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

ON THE FORMATION OF *O,O*-DIETHYL PHOSPHORYLATED AChE AND *O*-ETHYL METHYLPHOSPHONYLATED AChE AND THEIR REACTIVATION BY P2S

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Abstract—The *in vitro* reactivation profiles of *O,O*-diethyl phosphorylated ACh E and *O*-ethyl methyl phosphonylated AChE by P2S (2-hydroxy iminomethyl-1-methyl-pyridinium methane sulphonate) have been determined. Whilst re-inhibition of the reactivated AChE by phosphorylated oxime (POX) is not important in determining the reactivation profile of *O,O*-diethyl phosphorylated AChE, reinhibition of the reactivated AChE by phosphonylated oxime can, however, be important in determining the reactivation profile of *O*-ethyl methylphosphonylated AChE and the extent of this reinhibition is determined by the initial concentration of phosphonylated AChE.

Kinetic analysis of the reactivation profiles demonstrated that the generally accepted scheme for this reactivation process is incorrect and that phosphylated AChE cannot be considered as a single species although an adequate description of the present data is afforded by a model using a 1:1 mixture of two species each with its own rate of reactivation. In the case of *O,O*-diethyl phosphorylated AChE the main kinetic difference between these two species is found not in the formation or stability of the phosphorylated AChE-P2S complex but in its subsequent reaction.

From results with *O*-ethyl methylphosphonylated AChE prepared from two pairs of enantiomers as well as from the racemic fluoridate it was concluded that phosphonylation of AChE may not always occur via a mechanism involving inversion of configuration at phosphorus but can also occur with retention of configuration.

Reactivation by P2S of *O*-ethyl methylphosphonylated AChE prepared from (*S*) organophosphates proceeds with inversion of configuration at phosphorus. Inversion also occurs in the reinhibition of AChE by the POX produced in the initial reactivation.

To understand the importance of reactivation by oximes (OX) in the treatment of nerve agent poisoning an *in vitro* study of reactivation of phosphylated AChE (EP) by an oxime (OX) as shown in Scheme 1 was initiated (see Part I [1]). As stated elsewhere with such a complex scheme some breakdown into simpler units for investigation is necessary. This was done in Part I when all reactions other than 7 and 5 were evaluated.

$$EP + OX \underset{6}{\overset{7}{\rightleftarrows}} E + \text{phosphylated oxime (POX)}$$

$$\downarrow 5 \qquad\qquad \downarrow 3$$

aged phosphylated AChE (EP*) — breakdown products (for reactions 1, 2 and 4 see ref. 1)

Scheme 1

The majority of the work reported in the present paper was therefore designed to determine the rate of reactions 7 (and if appropriate 5) and to determine which of the reactions shown in Scheme 1 are likely to be important in *in vivo* systems. However, when analysis of the data was attempted it rapidly became apparent that Scheme 1 is inadequate in that it cannot quantitatively describe the reactivation processes. The purpose of the present paper is therefore to demonstrate the inadequacies of Scheme 1 and to modify and develop it so that a quantitative description of reactivation can be made. Two EPs have been used *O,O*-diethyl phosphorylated AChE and *O*-ethyl methylphosphonylated AChE and one oxime (P2S).

EXPERIMENTAL

*Summary of experimental procedures**

All reactions were at a pH of 7.40, $I = 0.10$ with NaCl, and at 37° (except for the preparation of EP which was done at 4°).

Preparation of EP. AChE (10 IU ml^{-1} = 1.68 × 10^{-8} M) was reacted with an excess of organophosphate. On completion of inhibition the excess of organophosphophate was removed by ultrafiltration.

Reactivation of EP in the absence of substrate. EP at initial concentrations ($[EP]_0$) of 10, 1 or 0.1 unit/ml was incubated with various concentrations of P2S. Aliquots were removed at various times and assayed for AChE activity using ACh as substrate.

Reactivation of EP in the presence of substrate. This is possible at $[EP]_0$ of 0.1 and 0.01 unit ml^{-1}. The substrate, ACh, was 2.5 × 10^{-4} M and AChE activity was thus assayed directly in the reactivation solution.

Materials and apparatus

These have been described previously [1].

* Because of the number and complexity of experimental procedures used this summary is given. It provides sufficient detail to allow an understanding of the main text.

Detailed experimental procedures

Preparation of inhibited AChEs (EP). A small volume (<1% of the volume of the AChE solution) of an isopropanol solution of the organophosphate was added to the AChE solution (10 IU ml^{-1}, I = 0.10 with NaCl, 5×10^{-3} M sodium phosphate buffer pH 7.40) and the resulting solution was allowed to react at *ca.* 4° for 15–30 min to give 100% inhibition. The concentration of organophosphate in the reacting solution was 10^{-7} M for the more active inhibitors and was increased as the inhibitory potency ($^2k_4$—see Ref. 1 Table 2) of the organophosphate decreased. On completion of inhibition excess inhibitor was removed by ultrafiltration through an XM50 membrane in an Amicon ultrafiltration cell using a pressure of 25–30 psi. The solution was reduced from 10 ml to 1 ml, made up to 10 ml with phosphate buffer and ultrafiltered again. This was repeated a further two times. Control experiments showed no loss of AChE activity was caused by the ultrafiltration procedure.

Kinetic procedures

All reactions were conducted in water at 37° with an I = 0.10 (NaCl) and pH of 7.40 unless otherwise stated. When reactivation occurred in the presence of substrate (acetylcholine iodide) and in enzyme assay solutions the substrate concentration was 2.5×10^{-4} M.

Before reactivation experiments commenced solutions of EP were checked for complete removal of excess inhibitor by the following technique: an aliquot (0.5 ml) of EP solution was mixed with an equal volume of free AChE solution and the activity of the resulting solution was assayed using the previously described pH stat technique [1]. This solution was then incubated for at least 2 hr and assayed again—no observable change in the rate of substrate hydrolysis was the criterion for complete absence of free inhibitor. Various procedures for measuring the rates of reactivation were used; the choice of procedure was determined by the initial concentration* of inhibited enzyme $[EP]_0$ in the reactivation solution and whether or not substrate was present.

$[EP]_0$ 10 and 1 units ml^{-1}. Reactivation experiments at these concentrations could only be done in the absence of substrate. P2S solution (previously adjusted to pH 7.40) was added to the EP solution (containing 5×10^{-3} M phosphate buffer pH 7.40) in a sufficient quantity (less than 1% of the volume of reaction solution) to give the required concentration of P2S (10^{-5}–10^{-3} M) and the resulting solution incubated at 37°. Aliquots of 0.15 ml were removed at various times and diluted immediately into 10 ml of 0.1 M NaCl. The resulting solution was assayed immediately as described previously.

$[EP]_0$ 0.1 unit ml^{-1}. In the absence of substrate the reactivations were conducted as for the higher EP concentrations except that 10 ml aliquots were used and the concentration of phosphate buffer was reduced to 2.5×10^{-4} M. Under these conditions reactivation could sometimes be observed in the assay solution and when this occurred only the initial rate was measured. In the presence of substrate reactivations were measured continuously in the assay solution.

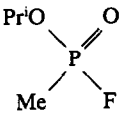
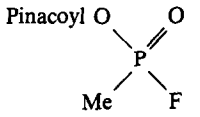
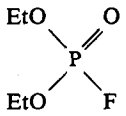
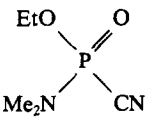
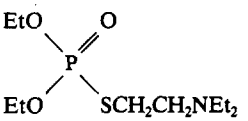
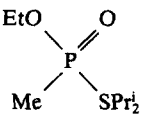
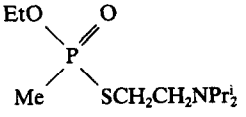
$[EP]_0$ 0.01 unit ml^{-1}. Reactivations at this concentration of EP could only be measured in the presence of substrate using the procedure given for an EP concentration of 0.1 unit ml^{-1} except that the solutions used in the Radiometer syringes were 10^{-3} M.

The rates of hydrolysis of substrate in the presence of the various concentrations of P2S arising from the reactivation experiment were measured using free AChE in order to obtain the rates of hydrolysis corresponding to 100% reactivation.

Analysis of kinetic data

Analysis of the reactivation profiles was done using analogue computing techniques. Analysis of profiles in which reincubation by POX was insignificant, and from which the scheme of a 50/50 ratio with two components was derived, was initially done using a least-squares curve fitting routine.

Table 1.

II	
III	
IV	
V	
VII	
VIII	
IX	

N.B. The above numbering is used in order to be consistent with Part I [1].

* The concentration of inhibited enzyme; 1 unit ml^{-1} of EP is that concentration of EP produced by inhibiting a solution of free AChE of concentration 1 IU ml^{-1}.

Table 2. Reactivation of *O,O*-diethylphosphorylated AChE by P_2S at various values of [P2S] and $[EP]_0$ at pH 7.4 and 37°

(A) $[P2S] = 10^{-4}$ M

	% Reactivation					
	$[EP]_0$ units ml^{-1}				Calc.* single component	Calc.† two component
Time (min)	10	10	0.1	0.1		
5	28.7	28.2	27.1	22.3	28.5	26
10	44.3	43.4	39.9	38.1	49	43
20	63.8	66.4	61.2	62.6	74	62
30	76.1	77.0	71.8	68.9	87	73
45	83.9	84.5	79.9	78.0	96	82
60	88.2	90.8	86.1	86.8	98	88

* Calculated using a $^1k_7$ of 1.1×10^{-3} sec^{-1}.
† Calculated using a $^1k_{7(1)}$ and a $^1k_{7(2)}$ of 17×10^{-4} and 4×10^{-4} sec^{-1} respectively.

(B) $[P2S] = 10^{-5}$ M

	% Reactivation			
	$[EP]_0$ units ml^{-1}		Calc.* single component	Calc.† two component
Time (min)	10	0.1		
10	9.7	11.1	10.4	6.4
20	15.1	13.6	20	12
30	20.2	16.6	28	17
60	32.1	28.6	48	30
90	39.2	38.2	63	39
120	46.6	45.5	73	46
180	58.2	60.2	86	56

* Calculated using a $^1k_7$ of 1.8×10^{-4} sec^{-1}.
† Calculated using a $^1k_{7(1)}$ $^1k_{7(2)}$ of 2.0×10^{-4} and 0.25×10^{-4} sec^{-1} respectively.

RESULTS AND DISCUSSION

O,O-*diethyl phosphorylated AChE*

O,O-diethyl phosphorylated AChE is the most simple EP studied as the organophosphates (**VII**) and (**IV**) (see Table 1) are achiral and no ageing of EP occurs (i.e. $k_5 \sim 0$). Superimposable reactivation profiles for EP are found indicating that the leaving group does not determine the site or sites of inhibition.

In Table 2 are given experimental data for reactivation at two values of $[EP]_0$ (10 and 0.1 unit ml^{-1}) and two P2S concentrations (10^{-4} and 10^{-5} M). The reactivation profiles are independent of $[EP]_0$ indicating that no significant reinhibition by POX occurs. This lack of reinhibition is expected from the rapid

Table 3. Reactivation of *O,O*-diethylphosphorylated AChE at various values of [P2S] $[EP]_0 = 10$ units/ml at pH 7.4 and 37°

[P2S] M											
10^{-5}		3×10^{-5}		5×10^{-5}		10^{-4}		2×10^{-4}		5×10^{-4}	
t*	%†	t	%	t	%	t	%	t	%	t	%
2	1.9	3	6.8	2	7.2	1	6.3	1.5	12	1	15
9	6.5	8	13	7	19	6	27	3	26	2	25
17	10	15	21	13	30	12	44	7	44	4	43
25	14	26	28	19	39	18.5	56	13	60	7	62
40	19	43	37	27	48	26.5	66	25	76	12	73
60	23	64	44	40	58	40	75	43	83	18	81
90	30			60	69	60	81			28	85
135	37			108	81					45	85
										120	88

* In minutes.
† % Reactivation.

breakdown of POX ($^2k_3$) and its relatively low rate of inhibition ($^2k_6$)[1].

In Table 3 are given data for the reactivation at an $[EP]_0$ of 10 unit ml^{-1} at several different P2S concentrations. At the higher P2S concentrations complete reactivation (i.e. > 95%) was usually observed within a few hours; at the lower concentrations complete reactivation was not observed. Control experiments in which uninhibited AChE and P2S were incubated at 37° for > 24 hr gave inconsistent results, and therefore it was not possible to show whether the incomplete reactivation was due to ageing of the inhibited AChE or denaturation. It was shown that ageing, denaturation, and spontaneous reactivation were not significant over the time course (2–3 hr) in which kinetic analysis took place.

The rates of inhibition by amiton (**VII**) of fresh AChE and of AChE which had been previously inhibited by amiton and then reactivated by P2S (the P2S etc. being removed by ultrafiltration subsequent to the reactivation) are identical as are the K_ms and V_{max}s using ACh as substrate. The reactivation profiles of the two **VII** inhibited enzymes are also superimposable. All of these observations demonstrate that no irreversible changes to the enzyme structure occur during inhibition/reactivation.

Although reinhibition by *O,O*-diethylphosphonylated P2S during the reactivation reaction does not occur to any significant extent attempts to fit the experimental data to the kinetics predicted by Scheme 1 (i.e. first order kinetics as $^1k_5$ and $^1k_6$ are insignificant) did not succeed. This is shown in Table 2 by a comparison of the columns headed "calculated for single component" with the experimental results. The first order rate coefficients used to calculate the first order reactivation profiles were calculated from the means of the first recorded reactivations, i.e. at 5 min for 10^{-4} M P2S and at 10 min for 10^{-5} M P2S. Although this lack of agreement between the observed data and first order kinetics is now attributed to EP being kinetically heterogeneous, other reaction mechanisms were also considered in an attempt to explain the experimental results. One such mechanism, in which oxime actually induces ageing and has been previously described [2–4], was discounted for two reasons: firstly, with both *O,O*-diethylphosphonylated AChE and *O*-diethyl methylphosphonylated AChE and P2S, complete reactivation is observed under some experimental conditions; secondly, in experiments not described in this paper, higher concentrations of P2S were added to reactions in which reactivation was proceeding slowly (e.g. 10^{-5} M P2S and *O*-ethyl methyl-phosphonylated AChE at 10 units ml^{-1} after 60 min in Fig. 2) to produce >95% reactivation (similar experiments with *O,O*-diethylphosphonylated AChE were not done). It was therefore concluded that EP cannot be considered as a single species in the kinetic analysis of the reactivation data.

An adequate mathematical representation of the reactivation profiles was made by considering the inhibited enzyme to be a mixture of two components, EP_1 and EP_2, each with its own rate of reactivation. Both the proportions of EP_1 and EP_2 and their rate coefficients ($^1k_{7(1)}$ and $^1k_{7(2)}$) were allowed to vary. The ratios of EP_1 and EP_2 were found to be scattered around 1:1. It was therefore arbitrarily decided to constrain the model to a 50/50 distribution as this was found not to affect the "agreement of fit" between the computed curves and experimental data due to the shallowness of the error surface. Thus no particular significance should be attached to the data being analyzed with a two equal component model. Two is merely the smallest number of components needed to give an adequate representation of the data. The significance of EP being a multicomponent system will be discussed later.

The adequacy of the two component model can be seen from a comparison of the final column of computed data in Table 2 with the experimental data. In Table 4 are given the rate coefficients obtained from analyzing the data in Table 3 according to the two component model.

Using the two component model it is interesting to analyze the dependence of the rate coefficients upon P2S concentration. The data were analyzed for agreement with Scheme 2 using Eadie type plots, i.e. $^1k_7$ versus $^1k_7/[OX]$

$$EP + OX \underset{}{\overset{K_{10}}{\rightleftharpoons}} EP\cdot OX \xrightarrow{^1k_{11}} E + POX$$

Scheme 2

for which reasonably straight lines were observed. The results are given in Table 5 together with the results of a similar analysis of the dependence of the initial rates of reactivation (calculated for either the first point or the first 10% and given in Table 4) upon oxime concentration. The affinity of EP with P2S ($\sim 5 \times 10^{-4}$ M) is very similar to that observed for the free enzyme (1.4×10^{-4}) suggesting no special features are involved in the formation of the precursor (i.e. EP·OX) to the reactivation step ($^1k_{11}$). This lack of special features in the binding of P2S to EP is also suggested by the affinity constants for EP_1 and EP_2 being similar, the lower rate of reactivation of EP_2 being attributable to its low $^1k_{11}$.

Table 4. Rate coefficients for the reactivation of *O,O*-diethylphosphorylated AChE at various values of [P2S] at pH 7.4 and 37°

	[P2S] M					
$^1k_7 \times 10^{-4}$ sec^{-1}	10^{-5}	3×10^{-5}	5×10^{-3}	10^{-4}	2×10^{-4}	5×10^{-4}
$^1k_{7(1)}$	1.8	5.5	8.7	17	30	48
$^1k_{7(2)}$	—	—	0.7	3	4.5	8.7
Calc. from 1st point	1.6	3.9	6.2	11	14	27
Calc. from 1st 10%	1.1	3.2	5.8	10	18	29

Table 5. Binding and reaction coefficients of P2S with *O,O*-diethyl-phosphorylated AChE at pH 7.4 and 37°

Origin of $^1k_7$	$K_{10} \times 10^{-3}$ M^{-1}	$^1/K_{10} \times 10^4$ M	$^1k_{11} \times 10^4$ sec^{-1}
$^1k_{7(1)}$	1.9	5.3	104
$^1k_{7(2)}$	2.0	4.9	17
1st point	3.1	3.2	42
1st 10%	3.3	3.0	52

O-ethyl methylphosphonylated AChE

This was produced by inhibiting AChE with the enantiomers and racemates of **VIII** and **IX** and with racemic **I**.

EP produced from (S)-**VIII** *and* (S)-**IX***

The data for $[EP]_0$s of 0.1, 1 and 10 units ml^{-1} with 10^{-4} and 10^{-5} M P2S are shown in Figs 1 and 2 respectively. Reactivation profiles for $[EP]_0$s of 0.01 and 0.1 unit ml^{-1} in the presence of substrate are superimposable on each other and on that observed for an $[EP]_0$ of 0.1 unit ml^{-1} in the absence of substrate. For reactivations at these low concentrations reinhibition by POX is unimportant and the reactivation data can be described adequately by the two component model previously developed.

Using the rate coefficients for reactivation obtained from this analysis and the rates of inhibition ($^2k_6$) and breakdown ($^2k_3$) of POX given in Part I [1] it should be possible to quantitatively describe the observed dependence of reactivation profile upon $[EP]_0$ shown in Figs 1 and 2. Although Scheme 1 must form the basis of a quantitative description various possible modifications to it need to be explored:

$$EP_1 + OX \underset{k_6}{\overset{k_{7(1)}}{\rightleftarrows}} E_1 + POX \xrightarrow{k_3} \text{Breakdown products}$$

$$EP_2 + OX \underset{k_6}{\overset{k_{7(2)}}{\rightleftarrows}} E_2 + POX \xrightarrow{k_3} \text{Breakdown products}$$

Scheme 3

$$EP_1 + OX \underset{k_6}{\overset{k_{7(1)}}{\rightleftarrows}} E + POX \xrightarrow{k_3} \text{Breakdown products}$$

$$EP_2 + OX \underset{k_6}{\overset{k_{7(2)}}{\rightleftarrows}}$$

Scheme 4

If POX is optically stable under these reaction conditions then the value of $^2k_6$ given in Part I [1] (obtained from the racemic fluoridate) must be doubled as the enantiomeric POXs can be assumed to have considerably different rates of inhibition. Both Schemes 3 and 4 were tested (using analogue computer simulation) against the data shown in Figs 1 and 2. Simulations from both schemes gave reasonably satisfactory visual agreement with the data at 10^{-5} M P2S but only simulations from Scheme 4 gave good agreement with the data at 10^{-4} M P2S. If the value of $^2k_6$ given in Part I [1] was used, i.e. POX was assumed to be racemised rapidly then simulations from neither Scheme 3 nor Scheme 4 gave satisfactory agreement with the data. It is therefore concluded that POX is effectively optically stable† and that Scheme 4 affords an adequately quantitative description of the experimental data. The full lines in Figs 1 and 2 are computed using Scheme 4.

EP produced from (R)-**IX**

For reactivations with $[EP]_0$s of 0.1, 1 and 10 unit ml^{-1} reinhibition by POX, henceforth designation (*R*)-POX,‡ is unimportant as the reactivation profiles are superimposable on each other and can be described adequately by the two-component model previously developed using rate coefficients with 10^{-4} M P2S of 8.3×10^{-4} and 1.5×10^{-4}/sec.§ Com-

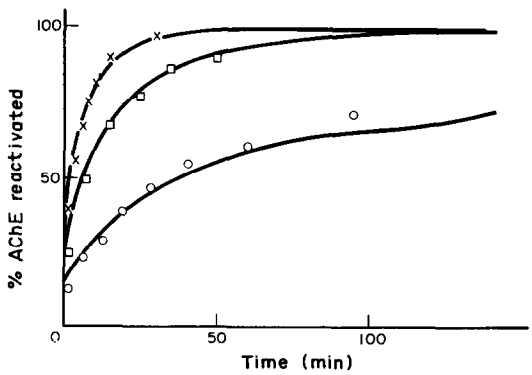


Fig. 1. Reactivation of AChE inhibited by (*R,S*)-**IX** 10^{-4} M P2S: × $[EP]_0$ 0.1 unit ml^{-1}; □ $[EP]_0$ 1 unit ml^{-1}; ○ $[EP]_0$ 10 units ml^{-1}; pH = 7.4, T = 37°. The full lines are calculated for Scheme 4 using values of $^1k_{7(1)}$ and $^1k_{7(2)}$ of 1.03×10^{-2} and 1.48×10^{-3} sec^{-1} respectively.

* Because of the high stereoselectivity of **IX** and **I** this EP is also produced by inhibiting AChE with (*R,S*)-**IX** and (*R,S*)-**I**. For the case of (*R,S*)-**VIII** *vide infra*.

† From Pt I it can be calculated that at pH 7.40 and 10^{-5} M P2S the ratio of P2S and hydroxide ion attack upon the phosphorus of **I** is ~1. Under similar conditions and at an $[EP]_0$ of 10 units ml^{-1} the first order rate of reaction of E with POX at 50% reactivation, i.e. $^1k_6$ is 2×10^{-2} sec^{-1}. If rapid racemisation occurs the first order rate of this racemisation, which can only occur by P2S attack upon the phosphorus of POX, must be at least ×10 greater, i.e. $>2 \times 10^{-1}$ sec^{-1}. Breakdown of POX under these conditions occurs via an hydroxide ion catalyzed elimination reaction with a $^1k_3$ of 1.1×10^{-2} sec^{-1}. Since breakdown via an S_N2P reaction by hydroxide would have been detected at >5% it follows that the rate of such a reaction is $<5 \times 10^{-4}$ sec^{-1}. Therefore the ratio of P2S and hydroxide ion attack upon the phosphorus of POX must be >500 if racemisation is to occur. In view of the observed ratio for the similar reactions with P–F, racemisation seems unlikely to occur.

‡ This designation will be justified later.

§ However the data are so few that an adequate kinetic analysis cannot be made and such data that are available can be described by first order kinetics.

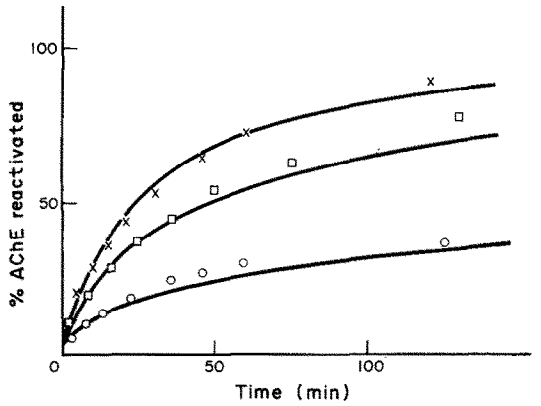


Fig. 2. Reactivation of AChE inhibited by (*R,S*)-**IX** 10^{-5} M P2S: × $[EP]_0$ 0.1 unit ml^{-1}; □ $[EP]_0$ 1 unit ml^{-1}; ○ $[EP]_0$ 10 units ml^{-1}; pH = 7.4, $T = 37°$. The full lines are calculated for Scheme 4 using values of $^1k_{7(1)}$ and $^1k_{7(2)}$ of 9.3×10^{-4} and 1.83×10^{-4} sec respectively.

puter simulation shows that despite the *ca.* 10 × lower reactivation rate than that observed for EP produced from (*S*)-**IX** if (*R*)-POX racemised and had a similar $^2k_6$ to (*S*)-POX considerable dependence of reactivation profile upon $[EP]_0$ would have been observed. Thus, as expected, (*R*)-POX has a much smaller $^2k_6$ than has (*S*)-POX and additional proof of the optical stability of POX under these reactivation conditions is provided.

EP produced from (R)-**VIII**

This EP has identical kinetic properties to that produced from (*S*)-**VIII**. This result, taken in conjunction with those discussed in the previous sections, implies that the stereochemical course for inhibition by (*R*)-**VIII** is different than that occurring during inhibition by (*S*)-**VIII** or by the enantiomers of **IX**, i.e. if, as will be shown later, (*S*)-**VIII** and (*R*)- and (*S*)-**IX** inhibit via inversion of configuration then (*R*)-**VIII** inhibits via a mechanism involving retention of configuration.

Although this conclusion may not have biochemical precedent it is perhaps not inconsistent with many stereochemical studies of phosphinothioates [5] where only small changes in the nature of the substrate, solvent, nucleophile concentrations, and general reaction conditions have been observed to markedly effect the degree of stereoselectivity in nucleophilic displacement. Thus a change in stereochemistry from inversion to retention of configuration on a complex enzyme surface may be possible.

Mechanism of inhibition of AChE

The model requiring two non-interconvertible forms of EP each with its own rate of reactivation is the most simple one that is required to describe the experimental data. More sophisticated models in which there are more than two forms of EP and in which the proportion of each type of EP varies according to the nature of the organophosphate cannot be excluded.

Consider a slightly more complex model in which whilst there are still two components their ratio is a variable dependent upon the organophosphate, reaction conditions etc. It is likely that EP_1 and EP_2 arise by inhibition of E_1 and E_2 but whereas EP_1 and EP_2 are non-interconvertible E_1 and E_2 are rapidly interconvertible.* Thus

$$\begin{array}{ccc} E_1 + PF & \xrightarrow{^2k_{4(1)}} & EP_1 \\ K_1 \updownarrow & & \\ E_2 + PF & \xrightarrow{^2k_{4(2)}} & EP_2 \end{array}$$

Scheme 5†

which on a kinetic analysis yields

$$^2k_4 = \frac{K_1\,{}^2k_{4(1)} + {}^2k_{4(2)}}{1 + K_1}$$

where $^2k_4$ is the observed second order rate constant for inhibition of $\bar{E}$ by PF, $K_1 = E_1/E_2$, and $\bar{E} = E_1 + E_2$ and the fraction of $\bar{E}$ going to EP_1 being

$$\frac{K_1\,{}^2k_{4(1)}}{K_1\,{}^2k_{4(1)} + {}^2k_{4(2)}}$$

Scheme 5 therefore allows EP_1 and EP_2 to be kinetically distinct whilst allowing E to behave in a kinetically homogeneous manner. It should be noted that the rate of interconversion of EP_1 and EP_2 does not have to be zero, merely slow compared to the time scale of reactivation. The reason for this slow (or non-) interconversion of EP_1 and EP_2 probably arises from the nature of E_1 and E_2.

E_1 and E_2 probably have slight differences in their tertiary structures which may be linked to a conjugate acid-base pair.‡ Phosphonylation of the enzyme may hinder an interconversion between such structures particularly if, for instance, hydrogen bonding of the serine hydroxyl is important in facilitating the interconversion or if hydrogen bonding of the enzyme to the phosphorus moiety strengthened the tertiary structure.

Such strong interactions are also indicated by the finding that one enantiomer of **VIII** phosphonylates with retention of configuration at phosphorus and the other with inversion of phosphorus configuration.

As discussed earlier [1] the high inhibitory potency of the alkyl methyl-phosphonylated oximes cannot be attributed unambiguously to either the oxime moiety being a good leaving group or to its binding to the enzyme via the quaternary nitrogen of the oxime (e.g. similar to **IX**). In either case it would be

* Although multiple reversible forms of AChE have been suggested before on the basis of non first order rates of inhibition by organophosphates [6] no such deviation from first order kinetics has been observed in the present work. This may be due to differences in the temperature of the inhibition experiments 5° in reference 6 and 37° in the present work.

† A similar scheme and kinetic analysis will obtain for reinhibition by POX. Separation of $^2k_4$ (or $^2k_6$) into an association step followed by covalent reaction is an unnecessary complication at present.

‡ Differences in secondary structure are much less likely due to the greater stability of the secondary structure compared to that of the tertiary structure.

expected that inhibition by POX will proceed with inversion of configuration at phosphorus. When reactivating the EP produced from the *S* enantiomer of **IX** it is found that the EP produced by reinhibition by POX is identical to that from which the POX is formed thus demonstrating that reactivation proceeds with inversion of configuration at phosphorus.

Implications for in vivo *studies*

Because of the presence in plasma of enzymes which rapidly hydrolyze the phosphonofluoridates (e.g. sarin (**II**) and soman (**III**) and related compounds (e.g. tabun (**V**)), reactivations, under conditions where reinhibition by POX is important ($[EP]_0$ 10 units ml^{-1}, [P2S] 10^{-5} M), were followed in the presence and absence of rat plasma (10% and 33%). In the presence of rat plasma the reactivation is markedly enhanced, that occurring at the higher plasma concentration being similar to that observed for an $[EP]_0$ of 1 unit ml^{-1}.

The reactivation experiments in the presence of rat plasma gave interesting results in that they indicated that although reinhibition by POX is a potential complication in understanding the mode of action of oximes *in vivo*, in practice it may not be so due to the rapid metabolisms of the POXs. This is particularly likely if the enzyme(s) responsible is the same as that responsible for hydrolyzing the phosphonofluoridates and related compounds as this is fairly widely distributed in tissue and is very important in determining the toxicity of the phosphonofluoridates. It is thus possible that reactivation *in vivo* is, in practice, less complex than that occurring *in vitro*.

Whilst two (or more) forms of EP occur *in vitro* with the soluble AChE preparation used in the present study it is a matter for speculation as to whether similar heterogeneity occurs with membrane bound AChE or whether the observed heterogeneity results from the solubilizing and purification of the enzyme. The possibility of similar heterogeneity occurring with carbamoylated AChE will be discussed in a future paper.

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