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IN VITRO STUDIES ON THE REACTIVATION BY OXIMES OF PHOSPHYLATED ACETYLCHOLINESTERASE—I

ON THE REACTIONS OF P2S WITH VARIOUS ORGANOPHOSPHATES AND THE PROPERTIES OF THE RESULTANT PHOSPHYLATED OXIMES

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Abstract—The rates of formation and decomposition of a series of phosphylated oximes derived from P2S (2-hydroxyiminomethyl-1-methylpyridinium methane-sulphonate) have been measured. The rates of inhibition of AChE by these phosphylated oximes and the parent (and related) organosphosphates have also been measured.

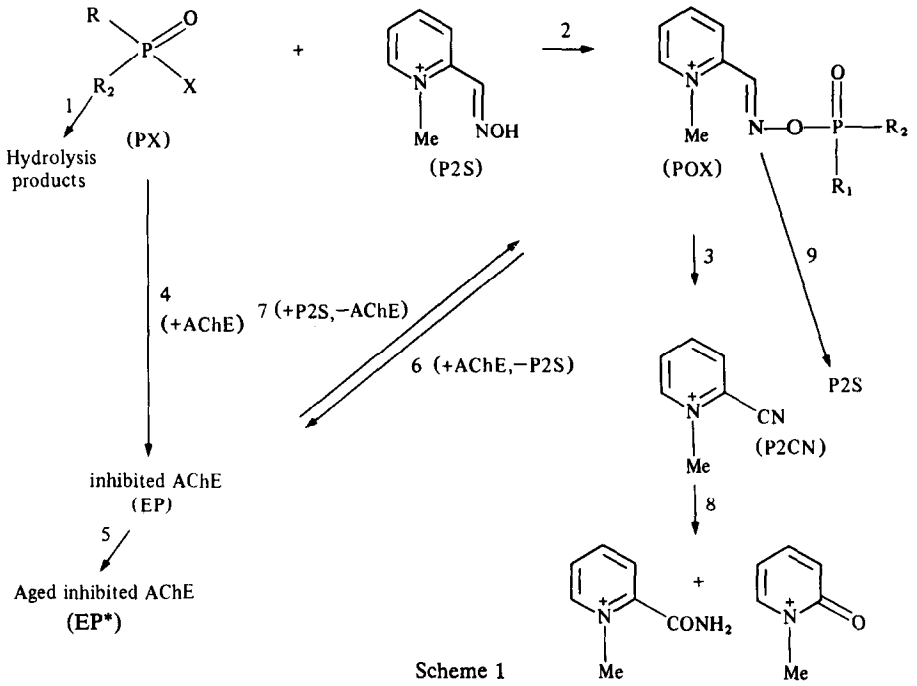
Possession of these rate data now permits a detailed analysis of the reactivation of phosphylated AChE by P2S to be made (see following paper).

Although oximes have been used in the treatment of organophosphate poisoning for many years there is no detailed comprehensive picture of their mode of action. In particular no correlation has been found between the *in vivo* potency of an oxime and the ability of that oxime to reactivate inhibited acetylcholinesterase (AChE). Reactivation *in vivo* is a complex process which depends upon the balance of a number of competing reactions; if the roles of oximes in the treatment of organophosphate poisoning are to be understood and if replacements for the oximes in current use are to be sought on other than an *ad hoc* basis, it is essential as a first step that the reactivation of inhibited AChE by oximes be understood in an *in vitro* system.

The reactivation of inhibited AChE by oximes and associated reactions have been investigated by many workers [1–5] over the past few decades but although there is a general consensus as to the nature of the main reactions, little agreement can be found in the detail of the reactions and in the quantification of the various rate processes.

The reactions that can occur between an organophosphate (PX), acetycholinesterase (AChE) and an oxime (P2S) are shown in Scheme 1.

Obviously such a complex scheme cannot usefully be investigated under conditions where all reactions are occurring: some breakdown of the system into simpler units for investigation is necessary. This can be arranged by appropriate selection of reactant



Scheme 1

concentrations or omission of reactants. This paper will deal with reactions 1, 2, 3, 4, 5, 6, 8 and 9, i.e. all reactions other than reactivation or ageing. The following paper will consider reaction 7 and to a lesser extent reaction 5 using the information gained in this present paper.

EXPERIMENTAL

Materials. Tabun (**V**) and the fluoridates (**I, II** (sarin), **III** (soman), **IV, VI**)* were synthesized by the standard techniques [6] (NB care must be taken in their preparation and handling due to their high toxicity) and were ≥98% pure as calculated from the stoichiometry of their hydrolysis (glass and specific ion electrode determination). **VII** and racemic **VIII, IX, X, XI** and **XII** were prepared from the appropriate thioacids and alkyl or dialkylaminoethyl halides [7]. The enantiomers were prepared similarly but using optically active thioacids [8]. Optical purities were checked by the NMR method using a chiral shift reagent [9] or by stereospecific synthesis [10].

P2S was recrystallised from ethanol and was >99% pure by potentiometric titration.

Bovine erythrocyte AChE (EC 3.1.1.7) was ex Sigma Chemicals.

P2CN (2-cyanomethylpyridinium methan-sulphonate) was prepared by heating a solution of 2-cyano-pyridine (13 g) and methyl methane–sulphonate (25 g) in toluene (39 ml) under reflux for 2 hr. The crude product was recrystallised from ethanol and its structure shown to be consistent with that expected using the usual spectroscopic techniques.

Apparatus. The apparatus for the non-enzymic and enzyme kinetic experiments has been described in refs 11 and 12 respectively. In addition a cyanide specific ion electrode (Philips type IS-5501) was used.

Analogue computer simulations were carried out using either a Solartron HS7-1 or HS7-3A analogue computer coupled with a Bryans 28000 XY A3 pen recorder.

Preparation of solutions. Organophosphates were dissolved in propan-2-ol (AR grade) and used throughout the day. Fresh solutions were made each day. P2S was dissolved in water and used throughout a week. Acetylcholinesterase and acetylcholine iodide (in water) were used throughout a week and stored in the fridge when not in use.

Kinetic methods. All reactions were conducted in water at 37° with an I of 0.10 (NaCl).

The rates of all the non-enzymic reactions were monitored by following hydrogen ion production using the pH stat and the appropriate specific ion electrode (fluoride or cyanide). Reactions 1 and 8 were studied in isolation from the other non-enzymic reactions. This facilitated analysis of the data from reaction mixtures in which reactions 1, 2, 3 and 8 were occurring.

Concentrations of reactants (other than hydrogen ion) were usually 10^{-3} M.

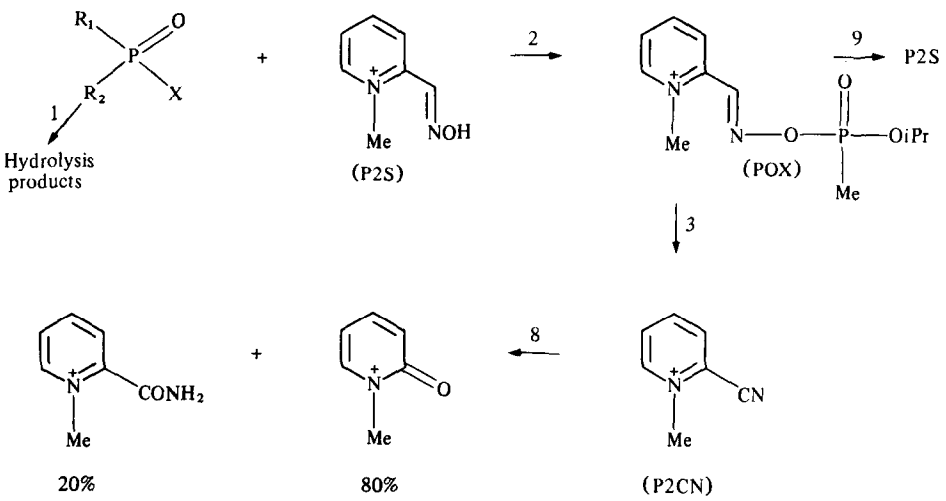
Rates of inhibition of AChE (0.1 IU/ml = 1.68×10^{-10} M) in the presence of substrate (acetylcholine iodide 2.5×10^{-4} M = 2.5 K_m) were determined as described previously [12]. Rates of inhibition of AChE by POX were determined similarly but inhibition was initiated by addition of aliquots (usually 1 or 10 μl) removed at various times from the reaction mixtures and used to determine the rate of breakdown of POX.

Analysis of kinetic data. First order rate coefficients (where appropriate) were obtained from the usual logarithmic plots. All other rate coefficients were obtained by manually matching the output of the analogue computer programmed to solve the differential equations describing the particular kinetic model being investigated with the experimental data on an X–Y recorder. The various differential equations used in analysis are shown in the Appendix.

RESULTS

The reactions of PX with water and P2S (Reactions 1, 2, 3, 8 and 9)

* For the structures of these and the other organophosphates used see Tables 1 and 2.



The rates of hydrolysis (by OH^-) of the organophosphates in the absence of oxime (reaction 1) were measured within the pH range 8.6–10.4 by a combination of pH stat and specific ion electrode techniques. Second order rate coefficients ($^2k_1$) and the first order rate coefficients ($^1k_1$) calculated for pH 7.40 are given in Table 1. Contribution to the overall rate of hydrolysis by H_2O was not significant.

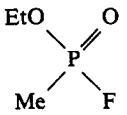
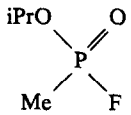
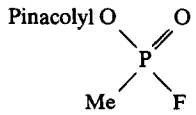
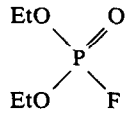
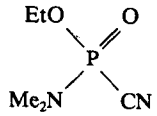
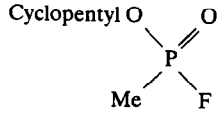
The hydrolysis of the nitrile (P2CN) was studied independently over the pH range 9.0–9.7, and was found to be hydroxide ion catalysed with a second order rate constant ($^2k_8$) of 290 M^{-1} sec^{-1} ($^1k_8$ for pH 7.40 being 1.7×10^{-1} sec^{-1}). A detailed study of this reaction was not made as the rate was slow and only marginally interfered with the determination of the rates of reactions 2 and 3. The products of the breakdown were not isolated but deduced by analogy [13], and by hydrogen ion and cyanide ion stoichiometries.

Due to its instability POX cannot be isolated and therefore its kinetic properties can only be determined indirectly by a kinetic analysis of the reaction between PX and P2S, and with knowledge of the values of $^1k_1$ and $^1k_8$ discussed above. From an analysis (using potentiometric titration and specific ion electrode techniques) of reaction mixtures of PX and P2S it was found that reaction 9 could not be detected and that the nitrile (P2CN) was the initial nonphosphorus breakdown product of POX; this reacted further to give the amide and the pyridone. The rate coefficients $^2k_2$ and $^2k_3$ were obtained by analysis of the pH stat and specific ion electrode data from the PX-P2S reactions using analogue computing techniques, and are shown in Table 1.

The inhibition of AChE by PX (Reaction 4)

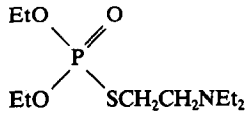
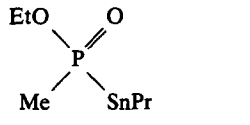
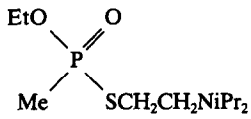
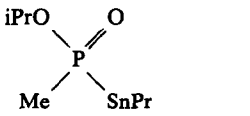
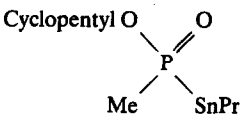
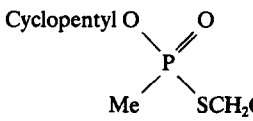
The rate coefficients ($^2k_4$) for inhibition of AChE by various organophosphates in the presence of substrate (ACh) are given in Table 2. All of the rates were measured under first order conditions, i.e. the inhibitor was in vast molar excess, and are not corrected for substrate protection. The reason for the study of some of these compounds will only become apparent in Part II of this series which will deal with reactivation of EP. However, it is convenient to

Table 1. Rate coefficients for the reactions of PX with water and P2S ($I = 0.1$ with NaCl, $T = 37°$)

PF		Reaction 1		Reaction 2	Reaction 3	
		$^2k_1$	$^1k_1 \times 10^6$	$^2k_2$	$^2k_3 \times 10^{-4}$	$^1k_3 \times 10^2$
	I	125 (±3)	75	22 (±1)	1.8	1.1 (±0.1)
	II	71 (±2)	43	8.0 (±0.7)	3.5	2.1 (±0.3)
	III	30 (±1)	18	3.0 (±0.4)	2.8	1.7 (±0.1)
	IV	5.8 (±0.4)	3.5	2.4 (±0.3)	4.5	2.7 (±0.3)
	V	22 (±0.6)	14	9.4 (±0.2)	2.8	1.7 (±0.1)
	VI	69 (±3)	41	7.3 (±0.5)	2.4	1.4 (±0.2)

Units of $^1k_1$ and $^1k_3$ are sec^{-1}.
Units of $^2k_1$, $^2k_2$ and $^2k_3$ are M^{-1} sec^{-1}.
$^1k_1$s are calculated for pH 7.40 ($[OH^-] = 6.0 \times 10^{-7}$ M).
The values shown are those calculated over a pH range.
Figures in brackets indicate upper and lower values.
$^2k_2$ values are those for oximate anion.

Table 2. Rate coefficients for the inhibition of AChE by various organophosphates in the presence of 2.5×10^{-4} M ACh

Organophosphate		$^2k_4$ M^{-1} sec^{-1}		
		racemate (*R*,*S*)	(*R*) enantiomer	(*S*) enantiomer
	I	$6.1\ (\pm0.5) \times 10^4$		
	II	$9.0\ (\pm0.2) \times 10^4$		
	III	$2.8\ (\pm0.2) \times 10^5$		
	IV	$3.1\ (\pm0.2) \times 10^3$		
	V	$3.1\ (\pm0.3) \times 10^4$		
	VI	$1.9\ (\pm0.5) \times 10^6$		
	VII	$1.6\ (\pm0.1) \times 10^4$		
	VIII	8.0 (±0.6)	1.0 (±0.1)	15.0
	IX	$3.2\ (\pm0.4) \times 10^5$	$4.6\ (\pm0.6) \times 10^3$	$6.7\ (\pm0.7) \times 10^5$
	X	2.7 (±0.3)	0.4	6.3 (±0.1)
	XI	180 (±12)	41	360
	XII	$1.0\ (\pm0.2) \times 10^6$	$1.6\ (\pm0.2) \times 10^3$	$1.7\ (\pm0.2) \times 10^6$

The results shown are mean values measured over a range of organophosphosphorus concentrations. Figures in brackets indicate upper and lower values.

include their rate coefficients of inhibition in the present section.*

The inhibition of AChE by POX (Reaction 6)

Obtaining the rates of inhibition of AChE by POX ($^2k_6$) is quite complex and in order to understand the results it is necessary to reiterate the experimental procedure.

P2S (10^{-3} M) and the PX (10^{-3} M) were allowed to react at pH 7.40 and the reaction profiles monitored using pH stat and ion selective electrode techniques. Using these profiles in conjunction with the previously determined values of $^2k_1$ and $^2k_8$ the concentration-time profile of POX in the reaction mixture was calculated.

Aliquots of this reaction mixture were taken at various times and injected into a reaction mixture containing AChE and ACh also being monitored in a pH stat. Inhibition of the enzyme occurred due to both the original PX and the corresponding POX. Analysis of this inhibition–time profile used analogue computing techniques to solve the appropriate differential equations. It required a knowledge of the initial concentrations of PX and POX in the aliquot of the first reaction mixture, the rate of inhibition of AChE by PX ($^2k_4$), the rate of breakdown of POX ($^2k_3$), and the initial concentration of enzyme $[E]_0$ in the second reaction mixture. Reactivation of EP by P2S did not occur in the second reaction mixture due to the large dilution (usually 10^3) of the aliquot. From a knowledge of these parameters and using the usual analogue computing techniques, $^2k_6$s for

* In studying the inhibitions by the various enantiomers in which each isomer in a pair can have very different activity, it is always a potential problem to ensure that the observed inhibition by the less active enantiomer is not due to traces of the more active enantiomer. In the case of the compounds with a relatively low ratio of activity between the two enantiomers the level of contamination of the less active enantiomer would have been discovered by the usual analytical techniques (see Experimental). With IX and XII where the more active enantiomer is very potent the necessary levels of contamination are too low for these techniques but could be determined and removed by incubation of the less active enantiomer with a concentration of AChE in excess of that calculated to be necessary to react with the more active enantiomer had all the observed inhibition been caused by it.

Table 3. Rate coefficients for the inhibition of AChE by POX in the presence of 2.5×10^{-4} M ACh

(A) POX formed from I: Initial AChE concentration 1.6×10^{-10} M

Time of aliquot (min)	[I] $\times 10^8$ M†	[POX] $\times 10^9$ M†	$^2k_6 \times 10^{-6}$ M^{-1} sec^{-1}
8	2.2	3.0	2.3
17	5.5	4.2	2.1
36	5.0	2.8	1.9
36.5	5.0	3.3	1.6
2	2.6	6.7	2.2
9	5.6	3.7	2.5
17	5.9	3.3	2.1
30	6.0	7.6	2.9
1	3.4	6.4	2.5
6	5.5	10.0	2.3
13	4.3	4.0	3.4
21.5	8.6	6.7	1.9
2	2.6	6.7	2.4
7.75	4.6	6.7	2.3
18	5.2	3.6	2.5
4	3.7	8.9	2.9
10	4.0	4.0	4.3
21.25	4.3	3.3	2.9
			Mean 2.5

* Results sometimes from more than one reaction.
† Concentration in enzyme solution immediately after addition of aliquot. Aliquot size may increase as time increases.

(B) POX formed from II: Initial AChE concentration 1.6×10^{-10} M

Time of aliquot (min)	[II] $\times 10^8$M	[POX] $\times 10^9$M	$^2k_6 \times 10^{-7}$ M^{-1} sec^{-1}
2	7.8	7.2	0.96
4	6.4	5.2	1.1
9	4.4	2.4	1.3
11	11.9	5.7	1.5
13.5	6.9	2.9	1.4
19	5.4	1.8	1.3
			Mean 1.3

(C) POX formed from III: Initial AChE concentration 2.4×10^{-10} M

Time of aliquot (min)	[II] $\times 10^8$M	[POX] $\times 10^9$M	$^2k_6 \times 10^{-6}$ M^{-1} sec^{-1}
2	9.0	4.0	5.1
4	8.3	3.8	6.2
8	7.0	2.8	5.9
12.75	5.9	2.0	7.0
19	5.0	1.4	7.1
27.5	4.0	0.90	6.5
40	3.1	0.55	9.1
			Mean 6.7

(*continued*)

Table 3 (*continued*)

(D) POX formed from **IV**: Initial AChE concentration 1.6×10^{-10} M

Time of aliquot (min)	[IV] $\times 10^6$ M	[POX] $\times 10^8$ M	$^2k_6 \times 10^{-5}$ M^{-1} sec^{-1}
1	5.8	13.2	1.5
2	5.5	14.7	1.3
4	5.1	13.5	1.2
8.5	4.4	9.9	1.0
11	4.0	8.4	1.1
14	3.7	7.2	1.1
22	3.0	4.8	1.5
			Mean 1.2

(E) POX formed from **VI**: Initial AChE concentration 1.6×10^{-10} M

Time of aliquot (min)	[VI] $\times 10^9$ M	[POX] $\times 10^{10}$ M	$^2k_6 \times 10^{-7}$ M^{-1} sec^{-1}
1.75	9.0	7.9	1.1
9	4.9	6.6	1.1
16	6.0	7.6	1.5
25	4.8	6.0	1.8
0.83	9.3	5.1	1.2
7	11.4	15.6	1.1
13.5	7.1	9.2	1.4
3.25	7.6	9.0	1.1
5	6.7	8.8	1.4
			Mean 1.3

individual aliquots can be determined. The results for the various PXs and POXs are given in Tables 3A–3E and are summarised in Table 4.

Under the reaction conditions used the initial concentration of PX in the enzyme solution was always in such large excess over $[E]_0$ in that first order inhibition of AChE by PX occurred. However, with the organophosphates **II**, **III** and **VI**, the initial concentration of POX was so low that it became comparable to $[E]_0$, i.e. second order reaction conditions obtained. This produced potential complications as when second order conditions obtain, kinetic analysis of the data requires (or produces) knowledge of the relative molar concentrations. In the present instance kinetic analysis of the data from the first reaction mixture of PX and P2S gives the concentration of POX at any instant. But when PX is chiral, i.e. is a mixture of enantiomers (or, in the case of soman, diastereoisomers) and it is likely that the POX enantiomers have different inhibitory potencies (cf. the enantiomeric pairs of organophosphates in Table 2) this concentration of POX may need to be reduced by a factor of 2 before it is used in the computer analysis of the inhibition profiles.* Good agreement between observed and computed inhibition profiles was observed even when the ratio of $[E]_0$ to POX concentration was high and this goodness of fit was not changed when this ratio was doubled by reducing the initial POX concentration in the computation by a factor of two, i.e. the limiting case when only one POX enantiomer is active. The complexity of the system is such that under the experimental conditions used (and these are limited by the techniques available) the system is insufficiently sensitive to allow separation of the inhibitory potencies of the POX enantiomers. Thus the values of $^2k_6$ reported are the values for racemic POX.

With **V** no inhibition by the corresponding POX was observed. This is due to its poor inhibitory potency not its low concentration.

* This is a limiting case although it is expected in practice to occur. The general case is for separate pathways for inhibition by each POX enantiomer to be included each with its own $^2k_6$ but with identical initial concentrations of POX equal to half that calculated for racemic POX in the racemic reaction mixture.

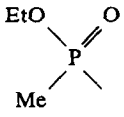
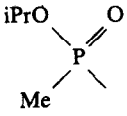
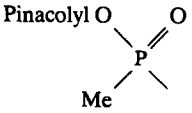
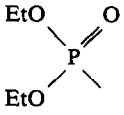
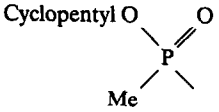
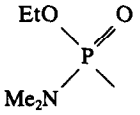
DISCUSSION

The reactions of PX with water and P2S

There is little correlation between the rates of fluoride (or cyanide) ion displacement by hydroxide ion and by P2S anion; the ratio of the two rate coefficients varying from 10 for **III** to 2.3 for **V**. This variation is sufficient to preclude agreement even between the rank orders for the two reactions.

The breakdown of the phosphylated oxime is, in all cases, by a hydroxide ion catalyzed elimination reaction to produce 2-cyano *N*-methyl pyridinium ion (P2CN). This is in partial agreement with the work of Blanch [14] except that he also claimed a general base (oximate anion) catalyzed elimination reaction (for the case when PX is II). Although

Table 4. Rate coefficients for the inhibition of AChe by various phosphylated P2Ss (POX) in the presence of 2.5×10^{-4} M ACh

Phosphorus moiety	$^2k_6$ M^{-1} sec^{-1}
	2.5×10^6
	1.3×10^7
	6.7×10^6
	1.2×10^5
	1.3×10^7
	Not measurable

Blanch found that the breakdown was completely by elimination he did not observe the subsequent reactions of P2CN. This breakdown by elimination is superficially in contrast to that found by van Hooidonk *et al.* [15] who studied a series of *O*-phosphylated pyridine-2-aldoximes. With the *O*-alkyl methylphosphonylated oximes they found beween 50 and 70% of the reaction was by nucleophilic substitution to regenerate oxime. However, the elimination reactions of the present phosphonylated quaternized oximes are ~10^4 times faster than that of their unquaternized analogues and although quaternization is expected to accelerate both elimination and substitution reactions, it is likely to affect elimination more than substitution due to the closer proximity of the quarternary nitrogen to the azomethine hydrogen than to the phosphorus moiety. A consequence of this reasoning is that breakdown via substitution is unlikely to be observed with the present compounds even though the phosphyl-oxime bond is labile.

Inhibition of AChE by POX

From a comparison of Tables 2 and 4 it can be seen that with the exception of the phosphoramido compounds the phosphylated oximes are between ×10 and ×100 more potent inhibitors of AChE than the corresponding fluoridates or thiocholine type analogues.

As the phosphylated oximes breakdown exclusively (within the limits of observation) by elimination and not by nucleophilic attack upon phosphorus, it is not possible to get a direct measure of the reactivity of the phosphyl-oxime bond. However, it is possible to estimate an upper limit for this reactivity by assuming that 1% of the breakdown could go via nucleophilic attack and not by elimination and would not be observed. This would then make the phosphorus centre up to *ca.* 100 times more electrophilic than the corresponding phosphorus centre in the fluoridates. This potentially greater reactivity than that of the fluoridates could account for the inhibitory potency of the phosphylated oximes but the possibility that the intrinsic reactivity is low and that the quaternary nitrogen serves a similar function to the nitrogen in the thiocholine type organophosphates cannot be excluded.

The inhibitor potency of the phosphylated oxime derived from **V** was not measurable and must have a low value as the rates of formation and breakdown of the phosphylated oxime are similar to those of the other phosphylated oximes. From a superficial comparison with the other compounds it can be concluded that the inhibitor potency of the phosphylated oxime derived from **V** is ≤ to that of **V**, i.e. $^2k_6 \leq 3 \times 10^4$ M^{-1} sec^{-1}.

For the phosphono compounds $^2k_6$ was between ×10 and ×100 greater than $^2k_4$. The fluorine analogue of **V**, i.e. *N,N*-dimethyl-*O*-ethyl phosphoramido fluoridate has a $^2k_4$ of ~80 M^{-1} sec^{-1} so if the analogy holds for the phosphoramido compound the $^2k_6$ for the phosphoramido oxime would be $<2 \times 10^3$ to 2×10^4 M^{-1} sec^{-1}. This is consistent with the estimated upper limit of 3×10^4 M^{-1} sec^{-1}.

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APPENDIX

The differential equations used to analyse Scheme 1 are shown below. Rate constants are numbered as per the reaction step.

$$-\frac{d[PX]}{dt} = {}^2k_1[PX][OH^-] + {}^2k_2[PX][P2S]$$

$$-\frac{d[P2S]}{dt} = {}^2k_2[PX][P2S]$$

$$\frac{d[POX]}{dt} = {}^2k_2[PX][P2S] - {}^2k_3[POX][OH^-]$$

$$\frac{d[P2CN]}{dt} = {}^2k_3[POX][OH^-] - {}^2k_8[P2CN][OH^-]$$

When aliquots from the reaction between PX and P2S were added to AChE the following equations were used:

$$-\frac{d[AChE]}{dt} = {}^2k_4[AChE][PX] + {}^2k_6[AChE][POX]$$

$$-\frac{d[POX]}{dt} = k_3[POX][OH^-] + {}^2k_6[AChE][POX]$$

${}^2k_6$ values thus obtained are for racemic POX.