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INTERACTION OF SOME TRIALKYL PHOSPHOROTHIOLATES WITH ACETYLCHOLINESTERASE

CHARACTERIZATION OF INHIBITION, AGING AND REACTIVATION

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Key words Acetylcholinesterase, Trialkyl phosphorothiolate Inhibition, Aging Reactivation

The reaction of bovine erythrocyte acetylcholinesterase (acetylcholine acetylhydrolase, EC 3 1 1 7) with a set of structurally related phosphorothiolates was studied in order to investigate the properties of the phosphorylated enzymes and to identify the leaving group OOS- and OSS-trimethyl phosphorothiolates and their triethyl analogues inhibit acetylcholinesterase reversibly and by progressive inhibition, and the phosphorylated enzymes undergo both spontaneous reactivation and aging For each compound the enzyme-inhibitor dissociation constant, and the rate constants for inhibition (k_a), reactivation and aging have been derived The OSS-compounds are more potent inhibitors than the OOS-compounds, and the derived inhibited enzymes reactivate and age faster By comparing reactivation and aging rate constants with those obtained from phosphorylated enzymes of known structure it was concluded that the leaving group during phosphorylation is the *S*-alkyl SSS-trimethyl and -triethyl phosphorothiolates also form reversible complexes and inhibit the enzyme progressively With these inhibitors the phosphorylated enzymes did not reactivate either spontaneously or in response to oximes under conditions successful for the other inhibitors The k_a values (37°C, pH 7 4) range from 30 M^{-1} min^{-1} (OOS-trimethyl phosphorothiolate) to 6 7 10^3 M^{-1} min^{-1} (OSS-triethyl phosphorothiolate) as compared to 1 25 10^5 M^{-1} min^{-1} determined for isomalathion (*O*, *S*-dimethyl *S*-(1,2-dicarbethoxyethyl)-phosphorodithioate), which was used as one of the reference compounds If the inhibitory potency of the trialkyl phosphorothiolates is calculated from measurements made after a fixed preincubation time the results in k_a values will be misleading

Introduction

The interaction of inhibitory organophosphorus esters with cholinesterases has been characterized in terms of several partial reactions (see Ref 1 and Scheme I) For many potent inhibitors the formation of Michaelis complex cannot be demonstrated easily because phosphorylation (k_{+2}) is so rapid that under conventional conditions of measurement the level of complex is very low However for many compounds the rates of spontaneous reactivation (k_{+3}) and aging (k_{+4}) have been determined and are known to be influenced by the chemical structure of the groups attached to phosphorus Many organophosphorus insecticides in current use contain thiono- and/or thiolo- groups and may also contain directly inhibitory phosphorothiolate impurities (isomers of the major component as well as trialkyl phosphorothiolates) The effect of such impurities on the toxicity of technical malathion has been shown recently [2,3] and several of the malathion impuri-

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Abbreviations for abbreviations of the organophosphorus compounds used in the study please see Table I

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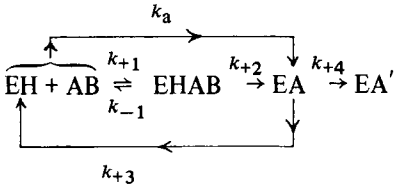
ties have been shown to inhibit acetylcholinesterase and carboxylesterase However we are not aware of detailed studies of the partial reactions of phosphorothiolates with esterases, only I_{50} values were derived from fixed-time inhibition measurements with enzymes in some rat tissues [4]

We have studied a series of structurally related trimethyl and triethyl phosphorothiolates (Table I) in order to establish which partial reactions in Scheme I do occur and which is the leaving group during inhibition We have shown that all compounds form a significant amount of Michaelis complex prior to phosphorylation, and in many cases the rates of spontaneous reactivation and aging could be determined Comparison of k_{+3} and k_{+4} constants with those obtained from enzymes inhibited by isomalathion, or by other typical dialkyl phosphate inhibitors, shows that the leaving group during inhibition by trialkylphosphorothiolates is the thioloalkyl and not an alkyloxy group



Scheme I Partial reactions in the interaction of cholinesterases with an organophosphorus ester EH, active enzyme, AB, inhibitor, EHAB, Michaelis complex, EA, phosphorylated enzyme, EA′ aged enzyme, k_{+1}, etc , rate constants for the appropriate reaction

Materials and Methods

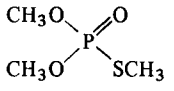
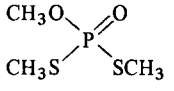
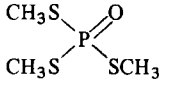
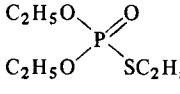
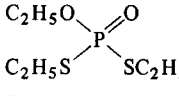
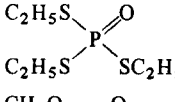
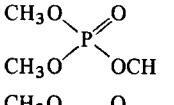
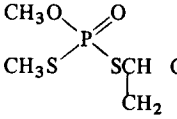
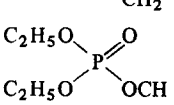
All experiments were carried out in 100 mM phosphate buffer, pH 7 4 Progressive inhibition, spontaneous reactivation and aging were done at 37°C, while non-progressive inhibition was done at 25°C The enzyme activity was determined at 25°C by the spectrophotometric method of Ellman et al [5] using acetylthiocholine iodide as substrate

Enzyme The enzyme was bovine erythrocyte acetylcholinesterase (acetylcholine acetylhydrolase, EC 3 1 1 7) from Sigma Stock solutions (usually 20 U/ml) were prepared in buffer and kept at −20°C until needed The enzyme concentration during assay was about 0 015 U/ml

Inhibitors (See Table I for structural formulae and abbreviations) DDVP and EDVP were obtained from Shell All other inhibitors were obtained as a gift from Dr J W Miles (U S A) Inhibitors I-VIII were 98% pure, IX was 77% pure Stock solutions (100 mM) of DDVP, IsoM and EDVP were prepared in acetone and further diluted with water, the acetone concentration during enzyme inhibition was less than or equal to 0 01% All other inhibitors were added to the enzyme either neat or dissolved in buffer

TABLE I

STRUCTURAL FORMULAE AND ABBREVIATIONS OF THE ORGANOPHOSPHORUS COMPOUNDS USED IN THIS STUDY

No	Structural formula	Abbreviation
I		OOS-Me
II		OSS-Me
III		SSS-Me
IV		OOS-Et
V		OSS-Et
VI		SSS-Et
VII		DDVP
VIII		IsoM
IX		EDVP

Reactivator TMB4 (*N,N'*-trimethylene-bis(4-hydroxyiminomethylpyridinium dibromide)) was a gift from Professor F Hobbiger (U K)

Progressive inhibition Enzyme (50 or 100 μl) and inhibitor (up to 100 μl) were added to 10 ml buffer and incubated at 37°C At intervals, samples (0 5 ml) were transferred to 1 0 cm photocells containing a solution (3 0 ml), at 25°C, of buffer, substrate (0 5 mM final) and DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) (0 33 mM final) The enzyme activity was determined by measuring the increase in absorbance at 412 nm for 1–2 min

Non-progressive inhibition Enzyme (250 μl) was added to a solution (3 25 ml) at 25°C containing buffer, substrate (concentration varied from 0 25 to 1 0 mM final), DTNB (0 33 mM final) and inhibitor The enzyme activity was determined by measuring the increase in absorbance at 412 nm for 1–2 min

Spontaneous reactivation Inhibitor (5–10 μl) was added to 1 0 ml enzyme and incubated at 37°C in order to obtain substantial enzyme phosphorylation (80–90% wherever possible) The inhibitor concentrations and time during this step were 7 9 mM OOS-Me (60 min), 3 0 mM OSS-Me (10 min), 3 6 mM SSS-Me (120 min), 0 8 mM OOS-Et (60 min), 50 μM OSS-Et (10 min), 1 8 mM SSS-Et (40 min), 3 μM DDVP (10 min), 5 μM IsoM (10 min) and 1 3 μM EDVP (10 min) An aliquot (0 1 ml) was then diluted 200-fold with buffer, and the enzyme was allowed to reactivate spontaneously at 37°C At intervals, samples (0 5 ml) were taken and the enzyme activity was determined at 25°C as described under Progressive inhibition The time of spontaneous reactivation is the time interval between enzyme dilution (zero-time) and withdrawal of samples for enzyme assay Reactivation was followed for 2–3 half-lives wherever possible

Aging Inhibitor (5–10 μl) was added to 1 0 ml enzyme and incubated at 37°C in order to obtain 80–90% enzyme phosphorylation During this inhibition step, the inhibitor concentrations and time of inhibition were the same as under Spontaneous reactivation At the end of the inhibition step, the first sample (50 μl) was withdrawn (followed at intervals by other 50 μl samples) and added to 5 0 ml TMB4, (100 μM) in buffer, at 37°C in order to reactivate the non-aged enzyme The time of reactivation was 3–5 min (when the inhibitors were IsoM, OSS-Me), 10 min (when the inhibitors were DDVP, OOS-Me, EDVP, OOS-Et) or 20 min (for OSS-Et) Acetylcholinesterase inhibited by the SSS-esters was not at all reactivated by incubation with TMB4 for periods up to 20 min At the end of this reactivation step samples (0 5 ml) of the reactivated enzyme were assayed for activity as described under Progressive inhibition The time of aging is the time interval between addition of the first sample (zero-time) and the subsequent samples into TMB4 solutions Under the assay conditions used the reactivator caused slight (not more than 5%) inhibition of enzyme

Evaluation of constants

The equations for the partial reactions between enzyme and inhibitor have been worked out in detail [1] The equations used to interpret our results are therefore summarized in this section, without giving details on how the equations were derived

k_{+2}, K_a and k_a from progressive inhibition

The differential equation which defines the rate of formation of the phosphorylated enzyme EA (Scheme I) is

$$\ln v_0 - \ln v_t = \frac{k_{+2}[AB]}{[AB] + K_a} t_1 \quad (1)$$

v_0 is the enzyme activity in absence of inhibitor, v_t is the activity of the inhibited enzyme, t_1 is the time of inhibition, [AB] is the inhibitor concentration and K_a is

$$K_a = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad (2)$$

At a given inhibitor concentration, $\ln v_t$ is a linear function of t_1 The slope of the line is the first-order rate constant of inhibition (k') which equals

$$k' = \frac{k_{+2}[AB]}{[AB] + K_a} \quad (3)$$

Eqn 3 can be rearranged into a Wilkinson-type equation [6]

$$\frac{[AB]}{k'} = \frac{K_a}{k_{+2}} + \frac{[AB]}{k_{+2}} \quad (4)$$

and a plot of $[AB]/k'$ against $[AB]$ enables k_{+2} and K_a to be obtained, when k' and $[AB]$ are known

The second-order rate constant of inhibition (k_a) can also be obtained from the following ratio

$$k_a = \frac{k_{+2}}{K_a} \tag{5}$$

When the concentration of the Michaelis complex EHAB (Scheme I) is small, Eqn 1 simplifies to

$$\ln v_0 - \ln v_t = k_a[AB]t_i \tag{6}$$

At a given inhibitor concentration, $\ln v_t$ is a linear function of t_i, and the slope of the line (k') equals

$$k' = k_a[AB] \tag{7}$$

wherefrom k_a is obtained When progressive enzyme inhibition is defined by Eqn 6, it is not possible to evaluate the constants k_{+2} and K_a

K_i and K_m from non-progressive inhibition

Reversible, competitive enzyme inhibition can be defined by the Hunter and Downs [7] equation

$$\frac{v_0[AB]}{v_0 - v_i} = K_i + \frac{K_i}{K_m}[S] \tag{8}$$

where v_0 and v_i are the enzyme activities (at a given substrate concentration) in the absence and presence of the inhibitor, K_i is the dissociation constant of the enzyme-inhibitor complex, K_m is the Michaelis constant for the enzyme-substrate reaction, and [AB] and [S] are the inhibitor and substrate concentrations, respectively The left-hand side of Eqn 8 is a linear function of [S], wherefrom the constants K_i and K_m can be derived (see Fig 3) The ratio $v_0[AB]/(v_0 - v_i)$ is the apparent dissociation constant of the enzyme-inhibitor complex in presence of a given substrate concentration

k_{+3} for spontaneous reactivation

The rate of spontaneous reactivation (reaction defined by k_{+3} in Scheme I) is given by

$$\ln \frac{100}{\%\text{ remaining inhibition}} = k_{+3}t_r \tag{9}$$

where percent remaining inhibition was calculated from

100% = (activity of enzyme without inhibitor) – (activity of inhibited enzyme at zero-time)

Remaining inhibition = (activity of enzyme without inhibitor) – (activity of inhibited enzyme at time t_r)

k_{+3} is the first-order rate constant of spontaneous reactivation and t_r is the time of reactivation 'ln percent remaining inhibition' is a linear function of t_r, wherefrom k_{+3} is calculated

k_{+4} for aging

The rate of aging (reaction defined by k_{+4} in Scheme I) is given by

$$\ln \frac{100}{\%\text{ reactivation}} = k_{+4}t_{ag} \tag{10}$$

'Percent reactivation' was calculated from

100% = (activity of reactivated sample at zero-time) – (activity of inhibited sample without TMB4 at zero-time)

Reactivation = (activity of reactivated sample at time t_{ag}) – (activity of inhibited sample without TMB4 at time t_{ag})

k_{+4} is the first-order rate constant of aging and t_{ag} is the time of aging 'ln percent reactivation' is a linear function of t_{ag} wherefrom k_{+4} is calculated

Results

Time-course of inhibition

Fig 1 shows the time-course of inhibition of acetylcholinesterase by the organophosphorus esters of Table I plotted as log percent activity against time For many organophosphorus esters such graphs comprise a family of curves with three features (1) lines are straight, (2) all lines intersect the y-axis at 2 0, and (3) slopes are linearly related to inhibitor concentration However, these characteristics were seen only for EDVP (Compound IX) for the remainder there was evidence of spontaneous reac-

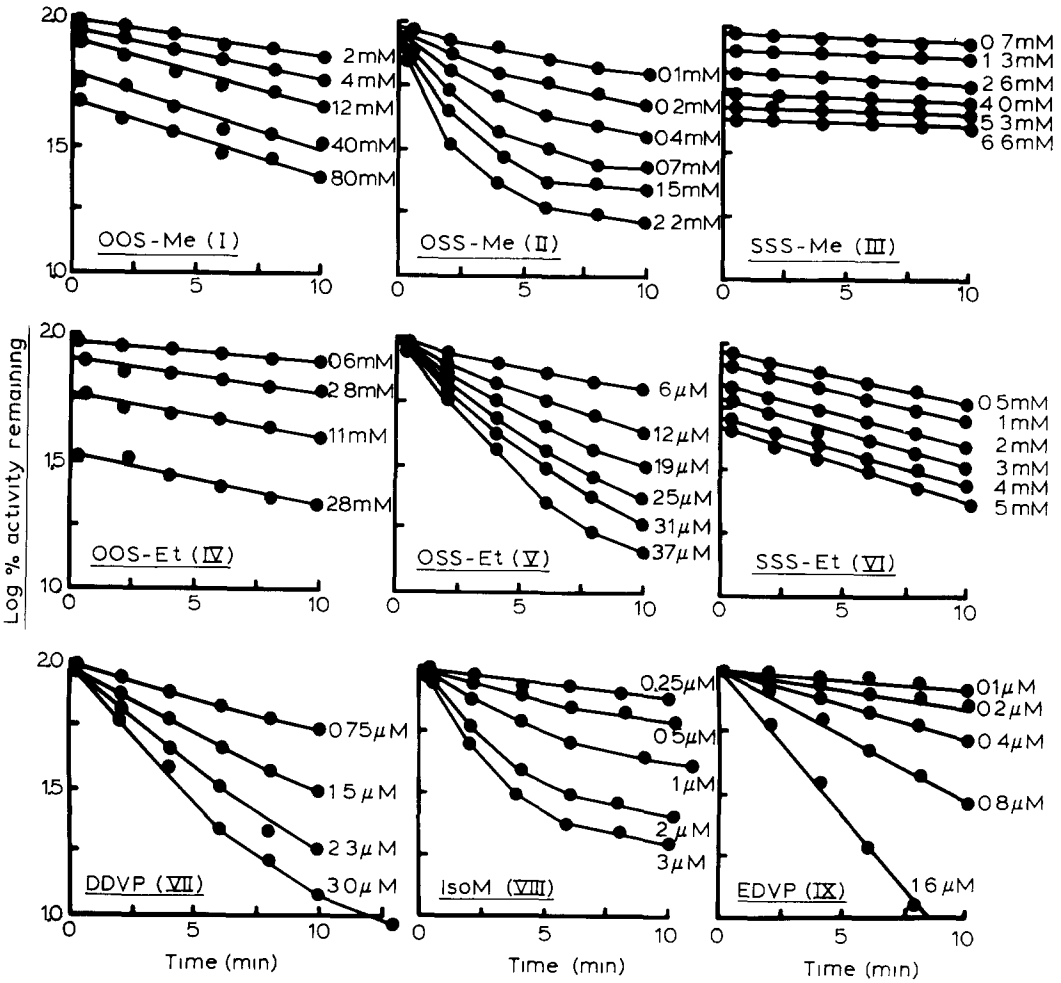


Fig 1 Time-course of inhibition of acetylcholinesterase by organophosphorus compounds (see Table I for abbreviations) Individual experiments are presented The inhibitor concentrations are indicated against each line

TABLE II

Constants (±S E) derived from progressive inhibition (K_a, k_{+2} and k_a) and non-progressive inhibition (K_i and K_m) of acetylcholinesterase by the organophosphorus compounds listed in Table I The evaluation of constants and number of experiments are described in the text

No	Compound	K_a (mM)	k_{+2} (min^{-1})	k_a (M^{-1} min^{-1})	K_i (mM)	K_m (mM)
I	OOS-Me	2 29 ±0 31	(6 90 ±0 20) 10^{-2}	30 1 ± 3 9	3 75 ±0 067	0 315 ±0 062
II	OSS-Me	0 73 ±0 11	0 597 ±0 027	(8 18 ± 0 12) 10^2	0 89 ±0 18	0 253 ±0 055
III	SSS-Me	0 34 ±0 18	(6 92 ±0 31) 10^{-3}	20 ±10	0 359 ±0 082	0 40 ±0 11
IV	OOS-Et	0 86 ±0 15	(3 949 ±0 085) 10^{-2}	46 2 ± 8 3	0 798 ±0 086	0 345 ±0 042
V	OSS-Et	0 284 ±0 088	1 92 ±0 59	(6 74 ± 0 20) 10^3	0 195 ±0 021	0 284 ±0 034
VI	SSS-Et	0 34 ±0 14	(6 86 ±0 31) 10^{-2}	(2 00 ± 0 79) 10^2	0 179 ±0 021	0 313 ±0 041
VII	DDVP			(5 91 ±0 15) 10^4		
VIII	IsoM			(1 252 ±0 032) 10^5		
IX	EDVP			(2 536 ±0 090) 10^5		

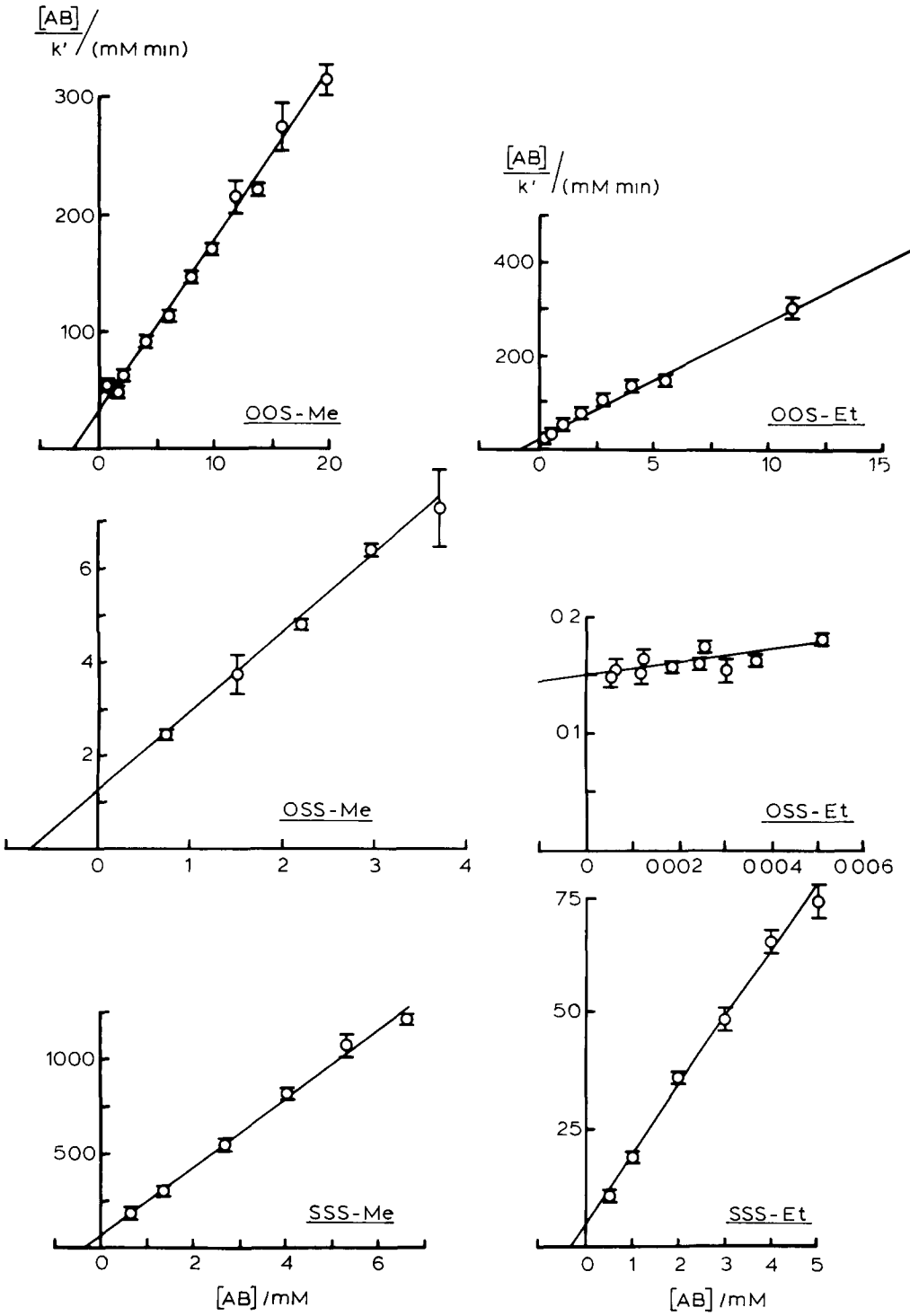


Fig 2 Wilkinson-type plots for progressive inhibition of acetylcholinesterase by organophosphorus compounds (see Table I for abbreviations) The lines were calculated by linear regression analysis AB is inhibitor, k' is the first-order rate constant of inhibition (see Eqn 3) derived from the time course of inhibition by linear regression The bars indicate S E

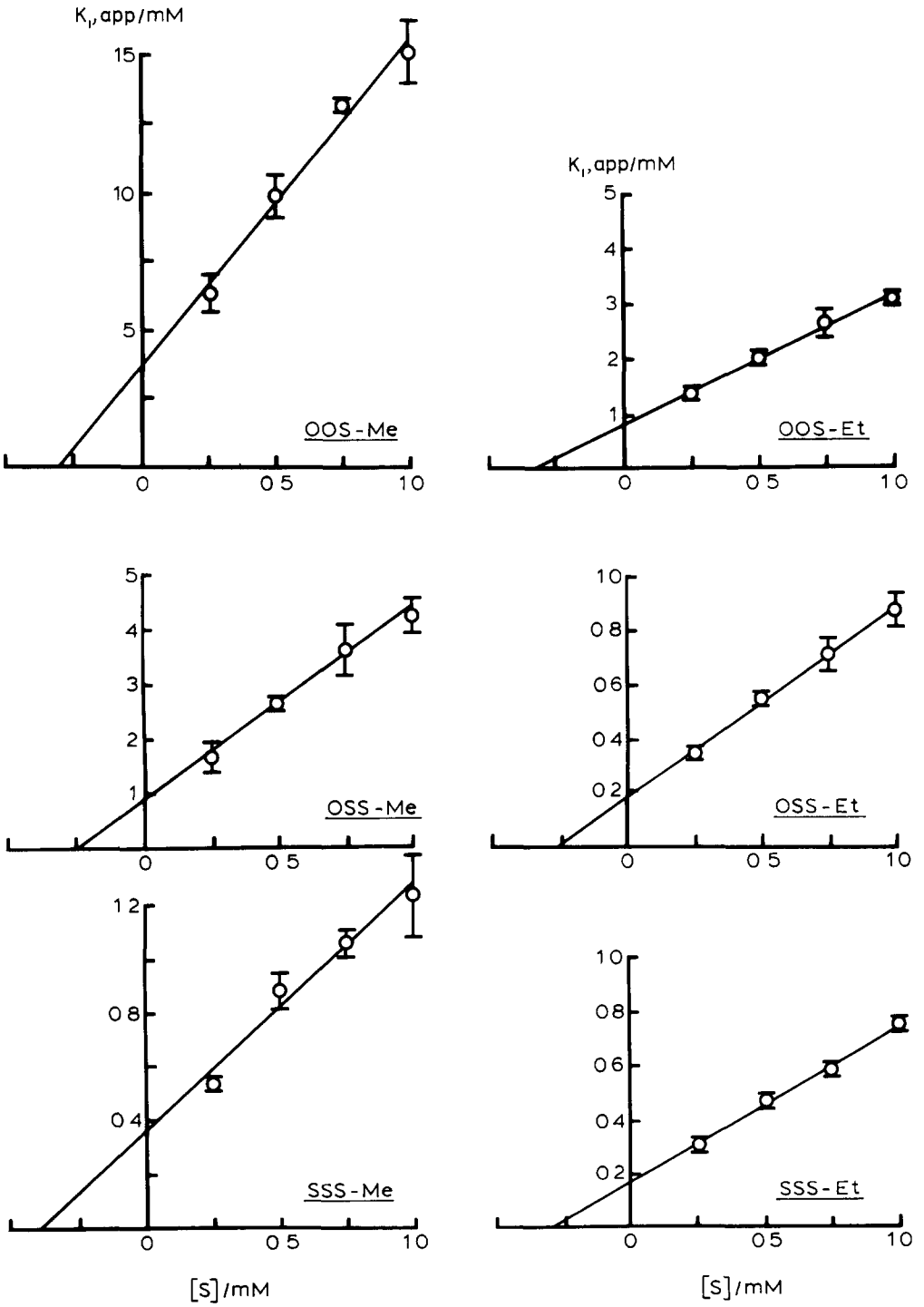


Fig 3 Hunter and Downs plots for non-progressive inhibition of acetylcholinesterase by organophosphorus compounds (See Table I for abbreviations) S is substrate, $v_0[AB]/(v_0 - v_i)$ is defined by Eqn 8 Each point is the mean of six separate determinations and the bars represent the S E The lines were calculated by linear regression

tivation (condition 1 not satisfied) or of Michaelis complex formation (condition 3 not satisfied) Failure to satisfy condition 2 also indicated formation of an inhibitor-enzyme complex

Determination of k_a, K_a and k_{+2}

Rate constants of phosphorylation were determined from the slopes (k') of progress lines such as are shown in Fig 1 For the curved lines more experiments at short inhibition times were performed than are represented on that figure, and k' values were determined from the initial slopes of the lines For compounds VII–IX the k' values were linearly proportional to inhibitor concentration [AB] and k_a values were derived using Eqn 7 and are listed in Table II For the other compounds, k' was not linearly proportional to [AB], but approached a maximum value (k_{+2}) at high inhibitor concentrations and for some compounds the maximum rates were actually obtained experimentally (compare Fig 1 and Table II) This is characteristic for the formation of a Michaelis complex between enzyme and inhibitor, which is described by Eqn 1–5 The data was therefore analysed by the Wilkinson-type plots (Eqn 4), shown in Fig 2, and the derived constants k_{+2}, K_a and k_a are recorded in Table II The k_a values are much lower for compounds I–VI, where appreciable Michaelis complex was detected, than for VII–IX for which no complex was detected The rate of progressive inhibition by III was extremely slow, the time of inhibition was therefore increased to 100 min (not shown on Fig 1) in order to evaluate the k' constants

Determination of K_i and K_m

Non-progressive inhibition by compounds I–VI was measured at four different substrate concentrations as described in Materials and Methods The ratios $v_0[AB]/(v_0 - v_i)$ were evaluated (see Eqn 8) and plotted against [S] (Fig 3) The values of K_i and K_m were derived from the slopes and intercepts of the lines according to Eqn 8 and are shown in Table II

The values of K_i for compounds I–VI are in fairly good agreement with the K_a constants derived from the rates of phosphorylation (Table II), indicating that both constants stand for the same enzyme-inhibitor complex

According to Hunter and Downs [7] the intercepts on the abscissa of Fig 3 should be equal to the K_m value (see Eqn 8) provided that the enzyme-inhibitor complex is exclusively a Michaelis-type complex Our intercepts were in the range 0 2–0 4 mM, the K_m for acetylthiocholine as substrate was 0 07 mM, determined by Lineweaver-Burk plot from the activities of the control samples Since the derived values were only slightly higher than 0 07 mM, it is likely that the enzyme inhibitor complexes are principally Michaelis-type complexes, but a small contribution from allosteric binding sites is probably also involved [8,9]

Determination of k_{+3} and k_{+4}

Rates of spontaneous reactivation and aging were determined as described in Materials and Methods The data was plotted according to Eqns 9 and 10, and two representative graphs are shown in Fig 4 No spontaneous reactivation was observed for enzyme inhibited by compounds III and VI and inhibited enzyme was refractory to TMB4, no rate constants can consequently be derived for enzyme inhibited by the SSS-esters

Rate constants for spontaneous reactivation (k_{+3}) were derived graphically from lines such as those presented in Fig 4a Many lines were curved, which we attribute to a combination of aging and reinhibition Rate constants were therefore derived from the initial slopes, within the initial time period reinhibition of the enzyme and aging of the inhibited enzyme were each less than 10% The k_{+3} constants are recorded in Table III, those for DDVP and EDVP are consistent with data from other sources [1,10]

Rate constants for aging (k_{+4}) were also derived graphically and are also recorded in Table III All lines (such as those in Fig 4b) were straight, but the precision of measurements was restricted by several factors (1) In cases when reactivation rates are greater than aging rates, the enzyme is inhibited for less than 100% of the period of aging, the measured aging rates will therefore be erroneously low, but the error was probably not large in the cases we studied (2) In the aging experiments, the inhibited enzyme was left to age in the presence of inhibitor (see Materials and Methods) At zero-time not all of the enzyme has been inhibited, and inhibition therefore proceeded during the period of aging, we have

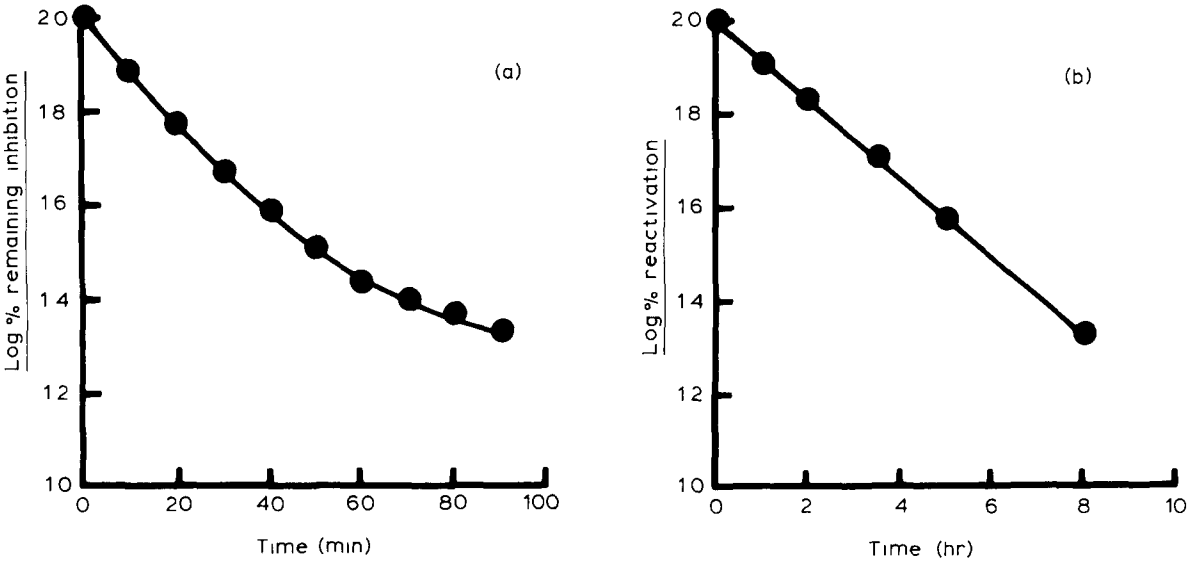


Fig 4 Time course of (a) spontaneous reactivation and (b) aging of bovine erythrocyte acetylcholinesterase inhibited by OSS-Et Each point represents a single determination The lines are drawn by eye

estimated however that the error in the least accurate measurement would be within a 2-fold factor, which we consider acceptable for the purpose of the comparisons made in this study (3) The TMB4 (100 μM) did not reactivate the enzyme fully even at zero-time aging Enzyme inhibited with methyl substituents reached a reactivation plateau at 75–80% activity (within 10 min), with the ethyl substituents the reactivation was slower, but a higher plateau was reached (90% activity after 20–30 min) Effects of this type have been described before and are attributed to interactions between enzyme, inhibitor, reactivator and phosphorylated reactivator [10,11,12] This effect however only influences the measured activities and not the derived rate constants Higher TMB4 concentrations could not be applied, because the oxime itself inhibits the enzyme

TABLE III

Rate constants of spontaneous reactivation (k_{+3}) and aging (k_{+4}) of acetylcholinesterase inhibited by the listed compounds (see Table I for abbreviations) The constants were graphically derived and results from individual experiments are given

No	Compound	10^4 k_{+3} (min^{-1})	10^4 k_{+4} (min^{-1})
I	OOS-Me	120	13
		95	
VII	DDVP	120	13
		110	17
		110	
II	OSS-Me	970	96
		910	93
VIII	IsoM	880	200
		930	227
		722	
IV	OOS-Et	2	2
IX	EDVP	2	2
V	OSS-Et	270	32

Isomalathion as an inhibitor

So far as we are aware the second-order rate constant (k_a) for inhibition of acetylcholinesterase by isomalathion (VIII) has not been published previously This compound is present in impure formulations of malathion and potentiates malathion toxicity by inhibition of carboxylesterase [2,3] Table II shows it is a potent inhibitor of bovine erythrocyte acetylcholinesterase k_a is twice that found for the well-known insecticide dichlorvos (VII)

Discussion

The four partial reactions depicted in Scheme I (Michaelis complex formation, phosphorylation, reactivation and aging) have been studied in the interaction of acetylcholinesterase with six structurally related phosphorothiolates (Table I) All four reactions occur side by side in significant proportions when compounds I, II, IV and V react with acetylcholinesterase Only two reactions (Michaelis complex formation and phosphorylation) could be proved for compounds III and VI

In our experiments we adjusted conditions to isolate as far as possible the step under consideration from the other partial reactions This isolation was seldom complete so as a check on the validity of these procedures we have taken our experimentally obtained rate constants for each partial reaction and used them to derive the theoretical time-courses of (a) inhibition by compounds V, VII and VIII and (b) spontaneous reactivation after inhibition by the compounds listed in Table III The calculations involved computer-assisted numerical integration of the differential equations corresponding to Scheme I The calculated time-courses were then compared with the experimental The equations were as follows 11–14

$$\frac{d[EH]}{dt} = -k_a[AB][EH] + k_{+3}[EA] \quad (11)$$

$$\frac{d[EA]}{dt} = k_a[AB][EH] - (k_{+3} + k_{+4})[EA] \quad (12)$$

$$\frac{d[EA']}{dt} = k_{+4}[EA] \quad (13)$$

$$[E_0] = [EH] + [EA] + [EA'] \quad (14)$$

For progressive inhibition the maximum difference between calculated and measured activities was 10% during a 10 min reaction No calculation was done for compound II with which there was substantial reversible inhibition at zero time (Fig 1) Also no calculations were done for the other compounds, because inhibition lines did not deviate from linearity (Fig 1)

The theroretical time course of spontaneous reactivation was calculated over a period of two half-lives for compounds I, II, V, VII and VIII the difference between the theoretical and experimental values was again not more than 10% The enzymes phosphorylated by compounds IV and IX reactivated and aged very slowly, and we followed the time courses experimentally over 8 h, which amounts to about one-sixth of one half-life over that period only 6–9% of the inhibited enzymes underwent reactivation and the theoretical value at that point is 8 4%

We consider that the agreement between the experimental and theoretical time-courses is good, and this in turn indicates that Scheme I satisfactorily represents the reactions which occur between acetylcholinesterase and the phosphorothiolates

For compounds I–VI it was not obvious which would be the leaving group during phosphorylation However, inspection of Table III shows that compounds having similar values for both k_{+3} and k_{+4} can be grouped in three pairs (I and VII, II and VIII and IV and IX) We can presume therefore that the inhibited species from within each pair are identical For inhibition of acetylcholinesterase by VII, VIII and IX there is no doubt which groups leave and which remain so that we may conclude that for the three pairs the groups remaining attached to phosphorus are dimethoxy, methoxy/methanethio- and diethoxy, respectively It follows therefore that alkylthio groups were the leaving groups in the cases of inhibition by I, II and IV, and by analogy we presume that *S*-ethyl is the leaving group for compound V

From the evaluated rate constants and dissociation constants shown in Tables II and III, certain structure-activity relationships can be derived Compounds II and V, containing two sulphur atoms, have higher affinities (about 3-fold) and phosphorylation rates (10- and 50-fold) than compounds I and IV containing only one sulphur atom However, introducing a third sulphur into the molecule (III and VI) has little effect on affinity, while it markedly decreases the rate of phosphorylation Comparing methyl and ethyl derivatives (with the same number of sulphur atoms) shows that the affinities and phosphorylation rates are of the same order, except for a 10-fold difference between III and VI in the value of k_{+2} Spontaneous reactivation and aging is faster for the enzymes inhibited by the OSS-esters than by the corresponding OOS-esters (compare II with I, and V

with IV) The methyl derivatives have higher k_{+3} and k_{+4} constants than the corresponding ethyl derivatives (compare I with IV, and II with V) The failure to detect either spontaneous or oxime-catalysed reactivation for SSS-inhibited AChE may indicate that aging of the inhibited enzyme proceeds considerably faster than spontaneous reactivation, or that neither of these two reactions proceeds at all We were unable to distinguish experimentally between these two alternatives

The inhibition of esterases by organophosphorus esters is often defined by inhibitor concentrations causing 50% inhibition after a fixed time of reaction (I_{50}) This is adequate only if the inhibition is defined by Eqn 6, which, however, did not apply to any of the six phosphorothiolates (I–VI) studied here, evaluation of rate constants from I_{50} data would therefore have been erroneous Values for I_{50} for compounds I and II against rate plasma cholinesterase were determined by Talcott et al [4] using a 60 min inhibition time, without knowing the kinetics of the reaction these values cannot be compared with our data on acetylcholinesterase

Compounds I, II, IV and V have acute toxicity properties indicative of their acting as anticholinesterases in vivo [3,13] Our observation of rapid aging rates indicates that oxime therapy of acute poisoning is unlikely to be successful unless it is instituted promptly Certainly, combined oxime and atropine therapy does not alter the 24 h LD_{50} for compound II in the rat [13]

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