In Vitro and in Vivo Protection of Acetylcholinesterase against Organophosphate Poisoning by Pretreatment with a Novel Derivative of 1,3,2-Dioxaphosphorinane 2-Oxide†

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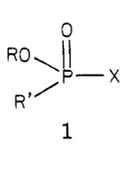
Covalent molecular combinations of a cyclic phosphate (dioxaphosphorinane) and a potential leaving group, such as 3-(trimethylammonio)phenol iodide (TMPH), suggested the synthesis of *O*-[3-(trimethylammonio)phenyl]-1,3,2-dioxaphosphorinane 2-oxide iodide (TDPI). TDPI inhibited acetylcholinesterase (AChE) ($k_i = 8.4 \times 10^3$ M^{-1} min^{-1}) via the formation of an unstable covalent intermediate. TDPI-inhibited AChE hydrolyzed spontaneously with $t_{1/2} \approx 10$ min. Butyrylcholinesterase (BuChE) was also inhibited by TDPI ($k_i = 1.8 \times 10^4$ M^{-1} min^{-1}), but the inhibited BuChE was more stable (>10 times) than the corresponding AChE–TDPI conjugate. Pretreatment of mice with TDPI conferred protection against 22 LD_{50}'s of paraoxon and 5 LD_{50}'s of soman, provided that treatment with anticholinergics and an oxime followed administration of these anticholinesterase poisons. Correlation between in vitro and in vivo observations suggests that the main protection of AChE conferred by TDPI results from temporary masking of the active site of the enzyme. The acute toxicity of TDPI was found to be 444 mg/kg (sc, mice), whereas analogous carbamates and a noncyclic phosphate also displaying antidotal properties are >170 times more toxic.

Standard treatment of organophosphate poisoning, due to compounds of the general formula **1**, involves a com-



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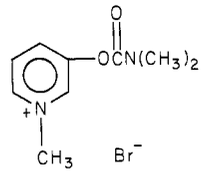
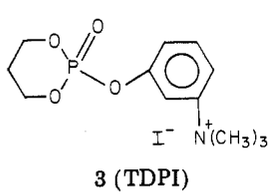
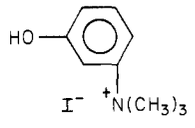


2 (pyridostigmine bromide)



3 (TDPI)

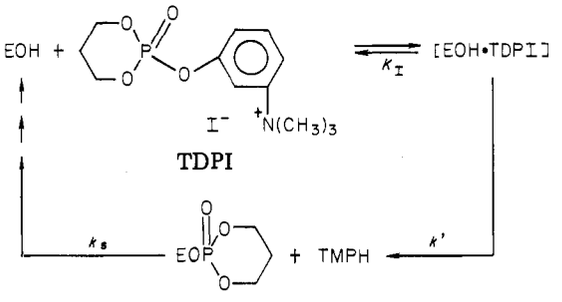


4 (TMPH)

1, R = alkyl or aryl; R' = alkyl, aryl, alkyloxy or aryloxy; X = F, *p*-nitrophenoxy, alkylthiolate, etc.

bination of an anticholinergic agent (atropine sulfate) and an oxime reactivator to dephosphorylate the inhibited acetylcholinesterase (AChE, EC 3.1.1.7).[1,2] However, these drugs exhibit low efficiency in animals poisoned with oxime-resistant organophosphates, such as 1,2,2-trimethylpropyl methylphosphonofluoridate (soman).[2-4] It is, therefore, of particular interest that a carbamate, such as pyridostigmine bromide, **2** (a quaternary drug that cannot cross the blood–brain barrier),[5] was found to confer antidotal protection against poisoning by soman and other organophosphates.[6,7] This protection was attributed to the partial inhibition of AChE activity and the slow hydrolysis of the carbamoylated enzyme.[6,8] Physostigmine[6,8,9] and several other carbamates[6,8] were also reported to be efficient in pretreratment against organophosphate poisoning. In all cases studied, protection was efficient, provided that the carbamate administered prior to the challenge and standard therapy (anticholinergic and oxime) followed the administration of the organophosphate poison.[6] Gordon et al.[6] report that the maximum sign-free doses of pyridostigmine and physostigmine are 0.1 and 0.16 mg/kg, respectively, and that their acute toxicities (LD_{50}, guinea pigs) range between 1 and 5 mg/kg. In 1971, Berry et al.[10] demonstrated that tetraethyl pyrophosphate

Scheme I[a]



[a] EOH = AChE

[TEPP, **1**, R = C_2H_5; R' = OC_2H_5; X = $OP(O)(OC_2H_5)_2$], when used prophylacticaly in conjunction with anticholinergic and oxime reactivator, protected animals against soman poisoning. However, the sign-free dose of TEPP was reported to be 0.2 mg/kg.[10]

The reactions of carbamates and organophosphorus compounds with AChE proceed by an identical mechanism,[4,11] i.e., covalent binding to a serine hydroxy at the active site. It is, therefore, of interest to explore the potential application of relatively nontoxic organophosphorus anticholinesterase as a pretreatment of animals against organophosphate poisoning. We report here on the synthesis and biological properties of the novel antidote, *O*-[3-(trimethylammonio)phenyl]-1,3,2-dioxaphosphori-

† A preliminary communication of this work has been presented at an ACS Symposium (Phosphorus Chemistry) held at Duke University, Durham, NC, in 1981. See *ACS Symp. Ser.*, **no. 171** (1981).

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nane 2-oxide iodide (TDPI, 3).

The design of 3 (TDPI) was based on the covalent binding of two moieties that have been reported to display biological properties that are pertinent to counteracting organophosphate poisoning. First, 1,3,2-dioxaphosphorinane 2-oxides have been shown to produce an unstable phosphoryl-AChE that undergoes spontaneous reactivation at pH 7.0 with $t_{1/2}$ = 12 min.[11,12] The anticholinesterase activity of the cyclic organophosphates is relatively poor,[11-13] and it therefore seemed worthwhile to search for an appropriate leaving group for increasing the inhibition potency of this series. This might allow reasonable rate of approach to steady state and adequate amount of AChE inhibition (Scheme I). It was assumed that recovery of enzyme activity after inhibition with any 1,3,2-dioxaphosphorinane 2-oxide inhibitor should be the same irrespective of the leaving group. Secondly, 3-(trimethylammonio)phenol (4) was selected as an appropriate leaving group for the following reasons: (1) Introducing a quaternary nitrogen should increase the rate of AChE inhibition,[4,14] while restricting phosphorylation of AChE in vivo to peripheral tissues. It has been previously shown[5-7] that good antidotal efficacy does not require the penetration of the protective drug through the blood–brain barrier. We assumed that by limiting the activity of 3 to peripheral tissues, we may obtain improved antidotal efficiency by not exposing the brain to additional stress. (2) Compound 4 and its structural analogues are extremely potent, reversible inhibitors of AChE.[15-17] These compounds were previously shown to protect in vitro eel[18] and rat-hemidiaphragm[16] AChE from irreversible phosphorylation. (3) Compound 4 is also the leaving group of neostigmine, a known powerful anticholinesterase carbamate.[4,19] (4) Phenyl esters, such as TDPI, were predicted to be relatively stable in neutral aqueous solutions at 25–37 °C.[11,14] Thus, we postulated that the newly designed drug would display useful effects as an antidote, exhibit low toxicity, produce a phosphorylated AChE subject to rapid spontaneous hydrolysis, and respond to oxime reactivators.

Experimental Section

Melting points were determined in open glass capillaries with a Thomas-Hoover Unimelt apparatus and are uncorrected. NMR spectra were recorded on Varian XL 100 and JEOL C 60 HL spectrometers for ^{31}P and ^{1}H, respectively. Chemical shifts are reported in parts per million relative to 85% H_3PO_4 and $(CH_3)_4Si$ (or DSS) as references for ^{31}P and ^{1}H, respectively. TLC was carried out on silica-coated glass plates (25 mm × 75 mm). All in vitro enzymatic studies were done in a Pye-Unicam SP 1800 UV spectrophotometer with a Unicam AR-25 linear recorder. Radioactive assays were conducted in a Packard Model B460 CD liquid scintillation counter. Hemidiaphragm stimulations were initiated with a Grass S88 stimulator, and contractions were recorded with a Myograph F-60 on physiograph recorder DMP-48 (Narco BioSystems).

Animals. Albino male mice, ICR strain (20–22 g), and Sprague-Dawley rats (250–350 g) were used throughout this work.

Materials. Electric eel AChE (EC 3.1.1.7, 1400 units/mg) and horse serum BuChE (EC 3.1.1.8, 6 units/mg) were purchased from Worthington Biochemicals Corp. A gift of 11S electric eel AChE (1 mg/mL), purified as described by Dudai et al.,[20] was granted by Dr. Amitai of this laboratory. Acetylcholine (ACh), acetylthiocholine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and diethyl *p*-nitrophenylphosphate (paraoxon) were obtained from Sigma Chemical Co. Tritiated acetylcholine ([^{3}H]ACh, 0.6 Ci/mmol) was a product of Amersham/Searl Corp. Atropine sulfate and benactyzine hydrochloride were obtained from Merck & Co. and Aldrich Chemical Co., respectively. Pyridostigmine bromide was a product of Hoffmann-La Roche. The following compounds were prepared in our laboratory according to published procedures: soman,[21] 2-(hydroxyiminomethyl)-1-methylpyridinium iodide and its β analogue (2- and 3-PAM, respectively),[22] 1,3-bis(4-hydroxyiminomethyl-1-pyridinium) propane dibromide (TMB-4);[23] 1,1'-[oxybis(methylene)]bis[4-[(hydroxyimino)methyl]-pyridinium dichloride (toxogonin).[24] 3-(Dimethylamino)phenol and methyl iodide were obtained from Fluka. The syntheses of 3 and the potential leaving group 4 are outlined below.

Methods. *O*-[3-(Trimethylammonio)phenyl]-1,3,2-dioxaphosphorinane 2-Oxide Iodide (TDPI, 3). A solution of 2-chloro-1,3,2-dioxaphosphorinane 2-oxide[25] (48.0 g, 0.3 mol) in dry benzene (50 mL) was added dropwise to a solution of 3-(dimethylamino)phenol (42.0 g, 0.3 mol) and triethylamine (freshly distilled, 30.3 g, 0.3 mol) in 250 mL of dry benzene. The mixture was refluxed for 4 h and cooled to room temperature, and the triethylamine hydrochloride was filtered off. After washing with cold water, followed by cold 10% aqueous NaOH, the benzene solution was dried over $MgSO_4$ and filtered, and the filtrate was concentrated under reduced pressure. The resultant viscous oil was chromatographed on silica ($CHCl_3$) to give 31.5 g of pure and homogeneous (TLC, 5% MeOH/$CHCl_3$), *O*-[3-(dimethylamino)phenyl]-1,3,2-dioxaphosphorinane 2-oxide: yield 41%; ^{1}H NMR ($CDCl_3$) δ 1.6–2.3 (m, 2 H, CCH_2C), 2.85 [s, 6 H, $N(CH_3)_2$], 3.90–4.60 (m, 4 H, CH_2O), 6.30–6.60 (m, 3 aromatic H), 7.0–7.2 (m, 1 aromatic H); ^{31}P NMR ($CDCl_3$) δ −13.3.

The foregoing preparation (11.0 g, 0.043 mol) and methyl iodide (20.0 g, 0.14 mol) were refluxed in 100 mL of acetone for 2 h. The precipitate was filtered, and the residue was recrystallized from 95% ethanol and dried over P_2O_5 to give 8.9 g of pale yellow rhombic crystals of 3: mp 178–181 °C dec; yield 52%. ^{1}H NMR (D_2O, DSS) δ 1.80–2.50 (m, 2 H, CCH_2C), 3.70 [s, 9 H, $N(CH_3)_3$], 4.40–4.90 (m, 4 H, CH_2O), 7.50–7.80 (m, 4 aromatic H): ^{31}P NMR δ −13.8 (Me_2SO-d_6), −11.3 (D_2O). Anal. ($C_{12}H_{19}INO_4P$) C, H, N. One equivalent of 4 (see below) was released upon hydrolysis of 3 in 0.1 N NaOH (see Methods). The concentration of 4, if present at all in the purified preparation of 3, was less than 0.2% as measured at 280 nm.

3-(Trimethylammonio)phenol Iodide (TMPH, 4). 3-Dimethylaminophenol and methyl iodide were mixed in ether and left at room temperature overnight. The precipitated crystals were collected and dried over P_2O_5: mp 185–187 °C (lit.[15] 183–185 °C).

Hydrolysis of 3. The rate of hydrolysis of 3 was measured by monitoring (280 nm) the formation of 4 at 25 °C, pH 7.0 (0.1 M phosphate buffer). At this wavelength, the absorption of 3 is well separated from the free phenol 4. Alkaline hydrolysis was monitored directly at 292 nm and followed to completion.

Enzyme Assay in Homogeneous Solutions. The activities of AChE and BuChE during the inhibition and reactivation studies in vitro were measured by the Ellman procedure,[26] at pH 7.0 in 0.1 M phosphate buffer, as described elsewhere.[11,12]

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AChE Determinations in the Hemidiaphragm and Whole Blood. Surface activity of the phrenic-nerve diaphragm preparation (rats) was assayed in accordance with a modified procedure outlined by Mittag et al.[16] and Johnson and Russel.[27] Tritiated ACh [$C^3H_3C(O)OCH_2CH_2N(CH_3)_3^+$]$I^-$ and cold ACh were added to a 30-mL bath at a final concentration of 0.055 mM (3.3 μCi/mL). Duplicates of 0.1 mL were removed at various time intervals and processed as described by Johnson and Russel.[27] Counts were corrected for background and for sponteneous hydrolysis of the diluted [^{3}H]ACh, at 32 °C, pH 7.4. Total activity of [^{3}H]ACh was determined by incubating 0.1 mL of bath solution (at t = 0) with 0.1 mL of electric eel AChE (1400 units/mL) for 10 min. Whole blood ChE activity was measured radiometrically by employing the procedure described above.

Inhibition and Reactivation of AChE and BuChE in Homogeneous Solutions. Inhibition of AChE or BuChE and the spontaneous or oxime-induced reactivation of the phosphorylated enzymes were performed as described earlier for 2-fluoro-1,3,2-dioxaphosphorinane 2-oxide.[11,12] Stock solutions of **3** (1–10 mM) were prepared in distilled water and diluted as required into the enzyme solution ($\sim 5 \times 10^{-9}$ M) at t = 0. The rate of inhibition was followed by extensive dilution (>200-fold) into the assay cuvette. Direct measurement of both the spontaneous recovery and the induced reactivity of the inhibited enzyme was initiated by dilution (>100) of an inhibited enzyme (5×10^{-8} M) (at t = 0) into the reactivation medium. Under these conditions, inhibition of enzyme by inhibitor residues (<0.1 μM) was found to be negligible. At suitable time intervals, 25–50 μL was diluted into 3.12 mL of phosphate buffer and assayed for 1 min as described above. The final concentration of oxime (1–10 μM) in the assay cuvette did not increase the spontaneous hydrolysis of acetylthiocholine. Detailed experimental conditions are included in footnotes to the tables and in the legends of the figures. We note that the concentration of either 3- or 2-PAM in the above-mentioned experiments were well above (>100) the concentration of AChE. Consequently, pseudo-first-order kinetics for these inhibition and reactivation reactions obtained.

Inhibition (and Reactivation) of Hemidiaphragm AChE by Either 3 or Soman. The inhibition of surface AChE activity by either **3** or soman was conducted in the presence of 0.055 mM ^{3}H-labeled ACh. Each diaphragm served as its own control by measuring the hydrolysis of acetylcholine during the first 30 min of the experiment (100% activity). At t = 0, either soman or **3** was added, and the hydrolyzed acetylcholine was measured at 15-min intervals, as described above. The rate of recovery of enzyme activity was measured immediately after the removal of the inhibitors from the hemidiaphragm preparation by extensive washing and adding of [^{3}H]acetylcholine to the bath, as described earlier. In control experiments, the hydrolysis of acetylcholine was found to be zero order over the time required to complete the experiment. The results are expressed as percent AChE activity relative to the initial 30 min, where only acetylcholine was present in the medium. Stock solutions of soman were prepared in propylene glycol and diluted at least 10^4-fold into the incubation bath. The specific experimental conditions are given in the legends to the figures.

Inhibition of AChE in Vitro by 4. The dissociation constant of the reversible complex formed between AChE and **4** was measured in the presence of acetylthiocholine (0.1–0.005 mM), and calculated in accordance with the equation of Hunter and Downs[28] as previously described.[12]

Aging of Inhibited AChE and BuChE. 3-inhibited enzyme was diluted extensively (>200-fold in the case of AChE) into the reactivation medium containing 0.5 mM 2-PAM. The degree of aging was defined as the fraction of enzyme that could not be reactivated after 24 h of incubation at 25 °C (pH 7.0, 0.1 M phosphate buffer). Suitable controls, not inhibited with **3**, were run in parallel and assigned 100% enzyme activity. The final concentration of **3** in the reaction medium (<0.1 μM) did not significantly inhibit either AChE or BuChE.

In Vitro Protection of AChE against Soman Inhibition by Pretreatment with Either 3 or 4. Eel AChE (~0.15 μM) was preincubated in 0.1 M phosphate buffer (pH 7.0, 25 °C) with various concentrations of either **3** or **4** for 25 min, followed by the addition of 0.27 μM soman (t = 0). The enzyme solution was diluted 500-fold at various time intervals into 0.5 mM 2-PAM (pH 7.0, phosphate buffer), and AChE residual activity was assayed by the method of Ellman[26] after 25 min of incubation. Under these conditions, soman-inhibited AChE was completely aged (see also ref 4).

Toxicity Determinations. Subcutaneous LD_{50} was based on 24-h mortalities in groups of four to six animals per each dose level, using the Weil method.[32]

Protection Experiments. Animals were injected subcutaneously with **2**, **3**, or **4** prior to the organophosphorus challenge (either soman or paraoxon), followed 10 s after the challenge by treatment as specified in the footnotes for the corresponding tables. The results, based on 24-h mortalities, were expressed in terms of a protective ratio (PR):

$$\mathrm{PR} = \frac{LD_{50}\text{ (of challenge) in treated animals}}{LD_{50}\text{ (of challenge) in untreated animals}}$$

Isolated Phrenic-Nerve Diaphragm Preparation. The left hemidiaphragm of a male rat was set up as described by Bülberg[29] and incubated in 30 mL of Ringer's solution at 37 °C. The phrenic nerve was continuously stimulated with supramaximal square wave pulses (0.3 ms) at a frequency of 0.2 pps. At 5-min intervals, the preparation was tetanized at a frequency of 50 Hz for 3 s. For purposes of this study, the maintenance of the tetanic contractions represented the level of neuromuscular function.[30,31] Compound **3** was added to the bath for 15 min prior to the addition of soman. When tetanus was reduced to 10% (or less) of its control value, the preparation was washed and continued to be tetanized and washed every 5–10 min for at least 90 min. The effect of either **3** or soman or a combination of both drugs on the tetanic tension was monitored and related to the activity of the muscle prior to the addition of the drugs and expressed as a percentage of tetanus height.

Measurement of Plasma Concentration of 3. After the administration of the initial dose of **3**, blood samples were drawn above heparin at varying intervals (usually 30–60 min) and then centrifuged, and the concentration of **3** was determined by diluting the plasma 5-fold into eel AChE solution where the inhibition of AChE was followed to steady state. The concentration of **3** in the plasma of the experimental animals was estimated by comparing the results obtained to a calibration curve constructed under the same experimental conditions by diluting known concentrations of **3** into plasma of naive animals. Less than 10% of **3** was hydrolyzed after 10 h of incubation in mice plasma at 25 °C.

Determination of Brain-AChE Activity in Mice Treated with Compound 3. Brain homogenates (1% Triton X-100 in 0.1 M phosphate buffer, pH 7.0) were centrifuged at 15000g for 30 min at 4 °C. AChE activity in the supernatant was measured before and after the incubation of the homogenate with 0.5 mM 2-PAM for 60 min at 25 °C. An increase in enzyme activity should represent the fraction of inhibited brain AChE that was reactivated by 2-PAM. 3-inhibited brain AChE did not significantly reactivate spontaneously at 4 °C during 30-min incubation.

Results and Discussion

Kinetic Studies in Homogeneous Solutions. Pseudo-first-order plots of enzyme residual activity vs. time in the presence of **3** revealed that the rate constants decreased with time for all enzymes that were tested in this study (Figure 1). AChE from either electric eel or rat brain homogenates displayed different profiles than that of BuChE. In the case of AChE, the development of steady state must be due to spontaneous hydrolysis of the in-

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Table I. Kinetic Parameters[a] for the Inhibition and Reactivation of AChE and BuChE by 3 in Accordance with Scheme I (pH 7.0, 28 °C, Phosphate Buffer, 0.1 M)

enzyme	source	K_I,[b] mM	k',[c] min^{-1}	k_S,[c] min^{-1}	k_i,[d] M^{-1} min^{-1}	k_r,[e] min^{-1}
AChE	eel	0.013 (±0.005)	0.11 (±0.03)	0.07 (±0.01) 0.08 (±0.005)[f]	8.4 (±0.80) × 10^3	0.26 (±0.02)
AChE	rat brain	0.031 (±0.01)	0.20 (±0.02)	0.015 (±0.003) 0.013 (±0.002)[f]	6.4 (±0.35) × 10^3	0.11 (±0.015)
BuChE	mouse serum	NM[g]	NM	<0.005[h]	7.3 (±0.75) × 10^3[i]	0.01 (±0.01)[k]
BuChE	horse serum	NM	NM	<0.005[h]	1.8 (±0.20) × 10^4[i]	0.06 (±0.01)[k]

[a] Kinetic figures are an average of at least three repetitions. Figures in parentheses are standard deviation. [b] Equation 2. [c] Equations 1 and 2. [d] $k_i = k'/K_I$. [e] 2-PAM, 0.5 mM, spontaneous reactivations subtracted. [f] Direct measurements after extensive dilution. [g] Not measured. [h] These values were not estimated accurately because of the aging that occurred during extended incubation. [i] Calculated for the fast component from the initial slopes as shown in Figure 1 (dashed lines). [k] Calculated for the fast component in the presence of 0.5 mM 2-PAM, from the initial slopes of the reactivation profiles. Not corrected for aging.

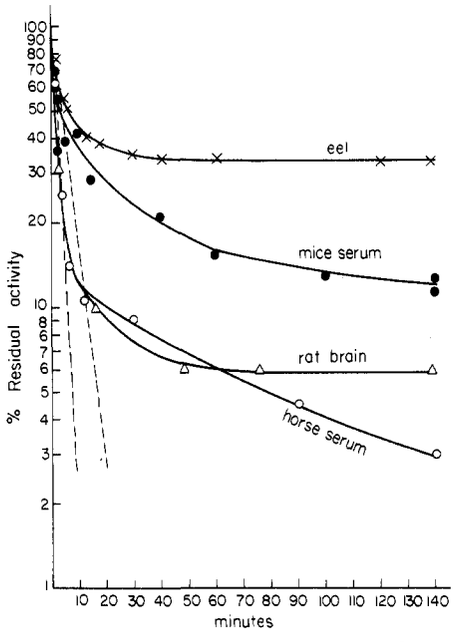

Figure 1. Semilogarithmic plot of percent residual activity of either AChE or BuChE vs. time, in the presence of 0.05 mM **3** at 28 °C (pH 7.0, 0.1 M phosphate buffer). Dashed lines represent the initial slopes from which the first-order rate constants for the inhibition of BuChE were calculated.

hibited enzyme, as the second alternative, i.e., rapid hydrolysis of the inhibitor **3** can be excluded. Compound **3** was found to be relatively stable under all experimental conditions employed in this study. For example, 10 h of incubation in either mice plasma or 0.1 M phosphate buffer at pH 7.0 (25 °C) resulted in hydrolysis of only 9 and 3% of **3**, respectively. The activity of BuChE from either mouse or horse serum did not approach steady state in the presence of **3** (during the first 3.5 h), although the rate of inhibition decreased significantly with time (Figure 1). Thus, we assumed in accordance with previous studies,[11,12] that Scheme I represents the reaction mechanism underlying the inhibition of AChE by **3**.

The following two equations,[11] the approach to steady state (eq 1) and the steady-state level (eq 2), were therefore

$$k_{obsd} = \frac{1}{t} \ln \left[\frac{\epsilon_t}{E_0} - \frac{E_t'}{E_0} \left(\frac{\epsilon}{E'} \right)_{SS} \right] = \frac{k'}{1 + K_I/I} + k_S \quad (1)$$

$$\left(\frac{\epsilon}{E'} \right)_{SS} = \frac{k_S(1 + K_I/I)}{k'} \quad (2)$$

employed to determine the individual rate constants associated with Scheme I where ϵ_t is the measured AChE activity upon dilution into the assay medium (ϵ = [EOH] + [EOH·TDPI]) and E_0 and I are the concentration of the enzyme and inhibitor, respectively, at $t = 0$. E' is the concentration of the phosphorylated enzyme. Subscripts t and SS were used to indicate the concentrations at time t and at the steady state, respectively. Equations 1 and 2 were used for experiments with AChE (eel and rat brain) in the concentration range 7 to 100 μM. We found that by plotting either $(\epsilon/E')_{SS}$ vs. I^{-1} or k_{obsd} vs. $(1 + K_I/I)^{-1}$, straight lines were obtained (not shown) as reported previously for other members of the 1,3,2-dioxaphosphorinane 2-oxide series.[11,12] The individual rate constants that are presented in Table I were derived from the slope and intercept of the above-mentioned plots. When the concentration of **3** exceeded 0.2 mM, we observed a slight deviation from the predicted values that were calcuated for eel AChE from the data presented in Table I. We did not attempt to further analyze this observation. We note, however, that similar findings were reported with regard to several carbamates.[33]

Because the inhibition of BuChE did not approach steady state, Scheme I could not be used to estimate the kinetic constants for this system, and the pseudo-first-order rate constants for the inhibition were calculated from the initial slopes of the corresponding plots (see Figure 1, dashed lines). Possibly, the inhibition profile for serum BuChE represents the kinetics of more than one class of enzyme due to the heterogeneous enzyme preparations, to the result of parallel aging, or to a combination of both factors.

Spontaneous reactivation rate constants for all inhibited enzymes were measured directly by the dilution method[11,12] and are summarized in Table I. The spontaneous regeneration of inhibited AChE was also evaluated from eq 1 and 2 and was found to be in good agreement with the rates obtained from direct measurements, after extensive dilution. 2-PAM (0.5 mM) accelerated the rate of spontaneous reactivation of **3**-inhibited AChE or BuChE, whereas 3-PAM, which is known as an exceptionally poor reactivator,[35] had no significant effect. The dependence of the reactivation of the phosphoryl-AChE conjugate (electric eel) on 2-PAM concentration revealed a typical saturation curve (not shown), as expected by analogy with experiments in which induced reactivation of diethylphosphoryl-AChE conjugates were studied.[35] The second-order rate constant for reactivation of **3**-inhibited AChE was found to be 1.8 × 10^3 M^{-1} min^{-1}, whereas for the noncyclic analogue of **3**, namely, $(C_2H_5O)_2P(O)$-AChE, reactivation was 8 times faster (1.4 × 10^4 M^{-1} min^{-1}).[35] We note that for BuChE the first-order plots revealed a de-

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Table II. Aging of AChE and BuChE during Incubation with 5 μM 3 at 25 °C (0.1 M Phosphate Buffer, pH 7.0)

enzyme[a]	incubation time with 3, h	% act. prior to reactivation	% act. after 20-h reactivation at 25 °C: spontaneous	% act. after 20-h reactivation at 25 °C: 0.5 mM 2-PAM
AChE, eel	2	65	96	100
	4	65	94	98
	20	60	98	98
AChE, rat brain	5	30	96	98
BuChE, mouse serum	4	68	78	78
	20	52	60	60
BuChE, horse serum	2	17	39	78
	4	21	42	61
	20	10	6	31

[a] Enzyme diluted 10-fold into 0.5 mM 2-PAM solution (0.1 M phosphate, pH 7.0) and assayed after 20 h of incubation at 25 °C.

crease in the rate with time, whereas for AChE straight lines were obtained for both the spontaneous and the 2-PAM-induced reactivation. We ascribe this behavior to either loss of enzyme due to aging (see below), to the presence of more than one class of enzyme in the serum, or to a combination of both possibilities.

The results of the aging studies are summarized in Table II. Reactivation went gradually to completion for AChE irrespective of the time of incubation with 3 prior to the dilution into the reactivation medium. In contrast, BuChE from either horse or mice serum was converted within a few hours into a form that could not be reactivated. This aging was time dependent and proceeded at a relatively slow rate (see Table II).

Compound 4, the corresponding leaving group of 3, and several structural analogues of 4 have been reported to inhibit reversibly 50% of AChE activity at concentrations as low as 0.3 μM.[15,17] The dissociation constant (K_I) of the reversible complex between eel AChE and 4 was measured in the presence of acetylthiocholine (0.1–0.005 mM) in accordance with the Hunter–Downs equation,[12,28] and the following values were extracted for K_I and K_m (acetylthiocholine): 0.25 and 80 μM, respectively. These results are in accordance with previous finding indicating that 4 competes with the substrate at the same anionic site,[17] presumably the active center of AChE. Addition of 2 μM 4 (obtained either from authentic samples prepared in our laboratory or from the complete hydrolysis of 3 by 0.1 NaOH) decreased the rate of AChE approach to steady state 2-fold in the presence of 0.1 mM 3. These results are presented in Figure 2. We note that the steady-state level was independent of the sequence of addition of 4 to the AChE/3 mixture. Thus, for example, when 4 was added to an equilibrated mixture of AChE/3 (lower curve in Figure 2), the new steady-state level approached the same level obtained by treating the enzyme simultaneously with 3 and 4 (upper curve of Figure 2). Since 4 did not accelerate the rate of hydrolysis of the spontaneous reactivation of the phosphoryl-AChE conjugate (see insert, Figure 2), we suggest that the protection provided by 4 against covalent phosphorylation by 3 should be attributed to competition between 3 and 4 for the occupancy of the anionic portion of the active site of AChE. Indeed, the free phenol 4 binds to AChE ~52 times more strongly than 3 (see Table I). Furthermore, in two extreme cases (not shown), we could demonstrate that 0.5 μM 4 did not affect

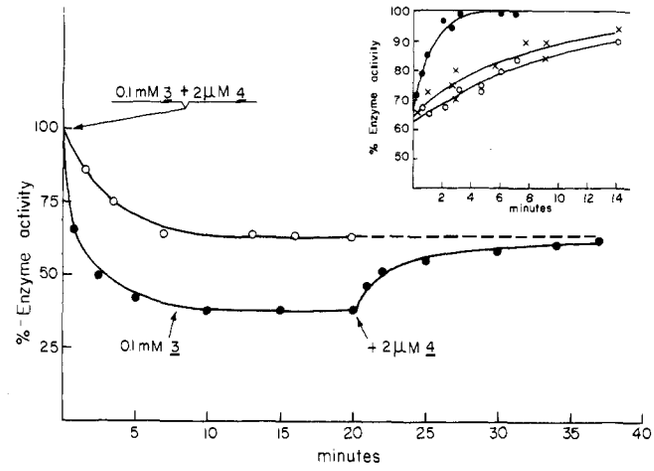

Figure 2. Inhibition profiles for the reaction of eel AChE with either 0.1 mM 3 or a combination of 0.1 mM 3 and 2 μM 4. The insert depicts the reactivation profile of 3-inhibited eel AChE that was diluted at $t = 0$ into either 0.5 mM 2-PAM (●–●) or 2 μM 4 (×–×). O–O represents the spontaneous reactivation (pH 7.0, 0.1 M phosphate buffer, 28 °C).

the reaction profile between 0.1 mM 3 and AChE, whereas 0.05 mM 4 completely prevented the phosphorylation of AChE by 3. Thus, 100% enzyme activity was regained immediately upon extensive dilution.

Thus far, inhibition of AChE activity in vitro by 3 has provided kinetic evidence supporting the hypothesis that the inhibition of the enzyme proceeds in accordance with Scheme I. The spontaneous rate constant for the hydrolysis of inhibited enzyme and the acceleration of reactivation by 2-PAM, but not by 3-PAM, substantiate the assumption that a covalent phosphoryl-AChE intermediate is formed, as is found for the reaction of AChE with carbamates.[4] In order to provide further evidence for the formation of a covalent 3–AChE conjugate, the following experiments were performed.

In three different experiments, we reacted 3, 6, and 9 μM (active-site concentration) purified eel AChE with 0.1 mM 3 in 0.1 M phosphate buffer (pH 7.0, 25 °C). In a control experiment, AChE was inhibited with paraoxon and then allowed to incubate with 3, as above. The rate of release of 4 was followed spectrophotometrically (at 280 nm, 4 could be distinguished from 3). The pseudo-first-order rate constant, calculated from the initial slopes of these runs, was found to be 0.12 min^{-1}. Under the experimental conditions described above, the ratio $[3]/K_I$ is ~8 and the pseudo-first-order rate constant may be approximated by k' (eq 1, when $t = 0$, $k_s = 0$). Indeed, k' was found to be 0.11 min^{-1} (Table I). When AChE was prephosphorylated by paraoxon, no increase in 4 concentration could be observed in the experimental cuvette. Interestingly, upon extensive dilution (×10^4), 70% of enzyme activity was recovered after 60 min and 95% after 16 h of incubation (with 6 μM AChE). After 16 h, only 70% of the theoretical amounts of 4 was released. The results from these experiments provided a qualitative direct indication that 4 is, indeed, released upon phosphorylation (i.e., inhibition) of AChE by 3.

The second-order rate constant (first order with respect to each reactant) for the hydrolysis of 3 in alkaline solutions (0.01–0.1 N NaOH, 25 °C) was found to be 2.3 M^{-1} min^{-1}. This value is in reasonable agreement with the predicted value (1.3 M^{-1} min^{-1}),[14] assuming a pK_a of 8.1[17] for the leaving group 4. In contrast, the relative increase in the bimolecular rate constant for the inhibition of AChE by 3, as compared to analogous cyclic phosphates,[14] in-

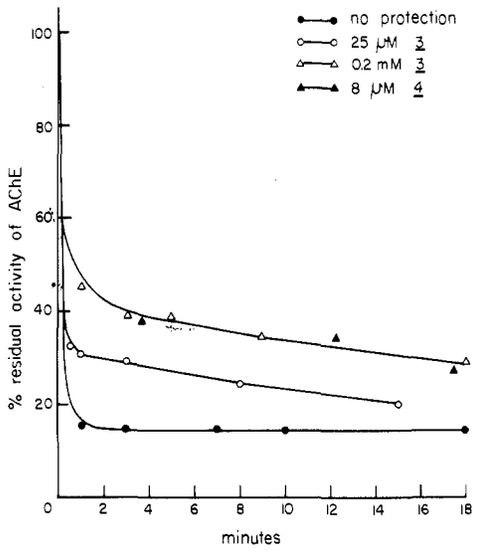

Figure 3. Percent residual activity of eel AChE that was pretreated with either **3** or **4** in 0.1 M phosphate buffer at pH 7.0 (25 °C) 15 min prior to the addition of 0.27 μM soman (t = 0). Each point represents enzyme activity after 25 min of incubation with 0.5 mM 2-PAM. The inhibited solution was diluted 500 times into the reactivation medium at the indicated time.

Table III. Changes in Tetanus Tension (Percent of Control) as a Function of Pretreatment with 3, Followed by Soman

3,[a] μM	soman,[a] μM	time of incubation after the application of soman, min	tetanus tension,[b] % of control act.
0	0		100
0.25	0.05	15	5
2.5	0.05	10	58
25	0.05	20	82
250	0.05	40	78
250	0.1	15	70
250	1.0	25	75
250	5.0	15	5

[a] The hemidiaphragm pretreated with 3 15 min prior to the addition of soman. [b] Measured 50 min after washout.

dicates that this compound is decidely more reactive (>100×) than would be anticipated from the pK_a of its leaving group.[14] Such a deviation from the corresponding Brönsted plot usually occurs when the leaving group of phosphorylating drugs that produce covalent conjugates contains a quaternary ammonium function.[14,18] This substantiates our assumption that AChE is phosphorylated to produce the covalent conjugate 3–AChE.

Protection of AChE against Soman in Homogeneous Solution and in Isolated Rat Hemidiaphragm. The protection of eel AChE in vitro against irreversible inhibition by soman is shown in Figure 3. Preincubation of enzyme with either 0.2 mM **3** or 8 μM **4** conferred identical levels of protection. However, the fact that to confer equivalent protection against soman, $[3]/K_I = 15$ while $[4]/K_I = 32$ indicates that **3** is more efficient. This is presumably due to the formation of the covalent phosphoryl–enzyme conjugate with **3**, which is not reflected in the dissociation constant K_I. The amount of protection was correlated with the concentration of **3**, as is demonstrated for two different concentrations in Figure 3.

Figure 4 indicates that pretreatment of the hemidiaphragm with 2.5 μM **3** for 15 min conferred adequate

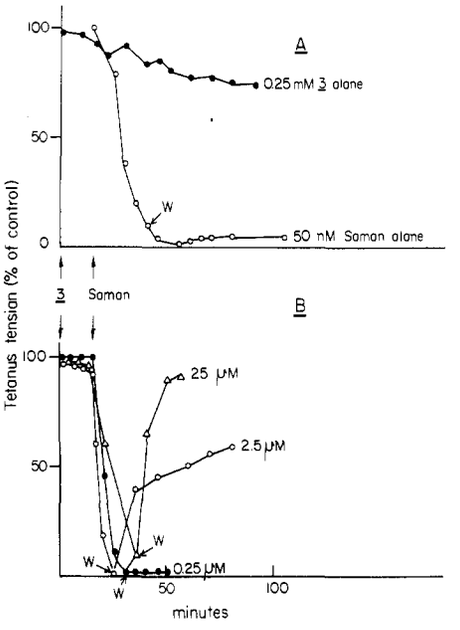

Figure 4. The effect of different concentrations of **3** on the tetanic tension depression caused by 50 nM soman. W denotes washout: (A) tetanic tension of control preparations; (B) the effect of **3** pretreatment, followed by soman (**3** concentrations are indicated for each curve).

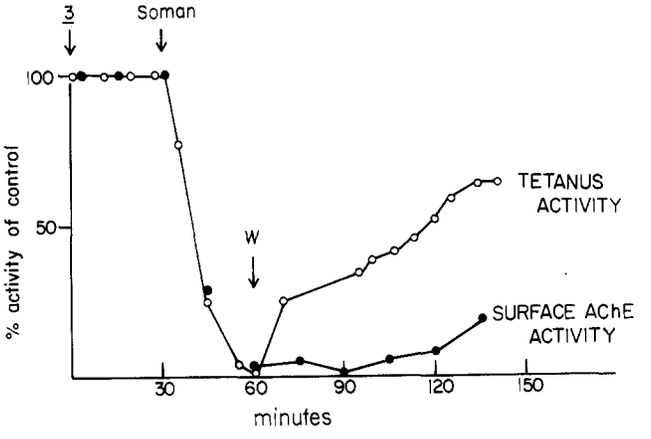

Figure 5. Correlation between surface AChE activity and maintenance of tetanic tension of a hemidiaphragm pretreated with 2.5 μM **3**, followed by 50 nM soman. W denotes washout. Both parameters were measured simultaneously in the same preparation.

protection against irreversible damage caused by 50 nM soman in terms of the tetanic tension test. The effect of the pretreatment was found to be dependent on the dose of both soman and **3**. The results are presented in Table III. The effects of **3** alone on the tetanus tension of control hemidiaphragm (Figure 4A) were found to be negligible. In several experiments, surface AChE activity was measured and compared with the depression of tetanic tension induced under the same experimental conditions (Figure 5). The addition of 2.5 μM **3** to the bath did not reduce surface AChE activity or tetanic tension during 30 min of incubation. However, the addition of 50 nM soman reduced both activities to 5% of control activity within 30 min. With removal of excess soman and **3**, tetanic activity returned gradually, reaching 65% of control within 60 min. When the hemidiaphragm was unprotected, 50 nM soman was sufficient to abolish irreversibly tetanic tension. Surface AChE activity was also restored upon removal of the drugs, but to only a minor extent (Figure 5). Similar

Table IV. Acute Toxicity of 3 and 4 in Mice and Rats

species	LD_{50}, mg/kg, sc		
	3	4	2
mouse	444.0 (384.0-513.0)[a]	59.9 (50.3-62.2)	2.6 (2.2-3.1)
rat	327	NM[b]	3.1 (2.8-3.5)

[a] 95% confidence limits. Four to six animals in each dose group. [b] Not measured.

Table V. Protection of Mice Pretreated with 3 against Soman, Followed by Different Therapeutic Mixtures[a]

therapy	pre-treatment	% survival (24-h observation)
toxogonin[b] + atropine[c]	3	5
	none	10
TMB-4[d] + atropine	3	0
	none	0
TMB-4 + atropine + benactyzine[e]	3	100
	none	30
atropine	3	0

[a] Conditions: 150 mg/kg of 3 administered sc 30 min before challenging with 3.8 times the LD_{50} (sc) of soman. Supporting treatment applied (im) 10 s after the injection of soman. [b] 60 mg/kg. [c] 11.2 mg/kg. [d] 12.5 mg/kg. [e] 8.0 mg/kg; ten animals in each group.

observations were reported by French et al.[30] with respect to pyridostigmine. These results are in agreement with previous conclusions that low levels of AChE are sufficient to maintain neuromuscular function.[30] We note that by increasing the concentration of **3** in the bath, surface AChE was regenerated to a greater extent. In contrast to **3**, **4** did not protect irreversible reduction of tetanus tension produced by soman, although the concentrations used (0.028 mM) were sufficiently high to completely inhibit surface-AChE activity.

The findings presented above and the results discussed in the previous section suggest that formation of the labile covalent conjugate **3**–AChE is the major mechanism providing antidotal protection.

In Vivo Experiments. Table IV summarizes the acute toxicity of **3** and **4** in mice and rats. Initial signs of **3** poisoning are characterized by muscular weakness within 1–5 min, followed by prostration and tonic clonic convulsions. Difficulties in respiration and cyanosis were observed before death. Animals dies ~30 min after the administration of the LD_{50} of the drug. A dose of 150 mg/kg of **3** (one-third the LD_{50}) in mice was found to be the maximum sign-free dose.

The degree of protection against soman by pretreatment with **3** was dependent on the antidotal treatment, as indicated in Table V. The efficacy of the therapeutic mixture of various oximes with atropine and benactyzine has been previously reported to be very effective for soman poisoning.[36,37] We also observed that the efficacy of the therapeutic mixture TMB-4, atropine, and benactyzine (TAB) was found to be superior to the other mixtures and, therefore, was used as standard treatment in further protection experiments. Protection ratios against soman and paraoxon were determined in mice; they are summarized in Table VI. It was found that pretreatment with **3**, followed by TAB therapy, protected against 5.2 times the LD_{50} of soman. We note that under the same experimental conditions, pyridostigmine (0.13 mg/kg) provided a protective ratio of 3.1. When paraoxon was the challenge, a protective ratio of 22.2 was recorded with therapy based on atropine alone (Table VI). The protection afforded against soman decreased slightly as the dose of **3** was reduced from 150 to 37.5 mg/kg (protective ratio 4.3). However, the signs and symptoms observed in animals protected with 150 mg/kg were less severe, and animals recovered more rapidly than those pretreated with 37.5 mg/kg. Administration of 10 mg/kg of **4** (maximum sign-free dose) either 5 or 30 min prior to the challenge, followed by TAB treatment, did not protect mice against 2 times the LD_{50} of soman.

Table VI. Protection of Pretreated Mice against Soman and Paraoxon Poisoning

challenge	pre-treatment[a] (sc)	therapy (im)	protective ratio[b] (95% CL)
soman	none	atropine	1.5
	3	atropine	2.0
	none	TAB[c]	2.0
	3	TAB	5.2 (4.8-5.6)
	4[d]	TAB	<2.0
	2[e]	TAB	3.1 (2.7-3.5)
paraoxon	none	atropine	3.0
	3	atropine	22.2 (17.8-27.8)

[a] 30 min prior to challenge. For detailed experimental conditions, see Table V, footnote *a*. [b] For definition, refer to Experimental Section. Four to six animals were used in each dose group. Each protective ratio was based on 16-20 animals. [c] TAB = TMB-4, atropine, and benactyzine. See Table V. [d] 10 mg/kg. [e] 0.13 mg/kg.

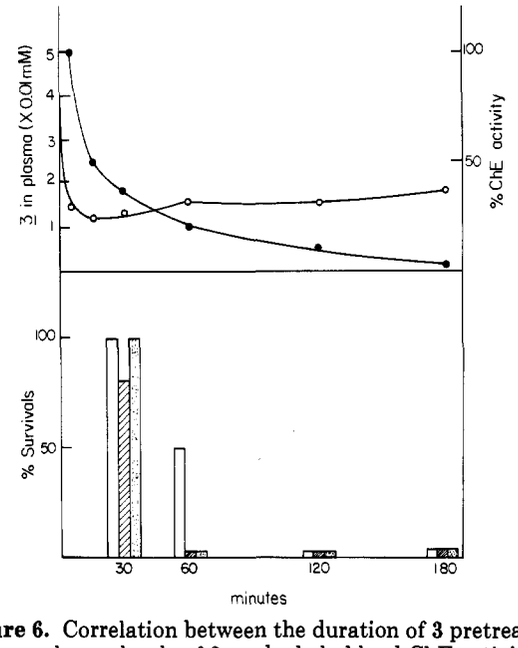

Figure 6. Correlation between the duration of **3** pretreatment efficacy, plasma levels of **3**, and whole-blood ChE activity, following sc administration of 150 mg/kg to mice. Soman was administered at different multiples of the LD_{50} does: (□) 4.3 × LD_{50}; (▨) 4.7 × LD_{50}; (▩) 5.3 × LD_{50}. (●–●) **3** concentrations; (O–O) whole-blood ChE activity.

Figure 6 depicts the duration of pretreatment efficacy after a single dose of **3**. The level of protection obtained 30 min after the administration of **3** decreased significantly after 60 min. The upper half of Figure 6 presents the time course for the plasma concentration of **3** and for whole-blood ChE activity in mice that received 150 mg/kg of **3**.

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The plotted data indicate that adequate protection was attained when a blood level of 0.01 mM 3 was maintained. Whole-blood ChE could not be correlated with the [3] in plasma. One explanation for these results may involve the aging of mice-serum BuChE (see Table II). The correlation between the duration of the pretreatment efficacy and the concentration of 3 in the plasma substantiates our suggestion that protection of AChE provided by 3, a result of temporary masking of the active site via the formation of a covalent phosphoryl-AChE conjugate, constituted the major antidotal mechanism.

We note that inhibition of mice-brain AChE could not be observed in animals receiving a lethal dose of 3 (450 mg/kg). Thus, AChE activity of mice-brain homogenates (six repetitions) was unchanged after incubation with 0.5 mM 2-PAM for 60 min at 25 °C. These results suggest that 3 does not cross the blood-brain barrier, and its activity is restricted to peripheral tissues.

Conclusions

The results of this study suggest that reasonable in vitro and in vivo protection against soman and paraoxon poisoning is provided by the newly designed cyclic phosphate ester via the reversible masking of AChE by covalent phosphorylation. Effective pretreatment was dependent on subsequent treatment with cholinolytics and TMB-4. The successful use of carbamates[6,8,30] or TEPP[10] as pretreatment against organophosphate poisoning has also been attributed to their ability to form unstable carbamoyl or oxime-reactivable diethylphosphoryl-AChE conjugates. Their protection is also dependent on additional treatment as mentioned above for 3.[6,8,10,30] However, in contrast to carbamates and tetraethyl pyrophosphate, the acute toxicity of 3 was found to be impressively low (LD_{50} = 444 mg/kg in mice). This low toxicity probably relates to the kinetic parameters for inhibition and spontaneous reactivation of 3-inhibited AChE. For example, 3 inhibits AChE ~25 times slower than does pyridostigmine,[33] and the inhibited enzyme hydrolyzes 2-3 times faster than the corresponding carbamoyl-AChE conjugate. Thus, it is possible to rationalize the differences between the acute toxicity of 3 and pyridostigmine in kinetic terms. Nevertheless, it should be emphasized that other factors, such as absorption, distribution, excretion, and metabolism, cannot be entirely exluded for the observed differences between the toxicity of the two drugs.

Although 3 was found to relatively stable in experiments close to physiological conditions (serum and phosphate buffer, pH 7.0, 25 °C), the in vivo half-life and duration of its antidotal capacity are relatively short (in mice). It is believed that 3 is a nontoxic potential drug that may be applied for pretreatment of organophosphorus poisoning, in conjunction with cholinolytic and oxime reactivators. In view of the marked species differences in response to protection against soman and other organophosphorus compounds,[6] we are now studying the biological and the pharmacokinetic properties of 3 in monkeys.

Finally, it is worthwhile to mention here that one speculative significance of these findings may be a possible application of the 1,3,2-dioxaphosphorinane 2-oxide moiety as a carrier of biologically active molecules of cholinergic importance, provided that they can serve as convenient leaving groups in reactions where AChE is the attacking nucleophile.

Acknowledgment. We thank Mrs. B. Manisterski for her excellent technical assistance.

Registry No. 3, 80531-03-9; 4, 2498-27-3; AChE, 9000-81-1; BuChE, 9001-08-5; paraoxon, 311-45-5; soman, 96-64-0; 2-chloro-1,3,2-dioxaphosphorinane 2-oxide, 872-99-1; 3-(dimethylamino)phenol, 99-07-0; *O*-[3-(dimethylamino)phenyl]-1,3,2-dioxaphosphorinane 2-oxide, 83547-88-0.

Nucleosides. 123. Synthesis of Antiviral Nucleosides: 5-Substituted 1-(2-Deoxy-2-halogeno-β-D-arabinofuranosyl)cytosines and -uracils. Some Structure-Activity Relationships

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The syntheses of several 2'-halogeno-5-substituted-arabinofuranosylcytosines and -uracils are described, and relationships of structure to anti herpes virus activity in vitro were examined. Those arabinonucleosides containing the 2'-fluoro function exhibit, generally, more potent anti herpes virus (HSV) activity than do their 2'-chloro or 2'-bromo analogues. The importance of the fluorine in the 2'-"up" (arabino) configuration for enhancement of antiviral effectiveness is demonstrated by the superior activity of 2'-fluoro-5-iodo-*ara*-C [**3a**, FIAC] to that of 2'-fluoro-5-iodo-*ribo*-C. Of all the nucleosides tested herein, FIAC exhibited the most potent in vitro activity against HSV. 2'-Chloro-5-iodo- and -5-methyl-*ara*-C (**3b** and **4b**) were 37 to >500 times *more* effective in vitro activity against HSV type 2 than against type 1, suggesting that these latter derivatives might serve clinically as useful probes to distinguish between HSV types 1 and 2 in the diagnosis of HSV infections in man.

In previous reports,[1-3] we described the syntheses and potent anti herpes virus activity of several 5-substituted 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)cytosines and -uracils. Of the newly synthesized compounds, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (2'-

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