nonlinear regression analysis using the program Grafit (Erithacus Software Ltd., Staines, UK). All kinetic determinations were performed at least in duplicate. The results are recorded in Table I.

Preparative-scale reductions of 2-keto carboxylic acids and isolation of the 2-hydroxy acid products were carried out by the method of Luyten et al.,[41] as follows:

(2*S*)-β-Phenyllactic Acid (2i). Sodium β-phenylpyruvate (1i, 400 mg, 2 mmol), NADH (57 mg, 0.08 mmol), sodium formate (340 mg, 5 mmol), FDP trisodium salt (138 mg, 0.25 mmol), dithiothreitol (1.5 mg, 0.01 mmol), lyophilized formate dehydrogenase (40 mg, 18 U), and mutant BSLDH (27-50 mg) were dissolved in 20 mM piperazine·HCl buffer (pH 6.0) to a final volume of 50 mL and incubated at pH 6.0 under pH-stat control until no further acid uptake was recorded. The reactions yielded (2*S*)-β-phenyllactic acid (2i, 240-280 mg, 1.2-1.4 mmol, 60-70% yield) with ^{1}H-NMR spectra identical with authentic samples.[3] The enantiomeric excesses of the (2*S*)-3-phenyllactic acids (2i) were determined as >98% on their Mosher esters as described previously[3] and their 2*S* absolute configurations from the chemical shifts of the ^{1}H resonances relative to tetramethylsilane as internal standard, with reference to those of authentic samples.[3]

CD spectroscopy on the mutant BSLDHs was performed as described previously for WT, Q102R, and Q102R/C97G BSLDHs.[26] The spectra are shown in Figure 1.

Graphics Analyses of the Q102R/R171Y,W mutants were performed by the general strategy outlined previously.[26] However, the current analyses were based on new X-ray coordinates, at 2.5-Å resolution, of the quaternary complex of BSLDH, oxamate, NADH, and FDP.[36]

Acknowledgment. This work was supported by a Protein Engineering Network of Centres of Excellence Award (to J.B.J. and C.M.K.), by a Natural Sciences and Engineering Research Council of Canada (NSERC) Strategic Program Grant (to J.B.J., M.G., and J. D. Friesen), by a Medical Research Council of Canada Group Grant (to C.M.K.), and by a Deutsche Forschungsgemeinschaft Postdoctoral Fellowship (to H.K.W.K.). We are also very grateful to Dr. Dale B. Wigley for making the new WT-BSLDH X-ray coordinates available to us prior to publication, to Drs. Kodandapani and Michael N. G. James for providing X-ray data on the mutant BSLDHs prior to publication, to Drs. Thomas Keller and Bernhard Westermann for the preparations of cyclohexylpyruvic acid and D-phenyllactic acid, respectively, to Mr. Kimio Oikawa for his skilled technical assistance with the CD measurements, and to Dr. J. J. Holbrook for providing the initial pKK223-3 plasmid construct containing the cloned BSLDH gene.

Registry No. Gln, 56-85-9; Arg, 74-79-3; Tyr, 60-18-4; Trp, 73-22-3; L-LDH, 9001-60-9; oxalacetic acid, 328-42-7.

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Consequence of Phosphorus Stereochemistry upon the Postinhibitory Reaction Kinetics of Acetylcholinesterase Poisoned by Phosphorothiolates

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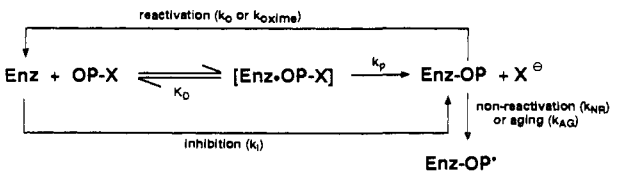
Abstract: Reaction of the chiral isomers of isoparathion methyl with solubilized rat brain cholinesterase (RBAChE) produced O,S-dimethylphosphorothiolated forms of the inhibited enzyme. The inhibited enzymes were evaluated for their individual rates of spontaneous reactivation, oxime-mediated reactivation, and non-reactivatability, and those rates were compared to those of RBAChE inhibited by the racemate. The rate constants for spontaneous reactivation were 1.13×10^{-2} min^{-1}, 1.02×10^{-2} min^{-1}, and 0.35×10^{-2} min^{-1}; for oxime-mediated reactivation were 4.10×10^{-2} min^{-1}, 4.57×10^{-2} min^{-1}, and 1.16×10^{-2} min^{-1}; and for non-reactivatability were 1.42×10^{-2} min^{-1}, 1.37×10^{-2} min^{-1}, and 3.07×10^{-2} min^{-1} for RBAChE inhibited with (−)-, (±)-, and (+)-isoparathion methyl, respectively. RBAChE inhibited by (−)-isoparathion methyl underwent spontaneous reactivation 3.23-times faster and oxime-mediated reactivation 3.53-times faster than RBAChE with the (+)-isomer. In contrast, RBAChE inhibited with (+)-isoparathion methyl underwent a time-dependent decrease in reactivatability twice that of the (−)-isomer. The postinhibitory rate constants obtained from RBAChE inhibited with racemic isoparathion methyl were similar to those obtained for inhibition with (−)-isoparathion methyl. These data correlate with the ability of the (−)-stereoisomer to inhibit the enzyme approximately 8-times faster than the (+)-isomer, leading to a larger mole fraction of RBAChE inhibited by (−)-isoparathion methyl. Chemical model studies investigating the mode of non-reactivatability by O,S-dimethylphosphorothiolated RBAChE suggest that the mechanism probably involves hydrolysis rather than dealkylation.

Introduction

Certain organophosphates (OP) inactivate acetylcholinesterases[1] and other related proteases by reaction at a nucleophilic serine hydroxyl to form a covalently modified enzyme (ENZ-OP) concomitant with the ejection of a leaving group (X^-) according to eq 1.[2] The inhibited enzyme is then incapable of hydrolysis, leading to an abnormal persistence and accumulation of the neurotransmitter acetylcholine in cholinergic nerve synapses and neuromuscular junctions.[3] The biological effects, which range from hyperexcitability to respiratory collapse, may result from exposure to insecticides[4] or nerve gas agents.[5]



(1)

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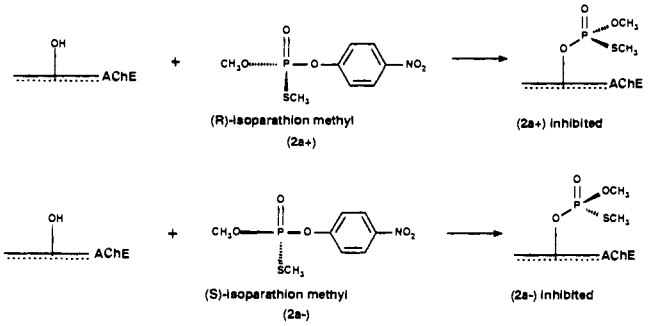
Exposure to a reactive organophosphate may occur at a low, sublethal level, and the organism generally can recover from acute poisoning via "reactivation". Reactivation may proceed spontaneously, where water is presumed to aid the displacement of the phosphoryl moiety (scission of the phosphoryl–serine bond)[2a] from the active site.[5] Alternatively, oxime-based antidotes may mediate the reactivation process.[6] Sometimes, reactivation by these pathways does not occur, and a process termed "aging" has been described, where dealkylation of one phosphorus ligand occurs (generally a secondary carbon ester) to yield a phosphate anion.[7] When aging occurs, charge repulsion and/or reorganization of electron density at the phosphate moiety render(s) the protein [Enz-OP*; where P* = P=O(O$^-$)(R)] refractory to further reactivation.

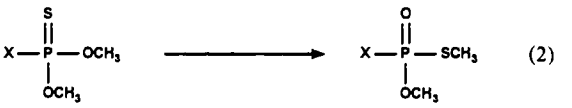
Besides aging, reactivation may be impeded due to an inability of the protein to regain the conformationally active form following inhibition.[8] A third possibility is that hydrolysis of a phosphate ester linkage occurs rather than an aging-type dealkylation (Enz-OP*; eq 1).[9] Other possibilities include (a) that the (phosphorylated) active site of some proteins is less accessible to reactivating nucleophiles owing to steric congestion, (b) denaturation, and (c) covalent modification of an essential residue other than serine.[10] These latter postinhibitory processes that lead to inactive enzyme but do not completely satisfy the mechanistic description of an aging reaction are best categorized as an inability to reactivate or "non-reactivatability". Therefore, non-reactivatability is a more broad description and accounts for the total amount of inactive enzyme, which may result from one or more biochemical factors in addition to aging. The physiologic consequence of aging and/or non-reactivatability is not well characterized for cholinesterases, but certain neurochemical lesions have been associated with the aging of neurotoxic esterase.[7c,d,11]

The extent of inhibitory (phosphorylation) and postinhibitory (reactivation, aging and non-reactivatability) processes depends greatly upon the nature of the phosphorus ester inhibitor and the particular enzyme studied. OP's have been investigated that test the demands of steric, electronic, and configurational aspects of cholinesterase inhibition[4a,12] and, to a somewhat lesser extent, postinhibitory phenomena.[7,9,13] In particular, stereochemistry at phosphorus influences the reactivation rate although much of the research has been dedicated to phosphonates (P–C linkage).[14] This investigation was initiated to study the role of phosphorus configuration upon postinhibitory reactions of cholinesterases poisoned by phosphorothiolates.

Scheme I

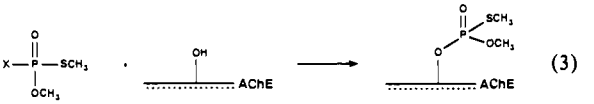


Phosphorothiolates, OP esters that bear one thiolester ligand (P–S–R), are impurities formed from phosphorothionate (P=S) insecticides via a thermal isomerization.[15] This rearrangement occurs most readily with *O,O*-dimethyl phosphorothionates (**1**) to form the corresponding *O,S*-dimethyl phosphorothiolates (**2**) (eq 2). We, and others, reported that phosphorothiolates are far



(2)

more potent anti-cholinesterase agents than the parent thionates,[15,16] presumably due to enhanced reactivity at phosphorus imparted by P=O linkage. A second and equally important consideration of the thermal isomerization of phosphorothionates is that a new center of asymmetry at phosphorus is formed. Chiral phosphorus esters, including certain phosphorothiolates, are known to react stereoselectively with cholinesterases.[4a,12c,e,14g,17] For isoparathion methyl (**2a**; X = *p*-nitrophenoxy), we showed that the (−)-isomer (**2a−**) was a 3–15-fold better inhibitor than the (+)-isomer (**2a+**) when tested against four different cholinesterases,[18] suggesting a strong reliance upon configuration at phosphorus. Because phosphorothionate insecticides such as malathion, fenitrothion, guthion, and parathion methyl all yield phosphorothiolate impurities that differ only at the X leaving group, a common cholinesterase inhibition pathway is anticipated, namely, formation of an O,S-dimethyl phosphorothiolated serine hydroxyl (eq 3). The stereochemical course of the phosphorylation



(3)

also should be conserved, despite the process (inversion or retention) or X-group, to provide a unique chiral phosphorylated enzyme upon reaction with a single enantiomer (Scheme I). Thus, an investigation of the role of phosphorus stereochemistry upon postinhibitory events of phosphorothiolate-poisoned AChE using chiral isoparathion methyl as a representative probe was realized.

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(7) Dealkylation is usually more pronounced in cases where the OP-conjugate contains a secondary alkoxy group such as isopropyl (sarin) or pinacolyl (soman) rather than methyl. The mechanism has not been investigated when a thiomethyl linkage is present. Owing to the polarizability of the sulfur atom, thioalkyl bond scission may compete. (a) Bender, M. L.; Wedler, F. C. *J. Am. Chem. Soc.* **1972**, *94*, 2101. (b) Toia, R. F.; Casida, J. E. *Biochem. Pharmacol.* **1979**, *28*, 211. (c) Johnson, M. K.; Read, D. J.; Yoshikawa, H. *Pestic. Biochem. Physiol.* **1986**, *25*, 133. (d) Schumacher, M.; Camp. S.; Manlet, Y.; Neuton, M.; MacPhee-Quigley, C.; Taylor, P. *Nature* **1986**, *319*, 407. (e) Clothier, B.; Johnson, M. K.; Reiner, E. *Biochim. Biophys. Acta* **1981**, *660*, 306.

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Specifically, a study to delineate the relative rates of spontaneous reactivation, oxime-mediated reactivation, and non-reactivatability of isoparathion methyl-inhibited RBAChE was undertaken. The reaction of isoparathion methyl and *O,O,S*-trimethyl phosphorothiolate with 2-pyridinealdoxime methiodide (2-PAM) also was studied to aid the mechanistic interpretation.

Experimental Section

1. General Information. Kinetic studies were conducted on a Beckman DU-40 UV-visible spectrophotometer equipped with a Kinetic Soft-Pac Module. Proton (TMS internal standard) and phosphorus (H_3PO_4 external standard) NMR spectra were recorded on a Varian VXR at 300 MHz and 121.4 MHz (broad band decoupled) in $CDCl_3$, respectively. Analytical thin-layer chromatography (TLC) was conducted on aluminum-backed silica plates (E. Merck). Visualization was done with an ultraviolet lamp and/or heating with a 5% ether solution of 2,6-dibromoquinone-4-chloroimide (DBQ) stain. Gas chromatography was performed on a Hewlett Packard 5890A fitted with a 30-m, DB-1 capillary column at gas flow rates of 300 mL/min (air), 30 mL/min (hydrogen), and 15 mL/min (helium). Samples were analyzed using a ramp progression from 50 to 250 °C at 20 °C/min with injector and detector temperatures at 250 and 275 °C, respectively. Reversed-phase HPLC was conducted on a Beckman Model 334 HPLC using a 30-cm, 10 μm ODS column (Regis; Morton Grove, IL) with MeOH/H_2O (50:50) at a flow rate of 2.0 mL/min and detection and 270 nm.

2. Materials. A. General Procedures. *CAUTION! Isoparathion methyl is toxic. One should not attempt to handle this compound without proper training and adequate laboratory facilities.* Acetylthiocholine iodide (ATCh-I), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2-pyridinealdoxime methiodide (2-PAM), and 1,1′-trimethylenebis(4-formylpyridinium bromide) dioxime (TMB-4) were obtained from Sigma Chemical Co. (St. Louis). *O,O,S*-trimethyl phosphorothiolate (**3**) and methyl paraoxon (**6**) were available from a prior study.[19] Solubilized rat brain acetylcholinesterase was obtained as follows. Excised rat brains (previously stored at −78 °C) were rinsed with phosphate buffer and homogenized briefly at 0 °C in 10 mL of phosphate buffer. Triton X-100 (10 mL; 1% w/v solution) was added, and the solution was mixed gently for 15 min and centrifuged for 1 h at 100 000 g. The supernatant containing the solubilized AChE stock solution was removed and refrigerated at −78 °C. The protein content varied between 8.0 and 9.0 mg/mL as determined by the Bradford assay.[20]

Compounds **2a+**, **2a−**, and **2a±** were prepared as previously reported.[19] Chemical purity was checked by GC (t_R = 8.8 min), TLC (R_f = 0.18 in petroleum ether/ether, 1:3), P-31 NMR (δ 27.9 ppm), and RPHPLC (t_R = 8.5 min),[16] whereas the optical purity was checked by rotation and comparison to literature values.[19,21] Solutions of 3.04×10^{-4} M, 7.98×10^{-5} M, and 1.67×10^{-4} M for **2a+**, **2a−**, and **2a±**, respectively, were prepared weekly in MeOH and stored at −78 °C.

All kinetic experiments were carried out in 0.1 M phosphate buffer, pH 7.6 at 37 °C. The rat brain enzyme activity was determined by the spectrophotomtric method of Ellman using acetylthiocholine iodide as a substrate and modified for RBAChE analysis.[22] RBAChE treated with excess **2** showed only negligible increase in absorbance over the time course of the experiments.

B. *O*-Methyl-2-pyridiniumaldoxime Methiodide (4).[23] To 2-pyridinealdoxime methiodide (0.10 g; 0.379 mmol) dissolved in 3 mL of phosphate buffer (0.1 M, pH = 7.6) at room temperature was added dimethyl sulfate (0.105 g; 0.832 mmol). The temperature was raised to 45 °C, and after being stirred for 1 h, the mixture was lyophilized, and the remaining powder was extracted with 5-mL portions of warm CH_3CN to afford the methyl ether. ^1H NMR (D_2O): δ 8.53 (d, J = 6.4 Hz, 1 H), 8.45 (s, 1 H), 8.27 (t, J = 7.8 Hz, 1 H), 8.16 (d, J = 8.1 Hz, 1 H), 7.76 (t, J = 6.5 Hz, 1 H), 4.16 (s, 3 H), 3.49 (s, 3 H). ^{13}C NMR (D_2O): δ 148.56, 147.77, 146.70, 143.53, 129.04, 127.50, 56.95, 47.89.

3. Kinetics. All rate constants were determined through data analysis using a linear regression routine[24] from the slopes of the graphs obtained

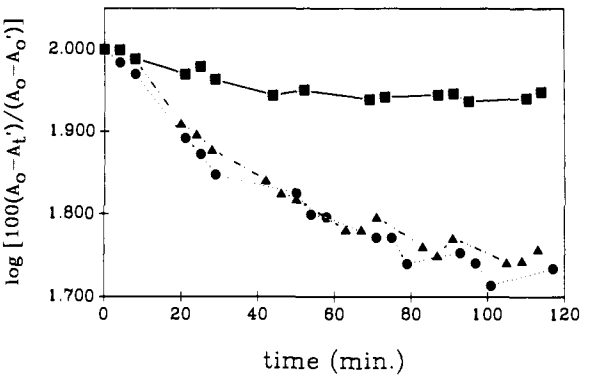

Figure 1. Spontaneous reactivation of rat brain acetylcholinesterase inhibited by chiral and racemic isoparathion methyl: (■) **2a+**; (●) **2a−**; (▲) **2a±**.

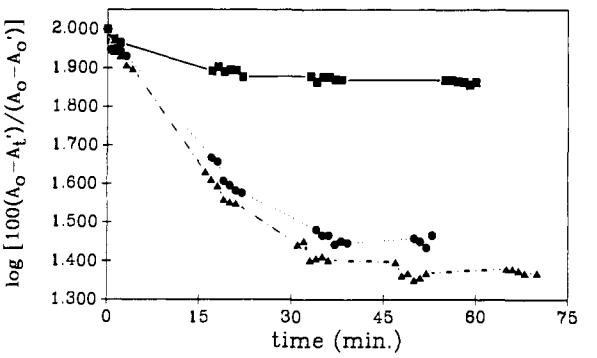

Figure 2. Oxime-mediated (2-PAM) reactivation of rat brain acetylcholinesterase inhibited by chiral and racemic isoparathion methyl: (■) **2a+**; (●) **2a−**; (▲) **2a±**.

(correlation coefficients > 0.98). The rate constants were expressed as statistical means with coefficients of variation from data sets of five to seven repetitions.

A. Spontaneous Reactivation. Control (A) and experiment (B) vessels were prepared as follows. Rat brain AChE stock solution (200 μL) was diluted to 1.0 mL with phosphate buffer. Methanol (5 μL) was added to vessel A, and 5 μL of an inhibitor solution that caused 90% inhibition was added to vessel B. Following a 20-min incubation at 37 °C, both vessels were diluted with 40 mL of phosphate buffer to halt further inhibition, and the enzyme activity was determined following a brief temperature equilibration. At selected time intervals, including a time point immediately following dilution (t = 0), 1-mL aliquots were transferred to cuvettes containing DTNB solution (3.33×10^{-4} M; 1.5 mL) and ATCh-I solution (7.5×10^{-2} M; 20 μL). Returned enzyme activity was followed for 2 h (occasionally to 24 h) by measuring the increase in absorbance at 412 nm. The rate constants of spontaneous reactivation (k_0) were calculated from the linear portion of the slopes (0–30 min) (Figure 1) according to eq 4:

$$2.3 \log [100(A_0 - A_t')/(A_0 - A_0')] = -k_0 t \qquad (4)$$

where $A_0 - A_t'$ = (AChE activity without inhibitor at time = 0) − (AChE activity of inhibited AChE at time = t) and $A_0 - A_0'$ = (AChE activity without inhibitor at time = 0) − (AChE activity of inhibited AChE at time = 0).

B. Oxime-Mediated Reactivation. Prior to the use of oxime reactivation agents, control studies were performed to establish the concentration limit of oxime use. Final concentrations of about 3×10^{-4} M for 2-PAM and 1×10^{-4} M for TMB-4[13a] caused interference in the colorimetric assay, and concentrations less than these values were used in the study. Control and experimental vessels were prepared as described for spontaneous reactivation. Following dilution with 40 mL of phosphate buffer, a 1-mL aliquot from each A (control) and B (experiment) was analyzed for residual activity (t = 0). The remaining solution from B was treated with 2-PAM (200 μL; 1×10^{-2} M) and vortexed at regular intervals for the duration of the experiment. At selected time points, 1-mL aliquots of B were transferred to cuvettes containing DTNB and ATCh-I and the enzyme activity was measured. The rate constants of oxime-mediated reactivation (k_{oxime}) were calculated from the linear portion of the slopes (0–20 min) (Figure 2) according to eq 5:

$$2.3 \log [100(A_0 - A_t')/(A_0 - A_0')] = -k_{oxime} t \qquad (5)$$

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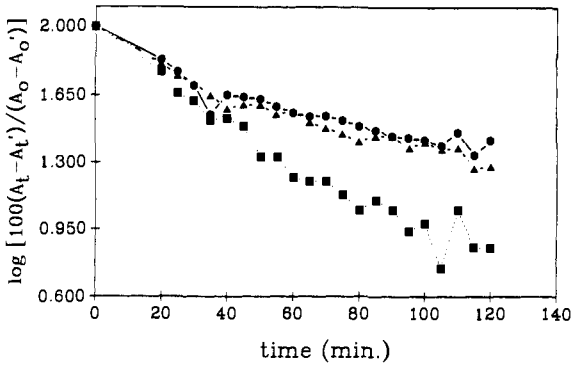


Figure 3. Decrease in reactivation versus time (non-reactivatability) following inhibition of rat brain acetylcholinesterase with chiral and racemic isoparathion methyl: (■) **2a+**; (●) **2a−**; (▲) **2a±**.

where $A_0 - A_t'$ = (AChE activity without inhibitor at time = 0) − (activity of reactivated AChE with oxime at time = t) and $A_0 - A_0'$ = (AChE activity without inhibitor at time = 0) − (activity of inhibited AChE without oxime at time = 0).

C. Non-Reactivatability. Rat brain AChE stock solution (200 μL) was diluted to 1.0 mL with phosphate buffer and incubated for 20 min with inhibitor to produce approximately 10–15% residual activity. Following the inhibition, the sample was diluted with 40 mL of phosphate buffer (t = 0), and at regular time intervals, 1-mL aliquots were withdrawn and added to a 1.52-mL solution containing DTNB and ATCh-I as noted previously to determine the enzyme activity that returned via spontaneous reactivation over the time course of this experiment. Concurrently, 2-mL aliquots were withdrawn and reacted with TMB-4 (10 μL; 2.0×10^{-3} M) or 2-PAM for 20 min. The enzyme activity was evaluated as described above to determine the amount of returned activity from oxime-mediated reactivation. The rate constants of non-reactivatability (k_{NR}) were calculated from the linear portion of the slopes (20–60 min) (Figure 3) according to eq 6:

$$2.3 \log [100(A_t - A_t')/(A_0 - A_0')] = -k_{NR}t \qquad (6)$$

where $A_t - A_t'$ = (activity of the reactivated enzyme with oxime at time = t) − (activity of the inhibited enzyme without oxime at time = t) and $A_0 - A_0'$ = (activity of the reactivated enzyme with oxime at time = 0) − (activity of the inhibited enzyme without oxime at time = 0).

D. Half-Life Calculations. All half-life ($t_{1/2}$) calculations were derived according to eq 7:

$$t_{1/2} = \ln 2/k(min^{-1}) \qquad (7)$$

4. Reaction of Phosphorothiolates with Nucleophiles. A. *O,O,S*-Trimethyl Phosphorothiolate and 2-PAM. Synthesis of *O,S*-Dimethyl Phosphorothiolate. To 1 mL of phosphate buffer was added 2-PAM (0.05 g; 0.189 mmol) and *O,O,S*-trimethyl phosphorothiolate (**3**) (0.029 g; 0.189 mmol) at 37 °C with stirring. Over three days, 100-μL aliquots were removed, diluted with D_2O, and analyzed via ^1H NMR. Examination of the ^1H NMR showed that *O,S*-dimethyl phosphorothiolate (**5**) had formed (cleavage of the P–OMe bond) in a yield of 43%. No 2-PAM methyl ether (**4**) was observed. ^1H NMR (D_2O): δ 3.42 (d, J = 12.5 Hz, 3 H), 1.97 (d, J = 13.6 Hz, 3 H).

B. Isoparathion Methyl and 2-PAM. To 1 mL of phosphate buffer was added 2-PAM (0.05 g; 0.189 mmol) and isoparathion methyl (0.05 g; 0.190 mmol) at 37 °C with stirring. The reaction was monitored by ^1H NMR. Loss of the *p*-nitrophenoxy moiety was seen to give the same NMR (doublets at 3.43 and 1.98 ppm) as experiment 4A.

C. Phosphorothiolate Hydrolysis. *O,O,S*-Trimethyl phosphorothiolate (**3**) (0.050 g; 0.320 mmol) or isoparathion methyl (**2a**) (0.050 g; 0.190 mmol) was dissolved in 1 mL of phosphate buffer at 37 °C. The hydrolysis was monitored by ^1H NMR over a period of 3 days for **3** or 8 h for **2a**.

Results

The chiral phosphorothiolates **2a−** and **2a+** are easily prepared and are reactive inhibitors of several different cholinesterases.[18,19,21] Racemic isoparathion methyl (**2a±**) also was studied as a substrate, since commercial mixtures would contain the racemate. For two reasons, RBAChE was chosen as the enzyme source for this study. First, warm-blooded mammals may be inadvertently exposed to phosphorothiolates via commercial insecticide application and the outcome of postinhibitory pathways could impact upon the resultant toxicology. Second, we showed previously that rat brain cholinesterase can best differentiate the stereoisomers of isoparathion methyl at 37 °C.[18]

Table I. Inhibitory[18] (M^{-1} min^{-1}) and Postinhibitory Rates (min^{-1})[a] for RBAChE Poisoned by the Stereoisomers of Isoparathion Methyl

process	2a−	2a±	2a+	ratio 2a−/2a+
inhibition (k_i, ×10⁴)	63.4 (0.04)	41.6 (0.01)	7.7 (0.02)	8.3
spontaneous reactivation (k_0, ×10⁻²)	1.13 (0.09)	1.02 (0.10)	0.35 (0.11)	3.23
oxime[b] reactivation (k_{oxime}, ×10⁻²)	4.10 (0.11)	4.57 (0.13)	1.16 (0.07)	3.53
non-reactivatability (k_{NR}, ×10⁻²)	1.42 (0.09)	1.37 (0.04)	3.07 (0.10)	0.46

[a] Coefficient of variation = SD ÷ $\bar{x}$ (in parentheses). [b] 2-PAM.

Table II. Total Percent (%) Reactivation[a] following Inhibition of RBAChE by the Stereoisomers of Isoparathion Methyl

reactivation	2a−	2a±	2a+
spontaneous	43.82 (0.22)	35.89 (0.28)	12.10 (0.14)
oxime-mediated			
(2-PAM)	70.79 (0.09)	73.91 (0.06)	29.67 (0.13)
(TMB-4)	87.54 (0.05)	88.11 (0.03)	30.05 (0.07)

[a] Coefficient of variation = SD ÷ $\bar{x}$ (in parentheses).

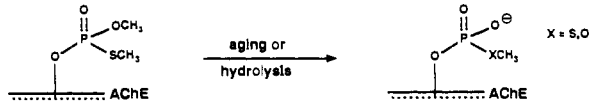
To precisely measure anticipated modest stereochemical differences, rigorous purification and analysis of the isoparathion methyl enantiomers were required. Chiral isoparathion methyl stereoisomers were prepared monthly by convergent routes using either the proline amide diastereomer method[19] or alkaloid salt.[21] Since isoparathion methyl is somewhat labile in base (and buffer), inhibitor solutions were closely monitored for decomposition.

In a typical kinetic assay, the enzyme solution was incubated with **2a+**, **2a−**, or **2a±** until 85–90% inhibition was achieved. The incubation mixture was then diluted 40-fold to terminate continued inhibition. Following this dilution, the enzyme was (a) monitored for activity versus time (spontaneous reactivation), (b) treated with oxime antidote and monitored for activity versus time (oxime-mediated reactivation), or (c) separated into aliquots and treated at various time intervals with oxime and the activity monitored versus time (non-reactivatability).

1. Spontaneous Reactivation. RBAChE inhibited by **2a+**, **2a−**, or **2a±** undergoes a time-dependent recovery of activity corresponding to the nature of the phosphorylating agent. Figure 1 shows the time course of spontaneous reactivation of RBAChE following inhibition by **2a+**, **2a−**, and **2a±**. The k_0 values, calculated according to eq 4, are shown in Table I. RBAChE inhibited by **2a−** reactivated 3.23-fold faster ($t_{1/2}$ = 61.3 min) than enzyme inhibited by **2a+** ($t_{1/2}$ = 198.0 min). The extent (total percent reactivation) that the inhibited enzyme reactivates depended upon the form of the inhibitor. After 2 h, only 12.1% of **2a+** inhibited RBAChE reactivated, whereas the **2a−** and **2a±** inhibited RBAChE reactivated to 43.8% and 35.9%, respectively (Table II). Further, the change in percent reactivation for the **2a+** inhibited species significantly decreased over the time course of the experiment, reaching a plateau value. The kinetic rate profile and k_0 value obtained from **2a±** inhibited RBAChE ($t_{1/2}$ = 68.0 min) closely resembled the results obtained from the **2a−** inhibited RBAChE.

2. Oxime-Mediated Reactivation. Figure 2 shows the time course of oxime-mediated reactivation of RBAChE inhibited by **2a+**, **2a−**, and **2a±**. The k_{oxime} values (Table I) were calculated according to eq 5, and the total reactivation restored is shown in Table II. As expected, the enzyme activity was restored to a greater extent and at an enhanced rate (3–4-fold) as compared to the case of spontaneous reactivation. Similar relative rate constants to the spontaneous reactivation were obtained, namely, **2a−** inhibited RBAChE reactivated 3.53-times faster ($t_{1/2}$ = 16.9 min) than the **2a+** inhibited species ($t_{1/2}$ = 59.8 min), and were identical when either 2-PAM or TMB-4 was used. The k_{oxime}

Scheme II

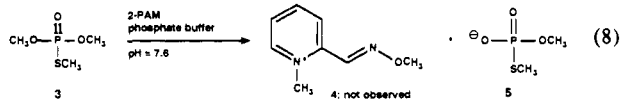


obtained from RBAChE inhibited by **2a±** was not significantly faster ($t_{1/2}$ = 15.2 min) than that for **2a−** inhibited RBAChE. The oxime reactivating agents restored 70–90% of the total activity (TMB-4 was slightly more effective) from **2a−** and **2a±** inhibited RBAChE, but only 30% from **2a+** inhibited RBAChE (Table II). Thus, the reactivation of RBAChE inhibited by *O,S*-dimethyl phosphorothiolates was influenced by the configuration of the phosphorylated enzyme but not the nature of the oxime. The oximes were unable to restore fully the enzyme activity.

3. Non-Reactivatability. RBAChE was inhibited with **2a+**, **2a−**, and **2a±** and treated with oxime reactivators following various incubation times. The amount of reactivity restored at a given point represents the amount of "reactivatable" enzyme species. Inactivated enzyme that had aged or undergone one or more of the aforementioned time-dependent, non-reactivating processes (e.g., protein conformational change, hydrolysis, etc.) does not contribute to the rate of substrate hydrolysis. Since the precise mechanism of the enzyme's overall refractory nature has not yet been established, it is defined as non-reactivatability, which takes all possibilities into account. The rate constants for non-reactivation (k_{NR}) were determined according to eq 6 and are shown in Table I. The **2a+** inhibited RBAChE underwent a non-reactivation rate twice that ($t_{1/2}$ = 22.6 min) of the **2a−** inhibited RBAChE ($t_{1/2}$ = 48.8 min), whereas RBAChE inhibited by **2a±** was similar in profile and rate ($t_{1/2}$ = 50.6 min) to RBAChE inhibited by **2a−**.

Since oxime nucleophiles reactivate the inhibited enzymes by nucleophilic attack upon the phosphorus atom followed by scission of the phosphorus–serine ester bond, it had occurred to us that cleavage of the thiomethyl moiety may be an alternate pathway. Previously, the thiomethyl group was shown to be a superior leaving group in the phosphorylation of electric eel acetylcholinesterase by methamidophos.[25] Also, certain oxime nucleophiles are known to dealkylate esters. If either path occurs, the serine moiety would now bear a phosphate anion (equivalent to an aged protein) and the enzyme would have been inadvertently rendered inactive. To test this possibility, we studied the chemical reaction between two different phosphorothiolates and 2-PAM to understand better the relative reactivity of the phosphorothiolate linkage.

4. Reaction of *O,O,S*-Trimethyl Phosphorothiolate and Isoparathion Methyl with 2-PAM. Since the phosphorothiolated enzyme bears two alkoxy and one thiolester bond, *O,O,S*-trimethyl phosphorothiolate (**3**) was selected to serve as a model for the mode of reaction with 2-PAM. Reaction of **3** with 2-PAM gives hydrolysis of the phosphorus–methyl ester linkage to form *O,S*-dimethyl phosphorothiolate (**5**) after 48 h at 37 °C (eq 8). The



(8)

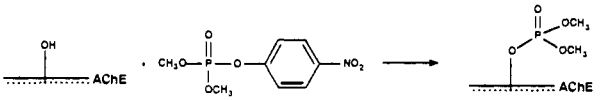
product was identified by ^{1}H NMR and comparison to standard material, which showed doublets centered at 3.42 and 1.97 ppm in D_2O in a 1:1 integration corresponding to OCH_3 and SCH_3 groups, respectively. To ensure that no dealkylation was taking place, 2-PAM methyl ether (**4**) was prepared separately (see Experimental Section), and the ^{1}H NMR was recorded and used as a standard. Particular attention was focused upon the methyl ether singlet (3.49 ppm). However, no peak corresponding to this moiety was observed. This result suggests that dealkylation is not a favored path. Further, 2-PAM methyl ether (**4**) was stable under the reaction conditions for 48 h, excluding the possibility that 2-PAM dealkylated the phosphorothiolate to afford **4**, followed by decomposition or hydrolysis back to oxime.

Discussion

Previous research showed that **2a+** and **2a−** were useful and representative irreversible inhibitors of the general class of *O,S*-dimethyl phosphorothiolates, materials found as impurities in certain organophosphate insecticides.[18] Compound **2a−** showed 3–15-fold greater inhibitory potency than **2a+** against four different sources of AChE.[18] The spontaneous reactivation rate constant was 3.23-fold greater for the **2a−** inhibited RBAChE than for the **2a+** inhibited RBAChE, and therefore, a single stereoisomer dominates both forward and reverse kinetic processes. Since spontaneous reactivation occurs by reaction of H_2O/OH^- at the phosphorus stereocenter, the stereochemical influence upon the rate constants was anticipated.

Clear differences between enantiomer k_0 rates were found, yet the k_0 for **2a±** was similar to that for the **2a−** kinetic profile. This result must be addressed because the lower reactivating ability of the **2a+** inhibited enzyme would go unnoticed in a kinetic study of the racemate. The behavior of the racemic-inhibited RBAChE can be explained by first consideration of the stereoselective inhibition of RBAChE by isoparathion where **2a−** is an 8-fold more potent anti-RBAChE than **2a+** at 37 °C (Table I). Therein, when RBAChE is inhibited by **2a±**, an 89:11 ratio of **2a−** to **2a+** inhibited RBAChE is obtained. Addition of the corresponding mole fractions of rate constants from **2a−** inhibited ($0.89(11.3 \times 10^{-3})$ min^{-1}) and **2a+** inhibited ($0.11(3.5 \times 10^{-3})$ min^{-1}) RBAChE affords a theoretical k_0 (**2a±**) of 10.4×10^{-3} min^{-1}, which compares favorably with 10.2×10^{-3} min^{-1} from our experiments. Accordingly, the k_0 obtained from **2a±** inhibited RBAChE preferentially reflects enzyme inhibited by **2a−** because this inhibited RBAChE is the dominant form entering the postinhibitory study. Similar stereochemical influences have been reported by Berman and Decker.[14i]

The k_0 values also are in good agreement with those found for methyl paraoxon (**6**; *O,O*-dimethyl *p*-nitrophenoxyphosphate), which reacts with RBAChE to form the achiral, O,O-dimethyl phosphorylated enzyme (eq 9) with a k_0 of 6.10×10^{-3} min^{-1}.[13a]



6: methyl paraoxon

(9)

The only difference between methyl paraoxon and **2a** is the single substitution of a sulfur for an oxygen ester atom. Paraoxon was found to deviate from linear first-order reactivation kinetics after 30 min, similar to **2a−** and **2a±** inhibited enzymes, which undergo a time-dependent decrease in reactivatability after 80 min.[13a] Since the **2a+** inhibited RBAChE only regains 12% of the initial activity, the importance of stereochemistry is reinforced even when a minor structural (atomic) alteration is being considered.

When a thiolate linkage is substituted for an ester linkage on the phosphoryl moiety in the active site, the overall effect is not easily judged. However, its relative contribution to the phosphoryl moiety itself can be estimated. Desktop minimization of *O,O,S*-trimethyl phosphorothiolate vs *O,O,O*-trimethyl phosphate revealed that a sulfur atom adds between 11 and 13 Å^2 to the water solvation shell (a 5% total increase). This difference alone does not appear significant enough to argue for a steric bias. A lack of steric bias between thionate (P=S) and oxon (P=O) inhibitors has been noted.[26]

Certain nucleophilic oximes are known to restore protein activity following irreversible inhibition by an organophosphate. Our results showed that RBAChE inhibited by **2a−** could have its activity almost fully restored whereas enzyme inhibited by **2a+** could only be restored to one-third of its initial activity. When subtracted from the total activity restored from spontaneous processes, the oximes could only restore a modest amount of activity. This feature was particularly noticeable in **2a+** inhibited

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RBAChE, which when treated with 2-PAM or TMB-4, recovered only an additional 18% of the total activity. These data suggest that irreversible, postinhibitory mechanisms are operative immediately following inhibition by **2a+**. Again, the oxime-mediated reactivation kinetics of **2a±** and **2a−** inhibited RBAChE were similar. Mole fraction calculations were conducted on the oxime-mediated process to give $k_{oxime} = 3.78 \times 10^{-2}$ min^{-1}, which compares reasonably with the observed (4.57×10^{-2} min^{-1}), although these values are not in as precise agreement as the k_0 values.

The percent spontaneous reactivation following inhibition of RBAChE by **2a+** was 12%, leaving 88% in the inhibited or non-reactivated form (Table II). This aspect is further highlighted by the fact that k_{NR} is approximately 9-fold k_0, indicating that RBAChE phosphorylated by **2a+** undergoes very little spontaneous reactivation in the first 30 min. In contrast, k_0 and k_{NR} values obtained from **2a−** inhibited RBAChE were similar, suggesting that spontaneous reactivation and non-reactivation were competing postinhibitory processes. The larger mole fraction of **2a−** inhibited RBAChE compared to **2a±** inhibited RBAChE contributed to making k_{NR} analogous to the k_0 and k_{oxime} values for racemic-inhibited RBAChE. The theoretical k_{NR} based on mole fraction for **2a±** inhibited RBAChE is 1.57×10^{-2} min^{-1}, comparable with the observed 1.37×10^{-2}.

Our chemical model studies on the mechanism of non-reactivatability showed that an $-OCH_3$ moiety was released in the reaction of 2-PAM with *O,O,S*-trimethyl phosphorothiolate. No 2-PAM methyl ether was observed, suggesting that the mechanism favored hydrolysis via first formation of the putative phosphorylated 2-PAM, over a dealkylation pathway. This result is consistent with hydrolysis data for methamidophos (*O,S*-dimethyl phosphoramidothiolate), which undergoes preferential P–OMe scission in aqueous base.[27] Therefore, a majority of the non-reactivation probably proceeds via hydrolysis of the O-methyl group rather than the S-methyl, which does not necessarily follow prediction based upon phosphorylation leaving group ability.[25]

Conclusions

The kinetic data indicate that phosphorothiolates bearing a center of asymmetry at phosphorus differ in their postinhibitory mechanisms and their inhibitory potency. Despite the greater sensitivity of RBAChE toward inactivation by **2a−**, the RBAChE could recover from poisoning by this stereoisomer. Conversely, RBAChE phosphorylated by **2a+** may be more susceptible to a cumulative inactivation, owing to inability of the enzyme to be reactivated.

Acknowledgment. This work was supported by the National Institute of Environmental Health Sciences (Grant ES04434), and this support is gratefully acknowledged. The authors thank Debra Quinn for technical assistance and Loyola University of Chicago for the purchase of the Varian NMR spectrometer used in this study and partial support through the BRSG.

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Intramolecular Ureido and Amide Group Participation in Reactions of Carbonate Diesters

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Abstract: The cyclization of ethyl and phenyl 2-ureidophenylcarbonates in H_2O at 30 °C involves two discrete steps with benzoxazolinone as the final product. The formation of benzoxazolinone is quantitative. Phenol is released in the initial step from both esters in an apparent OH^--catalyzed reaction, which shows that intramolecular nucleophilic attack by the ureido group is via an anionic species. The pH–rate constant profile for the second step in the reaction of the ethyl ester is sigmoidal with $pK_{app} = 8.9$. The initial reaction involves a rearrangement, and the neighboring phenoxide ion of the intermediate participates in the second step. In view of the D_2O solvent isotope effect ($k_{H_2O}/k_{D_2O} = 1.2$), this participation must be via a nucleophilic mechanism. The second step of the reaction of the phenyl ester, in which benzoxazolinone is formed, involves an apparent OH^--catalyzed reaction of a cyclic intermediate. This intermediate was identified as *N*-carbamoylbenzoxazolinone, which thereby indicates that the initial nucleophilic attack is by nitrogen through a 5-membered-ring transition state. *p*-Nitrophenol release from *p*-nitrophenyl 2-ureidophenylcarbonate is only 18-fold faster than phenol release from the corresponding phenyl ester. Ratios of k_{OH}(ortho) for phenol release from the 2-ureido-substituted esters to k_{OH}(para) for OH^--catalyzed hydrolysis of the corresponding 4-ureido-substituted esters are approximately 10^4 in all cases. In the intramolecular nucleophilic reactions of the ureido-substituted carbonate esters, a neutral species reaction is not observed, even at pH values as low as 3, in contrast with substituted benzoate esters having phenolic leaving groups. Also, in the apparent OH^--catalyzed reactions of the carbonate diesters, only one cyclic product is obtained, whereas both oxygen and nitrogen attack occur in the nucleophilic reactions of carboxylate esters. The neighboring amide group of *p*-nitrophenyl *o*-(carboxamido)phenylcarbonate participates with nitrogen attack and apparent OH^- catalysis. Intramolecular attack of nitrogen provides a rate enhancement of 10^3 in the release of *p*-nitrophenol over the OH^--catalyzed hydrolysis of the para-substituted compound. Thus, in the intramolecular nucleophilic reactions of the carbonate diesters, nitrogen anion attack takes place preferentially when such attack is sterically favored (five-membered-ring transition state) and when there is an equal opportunity for oxygen or nitrogen attack.

A number of important enzymes that catalyze carboxylation reactions require biotin as a cofactor.[1,2] All of these processes occur by two partial reactions in which biotin serves as a HCO_3^- acceptor and carboxyl carrier. The reactions require adenosine 5′-triphosphate (ATP) and involve a carboxybiotin intermediate (eq 1). *N*-Carboxybiotin was isolated from reactions catalyzed by β-methyl crotonyl-COA carboxylase[2] and will serve as a substrate in enzymatic reactions.[3] However, it was pointed out

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