this case the rate of reactivation of Sarin or of ^{37}S-N^+ inhibited BuChE by sodium fluoride is equal (Fig. 6). Addition of a preparation of phosphorylphosphatase (human serum fraction IV-1) during the incubation of BuChE from human serum fraction IV-6-3 with the inhibitors results in a small increase in the rate of reactivation of the Sarin inhibited enzyme (Fig. 6).

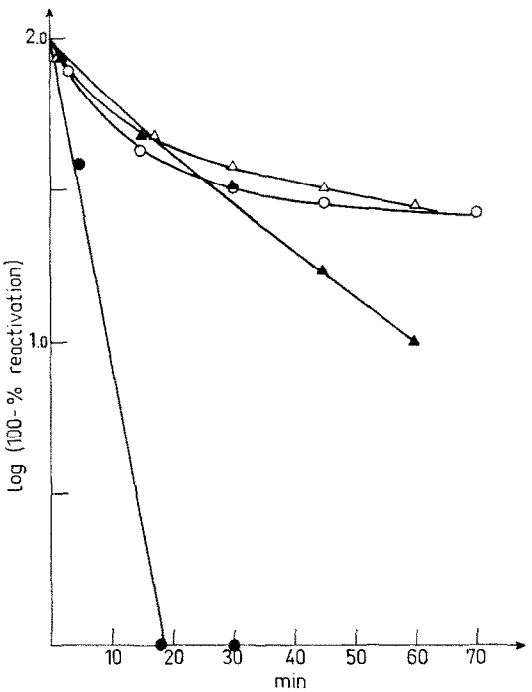

FIG. 5. Reactivation at pH 7·4, 37° of Sarin (●, ▲) or ^{37}S—N^+ (○, △) inhibited BuChE in human plasma. ●, ○ = reactivation with 5 × 10^{-3}M NaF, ▲, △ = reactivation with 1 × 10^{-4}M P2S.

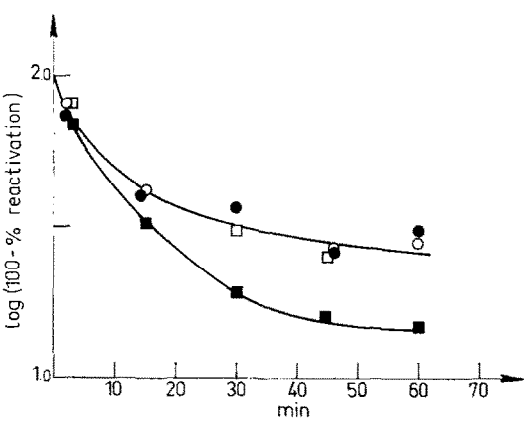

FIG. 6. Reactivation at pH 7·4, 37° of Sarin (●, ■) or ^{37}S—N^+ (○, □) inhibited BuChE in human serum fraction IV-6-3 with 5 × 10^{-3}M NaF. ● (○) = only ChE preparation present during inhibition, ■ (□) = human serum fraction IV-1 (phosphorylphosphatase) added during inhibition. Inhibition for 2 hr at 0° with 10^{-5}M Sarin, followed by dialysis.

Influence of pH

Sarin inhibited human plasma BuChE, completely reactivatable with 10^{-2} M P2S, was reactivated with sodium fluoride at pH 5·5–9·5 and 25°. No reactivation was observed at pH 9·5. The rate of reactivation increased with falling pH, showing a plateau at pH 7·5–7·0 (Fig. 7). Also initial reactivation of Sarin inhibited human erythrocyte AChE with sodium fluoride increases as the pH is lowered. Here the decrease in the rate of reactivation with time at each pH was noticed to be more marked as the pH was lowered (Fig. 8). Controls incubated at each pH with the same amount of sodium fluoride as the Sarin inhibited preparations showed no loss of enzyme activity with time.

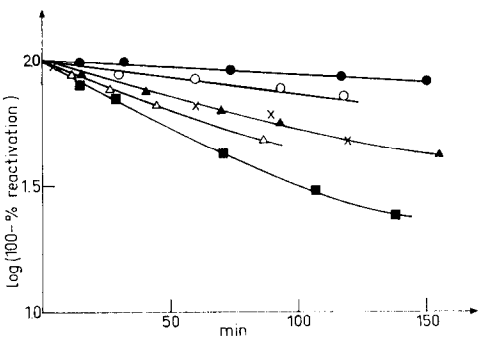

FIG. 7. pH-dependence of reactivation at 25° of Sarin inhibited BuChE in human plasma with 1×10^{-3}M NaF. ● = pH 8·5, ○ = pH 8·0, × = pH 7·5, ▲ = pH 7·0, △ = pH 6·5, ■ = pH 6·0.

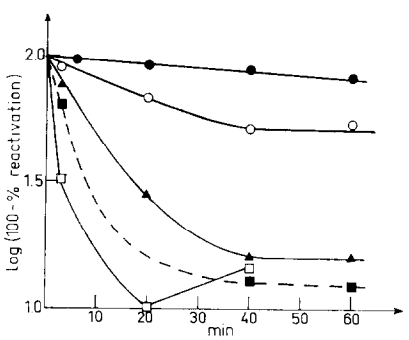

FIG. 8. pH-dependence of reactivation at 25° of Sarin inhibited AChE in human erythrocytes with 5×10^{-3}M NaF. ● = pH 9·0, ○ = pH 8·0, ▲ = pH 7·0, ■ = pH 6·0, □ = pH 5·0.

Influence of other compounds upon fluoride induced reactivation

For these experiments Sarin inhibited plasma BuChE was dialysed against distilled water and diluted with distilled water. Potassium chloride (titrator) was omitted during reactivation, but was present when the enzyme activities were determined. Table 2 shows that reactivation by 5×10^{-3} M sodium fluoride is very little influenced by cations. Choline bromide and trimethylammonium bromide speed up the reactivation by a few per cent, possibly due to their own reactivating properties.[7] They may also protect somewhat against ageing occurring during the course of reactivation. Bromide ions have little effect. Fluoride is known easily to form complexes, e.g. with iron ions[8].

As the presence of metal ions could not be completely avoided, EDTA, a metal complexing substance, was added in one experiment and found to have no appreciable effect. Also the rate of fluoride induced reactivation of Sarin inhibited erythrocyte AChE in the presence of ferric chloride was measured. Ferric chloride had no effect upon the rates of reactivation.

TABLE 2. THE INFLUENCE OF SOME COMPOUNDS UPON REACTIVATION OF SARIN INHIBITED HUMAN PLASMA BUCHE BY SODIUM FLUORIDE. CONCENTRATION OF ALL COMPOUNDS INCLUDING FLUORIDE 5×10^{-3} M. REACTIVATION IN % OF CONTROL WITH THE SAME SALTS ADDED

Time in min	—	% reactivation obtained with sodium fluoride and NaBr	EDTA	NH_4Br	trimethyl ammonium bromide	choline bromide
1–2	4	13	—	—	—	—
5	—	—	20	—	—	—
10–11	—	—	—	33	56	45
14–16	31	38	39	—	—	—
20–22	—	—	—	47	63	54
30	47	58	51	54	65	65
45	54	65	57	58	71	67
60	59	69	63	60	73	65
hours						
21–29	74	69	72	70	77	83

Finally the presence of 10^{-3} M sodium fluoride during reactivation of Sarin inhibited human plasma BuChE at pH 7·4 and 37° with 10^{-4} M P2S in 0·1 M potassium chloride was found to have practically no effect upon the rate of reactivation observed with P2S only (Table 3).

TABLE 3. THE INFLUENCE OF SODIUM FLUORIDE UPON REACTIVATION OF SARIN INHIBITED HUMAN PLASMA BUCHE BY P2S. REACTIVATION IN % OF CONTROL WITH THE SAME SALTS ADDED. INHIBITION WITH 10^{-5} M SARIN FOR 1 HR AT 0°

Time in min	% reactivation obtained with 10^{-4}M P2S and —	10^{-3}M NaF
1	21	22
15	60	63
29	75	80
44	83	86
57	—	92
170	90	89

Influence of temperature

Sarin inhibited human plasma BuChE was dialysed for 2 days. Reactivation with 1×10^{-3} M sodium fluoride was carried out in a thermostat at 15°, 25° and 35° in veronal buffer pH 7·4. As seen in Table 4 the rate of fluoride induced reactivation increases with the temperature.

TABLE 4. REACTIVATION WITH 1×10^{-3} M NaF IN VERONAL BUFFER pH 7·4 OF SARIN INHIBITED PLASMA BuChE AT VARYING TEMPERATURE

Temp. (°C)	% reactivated enzyme as function of time (hr) 0	3	6	21
15	10	38	58	83
25	10	62	78	94
35	10	76	87	94

Reactivation of ChE inhibited by Tabun or Soman

After inhibition of serum fraction IV-6-3 BuChE with 10^{-6} M Tabun for 1 hr at 0° followed by dialysis, or of human erythrocytes inhibited for 30 min, followed by washing, the inhibited enzyme could be reactivated both with 2×10^{-2} M sodium fluoride and with 10^{-2} M TMB-4 (Fig. 9). After 21 hr TMB-4 had reactivated 73 per cent, while fluoride had reactivated 71 per cent of the BuChE activity. Neither serum fraction IV-6-3 BuChE, inhibited with Soman for 1 hr and subsequently dialysed for 2 days at 0°, nor human erythrocyte AChE inhibited with Soman for 10 min and subsequently washed, could be reactivated with either 10^{-2} M sodium fluoride, 10^{-2} M P2S or 10^{-2} M Toxogonin.

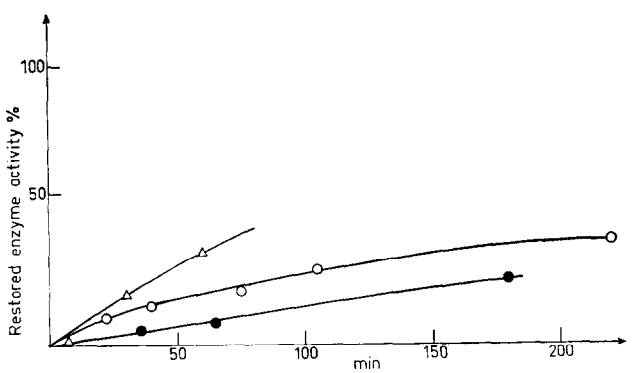

FIG. 9. Reactivation at pH 7·4, 25° of Tabun inhibited BuChE in human serum fraction IV-6-3 (○, ●) and AChE in human erythrocytes (△). ○ = 1×10^{-2}M TMB-4, △, ● = 2×10^{-2}M NaF.

DISCUSSION

It has been shown in a previous paper[3] that fluoride is a reversible inhibitor of ChE with a K_i of 0·4–2·8 $\times 10^{-3}$ M. This is a property fluoride has in common with well-known reactivators of phosphorylated ChE, the oximes, and demonstrates attraction to the enzyme. Oximes seem to be bound mainly to the nucleophilic anionic center of the enzyme and it has been shown that this bond is of importance for their activity as reactivators. Thus their tertiary analogues are less potent, especially when re-activating phosphorylated AChE.[9] Also the decrease of the rate of reactivation with decreasing pH is steeper for reactivators possessing a quaternary nitrogen group than for compounds lacking this group, where the pH-dependence at the acid side of pH 8 only seems to mirror the protonisation of the reactivating oxime group. The steeper

curve for the quaternary compound is explained with continuous protonisation of the anionic center ($pk_a \sim 6{\cdot}3$) of the enzyme as the pH is lowered, resulting in impaired attraction of the quaternary oxime to the enzyme. It has been suggested[3] that the fluoride ion, which was found to be a largely noncompetitive inhibitor of ChE, and the small amounts of HF and HF_2^-, present in the solution in the pH-range studied, is attracted to and bound by groups in the active center of the enzyme. Such bonds may be of help in the reactivation of the phosphorylated enzyme by fluoride.

In the experiments described in this paper an increase in the rate of fluoride induced reactivation with decreasing pH was observed, which parallels the earlier observed increase of fluoride inhibition of ChE with pH.[3] The obvious explanation is that HF ($K_1 = 6{\cdot}8 \times 10^{-4}$ M at 25°)[8] and/or HF_2^- is (are) the (main) reactivator(s), a theory somewhat strengthened by an observation of Thomas,[10] that Sarin binds hydrogen fluoride by dipole–dipole association. On the other hand, F^- occurs in an overwhelmingly higher concentration in the solution. If the fluoride ion is the reactivator, competition with hydroxylic ions at higher pH could disturb reactivation resulting in an increase of the rate of reactivation with decreasing pH. Furthermore the rate of reactivation could be influenced by the anionic center, if it is assumed that the approaching fluoride ion to a certain extent is repelled by the anionic center, as long as this center is dissociated. Probably also the s.c. acid group[9] (pk_a 9·5) in the enzyme has to be protonized for fluoride induced reactivation as for oxime induced reactivation, perhaps this also facilitates the attraction of the fluoride ion to the enzyme. This may explain why reactivation was not measurable above pH 9.

The experiments show that in the concentration range studied the initial rate of fluoride induced ChE reactivation is proportional to the concentration of the reactivator. This is in contrast to reactivation by oximes, where the above mentioned formation of a complex with the anionic site of the phosphorylated enzyme seems to be the rate determining step.[2, 11] Thus, fluoride is either not attached to the phosphorylated enzyme prior to the dephosphorylation of the enzyme or the formation of a complex between inhibited enzyme and fluoride is very rapid. Fluoride has been shown to be rather reactive towards phosphoryl groups.[12, 13] Assuming that the fluoride ion is the reactivator, it seems that a direct nucleophilic attack is made at the phosphorus atom of the phosphorylated enzyme, perhaps resulting in a transphosphorylation between enzyme and fluoride. In this case an anticholinesterase may be formed and eventually later be hydrolyzed by water. Such a reaction could explain the observed decrease in reactivation after about one hour of reactivation, more noticeable with increasing fluoride concentration. Rapid reactivation was, however, mostly followed by a further, slower reactivation. If a phosphoryl fluoride is formed more rapidly than it is hydrolyzed, reactivation should reach an equilibrium due to reinhibition of the enzyme. When discussing the shape of the reactivation curves, it has also to be kept in mind that the organophosphorus compounds used produce optical isomers of phosphorylated ChE. Reactivation of Sarin inhibited BuChE from human serum fraction IV-6-3 was found to occur in two steps with different rates of reactivation. This is in accordance with an observation by Behrends[14] on the 2-PAM induced reactivation of Sarin inhibited purified horse serum BuChE. Behrends has suggested that his curve represents the reactivation of the two optical isomers of Sarin inhibited BuChE, which seem to be reactivated at different rates. Though fluoride is a smaller compound than 2-PAM, steric differences in the phosphorylated enzyme may well

influence its effect. An eventual reinhibition may in such a case be preceded by racemisation of the detached organophosphorus residue.

Fluoride was found to be about nine times less active as reactivator of Sarin inhibited human plasma BuChE and about 45 times less active as reactivator of Sarin inhibited human erythrocyte AChE than an equimolar concentration of P2S, when initial rates of reactivation were compared at pH 7·4 and 37°. This shows that fluoride, which has no help of any cationic group and cannot be supposed to fit the enzyme surface particularly well, is a rather good reactivating group in itself, maybe better than the oxime group, whose effect has been shown by e.g. Wilson to be very dependent upon the complimentarines between enzyme and reactivator.[15] Neither fluoride nor oximes are, however, able to reactivate Soman inhibited ChE, probably due to rapid 'ageing' of ChE inhibited by this compound. Tabun inhibited ChE is reactivated by fluoride at a slow rate. AChE or purified BuChE inhibited by compounds which give the same phosphorylated enzyme (Sarin and ^{37}S-N[1]) were, as expected, reactivated at the same rate by fluoride.

During the study of fluoride induced reactivation it was observed that the rate of reactivation of Sarin inhibited crude plasma BuChE with either oximes or sodium fluoride is dependent upon the time of contact between inhibitor and enzyme and the concentration of the inhibitor during inhibition even when ageing seems not to have occurred. As neither reactivation of human erythrocyte AChE nor of purified serum BuChE was found to be sensitive to time or concentration of inhibitor, the explanation has to be sought in the crude plasma. It is known that phosphorylphosphatase destroys one isomer of optically active organophosphorus compounds more rapidly than the other one.[16] It has further been shown that in the case of Sarin the remaining isomer is the better ChE inhibitor.[16] Several possibilities exist; phosphorylphosphatase may aid in reactivation of the inhibited BuChE, as indicated by experiments with Tabun performed by Augustinsson.[17, 18] Possibly crude plasma BuChE is inhibited by only one optical active isomer, the one that is reactivated quicker. This is indicated by the difference in the reactivation after inhibition with ^{37}S-N[1] or Sarin, as ^{37}S-N[1] is not destroyed by phosphorylphosphatase.[6] On the other hand Hobbiger[7] has shown that human diethyl- or diisopropyl-phosphoryl BuChE is reactivated about 5 times quicker by nicotinehydroxamic acid methiodide than the corresponding types of phosphorylated AChE, a difference that was not found by Wilson[7] using human serum fraction IV-6-3. The authors, however, used different media for their studies. Neither diethyl- not diisopropyl-phosphoryl BuChE should be able to occur as optical isomers, but phosphorylphosphatase still may function as reactivator. Anomalies during inhibition with organophosphorus compounds and during reactivation of the phosphorylated BuChE occur frequently with crude human plasma and have been pointed out previously.[2]

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