Formation of an Unstable Covalent Intermediate during the Inhibition of Electric-Eel Acetylcholinesterase with 1,3,2-Dioxaphosphorinane 2-Oxides

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The kinetics of interaction of eel acetylcholinesterase (EC 3.1.1.7) with 1,3,2-dioxaphosphorinane 2-oxides were investigated. It was demonstrated that the rate of spontaneous re-activation as well as the re-activation profile in the presence of 2-pyridine aldoxime methiodide of the inhibited enzyme are irrespective of the leaving group of three inhibitors and exhibit the same values. The dissociation constant of the corresponding Michaelis complex was evaluated by two independent methods and the results were found to be in close agreement. It was shown that the active site is essential for interaction between the enzyme and the various dioxaphosphorinanes. The mixed anhydride of diethyl phosphoric acid and 2-hydroxy-1,3,2-dioxaphosphorinane 2-oxide behaves exactly as would be predicted from a typical diethyl phosphate inhibitor. Enzyme that was incubated with the cyclic acid or the corresponding methyl ester recovered immediately upon extensive dilution. Inhibition of enzyme in the presence of high concentrations of the corresponding 2-chloro and 2-fluoro derivatives decreased the regeneration rates as well as the maximal amount of the re-activated enzyme. This observation could not be explained in terms of a classical aging process. On the basis of the kinetics observations it is suggested that an unstable covalent phospho-enzyme intermediate is formed during the reaction between acetylcholinesterase and 1,3,2-dioxaphosphorinane 2-oxides.

It has been reported recently that the reaction of acetylcholinesterase (EC 3.1.1.7) and 2-fluoro-1,3,2-dioxaphosphorinane 2-oxide (I) is a progressive inhibition, characterized by a biphasic kinetic profile (Coult, 1976). It was explained by the author that the first phase represents slow formation of a reversible complex approaching a steady-state equilibrium, whereas the second phase is significantly slower (approx. by one order of magnitude), and represents the subsequent phosphorylation step. Consequently, according to Coult (1976), the slow return of enzyme activity on dilution is due to dissociation of a reversible complex, rather than hydrolysis of phosphorylated enzyme.

In contrast with Coult's (1976) findings, in a previous report, no biphasic profile could be detected during the inhibition of acetylcholinesterase by compound (I) provided that the inhibitor was pre-incubated in buffered aqueous solution for 20 min before the addition of the enzyme (Ashani *et al.*, 1972).

It is generally accepted that the formation of the enzyme-inhibitor reversible complex and its dissociation occur very rapidly. However, the rate constants expressing the velocities of the complex-

formation and its dissociation ($3 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $4 \times 10^{-2} \text{ min}^{-1}$ respectively) reported by Coult (1976) are surprisingly low. Such values suggest an extremely unusual property of a specific inhibitor (I) and perhaps one should reconsider the mechanistic interpretation of many other kinetic observations where the inhibition of acetylcholinesterase by various organophosphorus esters and carbamates were investigated extensively. It is difficult to refer in more detail to Coult's (1976) work owing to insufficient details with respect to the experimental procedures and the rationale behind the mathematical solution of the kinetic schemes.

In view of the unexpected results, it was worthwhile re-examining the details of the above-mentioned reaction. The clarification of the mechanism involved in this reaction is also very important for the understanding of the biological properties of several phosphorothiolates containing the 1,3,2-dioxaphosphorinane 2-oxide moiety (Amitai *et al.*, 1976).

In the present study we describe the preparation, purification and measurements of the kinetics of the reaction between eel acetylcholinesterase and the following compounds:

(I) $X = F$; (II) $X = Cl$;

(IIa) $X = OH$; (IIb) $X = OCH_3$;

Experimental

Materials

Purified 11S electric-eel acetylcholinesterase (EC 3.1.1.7; 1 mg/ml) was a gift from Mr. G. Amitai of this laboratory. The enzyme was purified as described by Dudai *et al.* (1972). Stock solutions were made in phosphate buffer (0.1M) containing 0.05% gelatine. 2-Hydroximinomethylpyridinium methiodide and the 3-analogue were prepared as described before (Ginsburg & Wilson, 1957). 2-Fluoro-1,3,2-dioxaphosphorinane 2-oxide (I) was prepared by the Fukuto & Metcalf (1965) method as described by Ashani *et al.* (1972). The 2-chloro analogue (II) was prepared as described by Lanham (1959) and used immediately. The synthesis of compounds (III) and (IV) is outlined below. The acid ($X = OH$; IIa) was used as its salt form or through hydrolysis of compound (II). Toxicity evaluation was performed as described by Amitai *et al.* (1976).

Methods

(a) *Synthesis.* Melting points were determined in open glass capillaries with Thomas-Hoover Unimelt apparatus and are uncorrected. N.m.r. spectra were determined on a Varian XL100 100MHz spectrometer with $[^2H]$ chloroform as solvent and tetramethylsilane and H_3PO_4 as references. T.l.c. was carried out on glass plates (25mm $\times$ 75mm) coated with silica.

2-Hydroxy-1,3,2-dioxaphosphorinane 2-oxide (IIa). This compound was used for the preparation of compound (III), by a somewhat different procedure from that reported in the literature (Khorana *et al.*, 1957). A mixture of acetone (10ml) and water (3.6ml) was added dropwise to a cold (5°C) solution of compound (II) (32.0g) and triethylamine (20.0g) in 100ml of dry acetone. The mixture was stirred for

2h at room temperature. The white precipitate was filtered off and the solvent was removed *in vacuo* (20mmHg). The crude residue was dissolved in chloroform (100ml), washed with water (2 $\times$ 50ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residual solid (32.5g) recrystallized from tetrahydrofuran. We noted that at this stage a considerable amount of the free acid undergoes condensation to form the symmetric pyro derivative (III) as indicated by the t.l.c. analysis [5% (v/v) methanol/chloroform as solvent]. Extensive hydrolysis in boiling water and evaporation to dryness resulted in the free acid (dicyclohexylammonium salt; IIa, m.p. 201–203°C) (Found: C, 56.3; H, 9.5; N, 4.5; calc. for $C_{15}H_{30}NO_4P$: C, 56.4; H, 9.4; N, 4.4%).

The corresponding methyl ester (IIb; $X = OCH_3$) prepared from compound (II) and methanol, was found to be identical with similar preparation described elsewhere (White *et al.*, 1971).

Bis(propane-1,3-diol) cyclic pyrophosphate (III). This compound was prepared from the foregoing acid by the procedure described by Khorana *et al.* (1957). The crude solid was recrystallized from diethyl ether/acetonitrile (m.p. 137–138°C; literature value 137–137.5°C). T.l.c. indicate that this preparation is pure and homogeneous. ^{31}P n.m.r. in $[^2H]$ chloroform give a multiplet with $\delta +20.8$ p.p.m.

2-(OO-Diethyl phosphoro)-1,3,2-dioxaphosphorinane 2-oxide (IV). Caution: this compound is extremely toxic and adequate safety precautions should be taken during its preparation and handling.

A solution of OO-diethyl phosphorochloridate (1.1g, freshly distilled) in dry benzene (10ml) was added to a solution of dicyclohexylammonium salt (IIa; 2.0g) in dry benzene (50ml). The mixture was stirred at 60°C for 5h, cooled and the white solid filtered off. The solvent was evaporated *in vacuo* (20mmHg). The residual semisolid was purified by column chromatography (silica) with chloroform as eluent. The final product was a viscous oil. The isolated compound was checked for purity on silica chromatoplates (5% methanol/chloroform) and found to be homogeneous.

N.m.r. studies gave the following results ($[^2H]$ -chloroform as solvent, δ values in p.p.m.). 1H : δ 1.40 (6H, t, CH_3-CH_2 , J 7.0 Hz), δ 1.75–2.6 (2H, broad m,

J_{H-H} 7.0 Hz, J_{P-H} 7.5 Hz), δ 4.4–4.75 (4H, m, OCH_2-CH_2). ^{31}P : $\delta +13.5$ [$P(OC_2H_5)_2$, double quintet, J_{P-O-P} 19 Hz, $J_{CH_3-CH_2-OP}$ 7.5 Hz], $\delta +20.8$

and P-H were obtained through a decoupling technique (Found: C, 30.7; H, 6.3; calc. for $C_7H_{16}P_2O_7$: C, 30.7; H, 5.8%).

The same compound was also obtained by reacting *OO*-diethyl phosphate dicyclohexylammonium salt and 2-chloro-1,3,2-dioxaphosphorinane 2-oxide.

The acute toxicity to mice (subcutaneously) was found to be in the range of 0.3 mg/kg.

(b) *Inhibition of the enzyme in the absence of substrate.* (i) Inhibition with compounds (I), (IIa), (IIb) and (III). To 1 ml of enzyme solution ($\sim 0.01 \mu\text{M}$) in phosphate buffer (pH 6.8, 0.1 M) was added 3–30 μl of freshly prepared stock solution of the inhibitor (1–300 mM) in water, except in the case of compound (II) where acetone was used as solvent. At time intervals of 0.5–2.5 min, 10–25 μl of the inhibited enzyme was diluted into 3.12 ml of the Ellman assay medium (Ellman *et al.*, 1961) and assayed for residual enzyme activity. All enzymic studies were done in a Pye Unicam SP.1800 u.v. spectrophotometer attached to a Unicam AR-25 linear recorder. It is important to note that, in the case of compound (I), the aqueous solution was left to react with 'unstable' inhibitors for 60 min before dilution to the enzyme solution.

(ii) Inhibition with compound (IV). The inhibition of the enzyme by this compound was carried out as described above, except that the stock solution concentrations were in the range of 1–10 μM . The hydrolysis of the pyrophosphate under the experimental conditions is negligible.

(iii) Inhibition of acetylcholinesterase with tetraethyl pyrophosphate and diethyl phosphorofluoridate. Stock solution of both inhibitors was made in water (10 μM), and used immediately. Final concentration of inhibitor was 0.2–0.5 μM . The inhibited enzyme (99% inhibited after 30 min) was further used to study the interaction of compounds (I) and (III) with the diethylphospho-enzyme. The inhibited enzyme was subsequently incubated with compound (I) or (III) and diluted 500-fold into 2-pyridine aldixime methiodide solution to study the rate of enzyme re-activation.

(c) *Inhibition of the enzyme in the presence of substrate.* To 3.15 ml of enzyme solution in phosphate buffer (0.1 M, pH 6.8), containing varied concentrations of acetylthiocholine iodide and 0.3 mM-5,5'-dithiobis-(2-nitrobenzoic acid), was added 3–30 μl of stock solution of 1–100 mM-compound (I) or (III) in water (freshly prepared before each experiment). Enzyme activity was monitored within 10 s for periods not exceeding 1 min. The final concentration of the inhibitors was set low enough so as not to produce any significant progressive inhibition within 1 min of assay. The recorded slopes of enzyme activity before and after the addition of the inhibitors were treated according to eqn. (3).

(d) *Direct measurement of the re-activation of inhibited acetylcholinesterase.* Enzyme solution (0.5

μM) was inhibited with the following compounds for periods of 5–10 min in phosphate buffer (0.1 M, pH 6.8): (I), (III) 0.5–2.5 mM; (II) 2–5 μM ; (IV) 0.5 μM . The inhibited enzyme was diluted 100-fold into the specific recovery medium. The rate of re-activation was measured by the Ellman procedure as described above by diluting 25 μl of the solution directly into the reagent mixture (3.12 ml).

Results

Inhibition of acetylcholinesterase by compounds (I) and (III)

The overall kinetic processes involved in the reaction of acetylcholinesterase and an organophosphorus inhibitor are:

where E is the free enzyme, I the inhibitor, EI the reversible complex and E' the phosphorylated enzyme. K_I is the dissociation constant of the reversible complex and k' and k_s the phosphorylation and spontaneous reactivation rate constants respectively. The fluoridate (I) and the cyclic pyrophosphate (III) were found to produce the same inhibition profile of eel acetylcholinesterase. Enzyme activity approached a steady-state equilibrium in the presence of both inhibitors with a rate depending on the inhibitor concentration. Fig. 1 demonstrates the importance of pre-incubation of compound (I) in water to allow the hydrolysis of unstable inhibitor, presumably the chloridate (II). As described earlier (Ashani *et al.*, 1972), two kinetic equations, the steady state (eqn. 1) and the approach to the steady

Fig. 1. Inhibition profile of eel acetylcholinesterase with compound (I) as a function of preincubation time in water at 25°C

Final inhibitor concentration was 0.11 mM. Incubation time: Δ , 10 min; $\bullet$, 60 min; $\circ$, 100 min; $\square$, 210 min.

state eqn. (2), were used to separate the individual constants K_1 , k' and k_s .

$$\left(\frac{\varepsilon}{E'}\right)_{ss} = \frac{k_s \left(1 + \frac{K_1}{[I]}\right)}{k'} \quad (1)$$

$$\frac{1}{t} \ln \left[\frac{\varepsilon_t}{E_0} - \frac{E'_t}{E_0} \left(\frac{\varepsilon}{E'}\right)_{ss} \right] = -\frac{k'}{1 + K_1/[I]} + k_s \quad (2)$$

where E_0 is the initial enzyme activity and ε_t is the activity of the enzyme at time t . E'_t is the amount of the phosphorylated enzyme at time t , and $(\varepsilon/E')_{ss}$ is the ratio of active to inhibited enzyme at the steady state. It was assumed that, by extensive dilution, the free enzyme (E) and the amount of the reversible complex (EI) are actually measured ($\varepsilon = [E] + [EI]$). Thus the curves obtained by semilogarithmic plot of ε_t versus t for compounds (I) and (III) converted to straight lines when the experimental results were treated according to eqn. (2). Straight lines were also obtained by plotting $(\varepsilon/E')_{ss}$ versus $1/[I]$. Fig. 2 presents a typical plot by which k' and k_s were separated for compound (III) (Ashani *et al.*, 1972). The values of K_1 , k' and k_s evaluated for compounds (I) and (III) are summarized in Table 1.

Inhibition of acetylcholinesterase by the mixed anhydride (IV)

The rates of the inhibition in the presence of excess inhibitor were found to be first-order and the rate constants were linearly related to the concentration

of the inhibitor. In addition all extrapolated straight lines to $t = 0$ passed through the origin. The inhibited enzyme did not show any appreciable spontaneous recovery upon dilution. More than 80% were reactivated within 2 min in the presence of 0.1 mM-2-

Fig. 2. Separation of k' and k_s for compound (III) in accordance with the equation $k_{obs.} = k'/(1 + K_1/[I]) + k_s$

Table 1. Kinetic parameters for the inhibition of eel acetylcholinesterase by 1,3,2-dioxaphosphorinane 2-oxides. The results are for assays performed in 0.1 M-phosphate buffer, pH 6.8, at 25°C. The results have an estimated error of $\pm 10\%$.

X	K_1 (mM)	K_m (mM)	k' (min^{-1})	k_s (min^{-1})	k_1 ($\text{M}^{-1} \cdot \text{min}^{-1}$)
Cl				0.06†	5.4×10^5 †
F	0.65‡		0.52§	0.05§	0.59×10^3
	0.45*	0.051			
	0.42†		0.54†	0.049†	1.27×10^3 †
	0.32‡		1.2§	0.06§	3.75×10^3
	0.20*	0.068			
					1.5×10^6 ¶
	9.15†		0.038†		4.15†

* Calculated from eqn. 3.

† Results from Ashani *et al.* (1972).

‡ Calculated from eqn. 1.

§ Calculated from eqn. 1 and the relationship $k_{obs.} = k'/(1 + K_1/[I]) + k_s$.

|| Calculated from k'/K_1 .

¶ Calculated from $k_1 = k_{obs.}/[I]$.

pyridine aldoxime methiodide. The bimolecular rate constant for the inhibition of acetylcholinesterase by compound (IV) was found to be $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ in 0.1 M-phosphate buffer (pH 6.8, 25°C).

Incubation of acetylcholinesterase with compounds (IIa) and (IIb)

In these experiments the enzyme was inhibited for periods of 10–30 min, before 100-fold dilution into the spectroscopic cell, containing the Ellman reagent. Dissociation was completed at least in 10 s (assay limitation) as could be judged from the recorded slopes. These findings indicate that compounds (IIa) and (IIb) do not confer unusual binding properties to the enzyme. The free acid ($X = \text{OH}$) in these experiments was used either as the dicyclohexylammonium salt (see under 'Synthesis'; IIa) or obtained through a complete hydrolysis of compound (II) ($X = \text{Cl}$) in water.

Inhibition of acetylcholinesterase by compounds (I) and (III) in the presence of acetylthiocholine

In order to demonstrate the rapid formation of the reversible complex, we have also measured the activity of the enzyme immediately after the addition of known amounts of inhibitor to a mixture of acetylcholinesterase and acetylthiocholine. The following equation (Hunter & Downs, 1945) was used to evaluate the nature of the competition between the inhibitor and the substrate and to separate the individual constants K_1 and K_m :

$$\frac{E[I]}{E_0 - E} = K_1 + \frac{K_1}{K_m} [S] \quad (3)$$

where E_0 is the enzyme activity without inhibitor (I) and E is the recorded straight slope measured within 0.1–1 min after the addition of the inhibitor to the enzyme substrate mixture. K_1 and K_m were calculated from the intercepts on the vertical and horizontal axes respectively. The relation between the reversible inhibition and the concentration of acetylthiocholine is shown in Fig. 3. The results are summarized in Table 1.

Direct measurement of the spontaneous regeneration of acetylcholinesterase inhibited with compounds (I), (II) and (III)

The pH-rate profile of the spontaneous re-activation rate constant for the inhibited enzyme was constructed from various measurements using the inhibitors (I), (II) and (III) (Fig. 4). In all cases, the experimental conditions were directed to obtain 95–100% inhibition of activity of the phosphorylated enzyme. We have extended the measurements in phosphate buffer up to pH 8.5. Negligible changes in pH were observed during the kinetic measurements. It is

Fig. 3. Evaluation of K_1 and K_m according to eqn. (3)

Fig. 4. pH-rate profile of the spontaneous re-activation of eel acetylcholinesterase inhibited by various cyclic phosphates

The profiles were constructed from direct measurements after extensive dilution at 25°C. ○, Compound (I); ●, compound (II); □, compound (III)

worthwhile mentioning that we observed a significant acceleration of the re-activation in the presence of Tris buffer, as compared with phosphate at the same pH and buffer concentration ($>0.01 \text{ M}$). For example, in one case (pH 7.8, 0.1 M) the rate was almost doubled. Only at 5 mM-buffer could we eliminate the differences between the two salts. Maglothín *et al.* (1975) reported recently on the specific acceleration of dephosphorylation of a diphenylphospho group from eel acetylcholinesterase by substituted quaternary ammonium cations, and our results provide a second example of such an effect.

Fig. 5 demonstrates the re-activation profile of inhibited acetylcholinesterase in 0.1 mM-2-pyridine aldoxime methiodide, and without the specific re-activator. First-order plots for the re-activation resulted in reasonable straight lines under the experi-

Fig. 5. Plot of percentage of re-activation versus time of inhibited eel acetylcholinesterase by compounds (I), (II) and (III) at pH 6.8 and 25°C

●, 0.1 M-Phosphate; △, 0.1 mM-2-pyridine aldoxime. Final inhibitor concentrations in the re-activation medium were: compound (I) (a), 5.1 μ M; compound (II) (b), 0.04 μ M; compound (III) (c), 4.0 μ M.

mental conditions carried out in this work. In addition, the rates of the re-activation were found to be independent of the initial degree of inhibition whenever compound (III) was used to produce the inhibited enzyme. We noted that in several experiments where the enzyme was inhibited by high concentration of compound (II) (50 μ M) the maximal re-activation obtained was 75–80%. In addition, the regeneration rate constants with or without 2-pyridine aldoxime methiodide were also decreased. A similar phenomenon was observed with regard to compound (I) whenever the inhibition periods were too long. The results of the re-activation of the inhibited acetylcholinesterase are summarized in Table 2, where the effects of quaternary ligands on the rate of the spontaneous re-activation are included. It was interesting to study the re-activated enzyme by

measuring kinetic parameters such as substrate hydrolysis or inhibition by organophosphorus compounds. Table 3 presents K_m values for acetylthiocholine and k_i for the bimolecular inhibition of acetylcholinesterase by tetraethyl pyrophosphate.

Acetone was present only where compound (II) was used to produce inhibited enzyme, and its maximal concentration in the re-activation media was less than 0.01 %. Nevertheless, in view of the interesting observations reported by Maglothin *et al.* (1975) regarding the organic solvent effect on the rate of the spontaneous re-activation of diphenylphosphoacetylcholinesterase, we measured the regeneration rate in the presence of dimethyl sulphoxide. We did find that the phosphorylated enzyme undergoes spontaneous re-activation at rates higher by a factor of 10 in the presence of 2% dimethyl sulphoxide.

Maximal re-activation of acetylcholinesterase inhibited by compounds (I), (II) and (III)

Table 4 summarizes the maximal re-activation of acetylcholinesterase previously inhibited with various 1,3,2-dioxaphosphorinane 2-oxides. The inhibited enzyme was incubated for very short periods with the inhibitors just to assure a significant amount of inhibition before its dilution (100-fold) into the re-activation solution. We measured the effect of two concentrations of 2-pyridine aldoxime methiodide, 0.1 and 1 mM. Although it was reported in the literature that 1 mM-2-pyridine aldoxime methiodide accelerated both spontaneous re-activation and aging of diphenylphosphoacetylcholinesterase (Maglothin *et al.*, 1975), the amount of re-activable enzyme was found to be independent of the oxime concentration.

Discussion

According to the pH-rate profile of the spontaneous re-activation for three different inhibitors (I, II and III) containing the same cyclic phospho moiety, we may conclude that the same inhibited enzyme is produced as far as its stability towards hydrolysis is concerned. Since it is not likely that different reversible complexes (EI) will exhibit the same (and slow) rate of dissociation, it is suggested that a common covalent phospho-enzyme intermediate is formed and can account for the slow return of enzyme activity. Such an intermediate is independent of the nature of the leaving group. However, a bell-shaped pH-rate curve cannot be used as the sole criterion for advocating dephosphorylation and ruling out slow dissociation from a reversible complex. Therefore, we have also considered the view that the cyclic ring might act as the dominant moiety of affinity in this particular series of organophosphorus esters. However, when we have carried out the same experiments described so far in the presence of the free acid (X = OH) or the corresponding methyl ester (X = OCH₃) we did not

Table 2. *Re-activation rate constants measured by dilution (pH 6.8, 25°C)*

Quaternary effector	Concentration (mM)	Compound ...	Reactivation rate constants (min ⁻¹)		
			(I)	(II)	(III)
0.10		0.27	0.287	0.315
0.25			0.092	0.085
	1.00		0.078	0.112	0.105
N ⁺ (CH ₃) ₄ Cl ⁻	0.25			0.105	0.085
	1.00		0.088	0.098	0.096
Tris	2.00			0.088	
None			0.086	0.101	0.092

Table 3. *Kinetic properties of re-activated acetylcholinesterase*
Results are from assays performed in 0.1 M-phosphate buffer, pH 6.8, at 25°C.

Inhibitor	Maximal re-activation (%)	K _m for acetylthiocholine (mM)	10 ⁻⁶ × k _i for tetraethyl pyrophosphate (M ⁻¹ · min ⁻¹)
No inhibitor		0.088	2.07
(I)	75	0.068	
(II)	75	0.078	2.05
(III)	100	0.071	1.93
		0.082	2.02

Table 4. *Maximal re-activation of inhibited acetylcholinesterase after 20 h at 25°C*

The inhibitor concentration is given before dilution into the re-activation medium (100-fold). The pH was adjusted with 0.1 M-phosphate buffer.

Inhibitor	Inhibitor concn. and initial activity upon dilution (%)	pH	Re-activation (% of control activity)		
			Spontaneous	0.1 mM-2-Pyridine aldoxime methiodide	1 mM-2-Pyridine aldoxime methiodide
Chloridate (II)	47 μM (6%)	6.10	78	92	90
		7.00	75	73	78
		7.30	83	85	85
		8.15	78	75	78
	4.7 μM (16%)	6.65	95	98	103
		7.00	98	98	99
		7.30	102	96	96
		7.90	97	94	97
		8.50	100	103	103
Pyrophosphate (III)	0.4 mM (15%)	6.65	94	96	96
		7.00	94	100	95
		7.50	97	100	100
		7.90	95		94
		8.50	92	94	94
Fluoridate (I)	3.5 mM (13%) 0.35 mM (40%)	6.8	72	78	75
		5.8	94	96	93
		6.5	92	95	95
		6.8	90	93	98
		7.3	92	97	92
		8.4	94	94	94

detect slow return of activity after extensive dilution. These compounds lack a fundamental requirement of an acetylcholinesterase phosphorylating agent, namely, a good leaving group. Therefore, one may expect only a reversible non-covalent binding to the enzyme and dissociation upon dilution. In these experiments dissociation was completed at least in 10s (assay limitation) and full enzyme activity was regained, even when the enzyme was inhibited for 30min with 1mM of compounds (IIa) or (IIb). The kinetic behaviour of compound (IV) may further rule out any unusual properties of the cyclic moiety by using the following rationale.

Convincing evidence has been provided, demonstrating the similar reactivity of a phosphorus atom towards nucleophilic displacements in aqueous solutions both for 1,3,2-dioxaphosphorinane 2-oxides and diethyl phosphates, the open-chain analogues $(C_2H_5O)_2P(O)X$ (Khan & Kirby, 1970; Ashani *et al.*, 1973). Nevertheless, the latter were found to be significantly more potent inhibitors of acetylcholinesterase in terms of rate of inhibition. For example, *OO*-diethyl phosphorofluoridate, $(C_2H_5O)_2P(O)F$, is 10^3 times more powerful than compound (I), the cyclic analogue (Ashani *et al.*, 1972). Thus it was interesting to prepare a mixed anhydride, comprising molecular moieties of both species. The new derivative (IV) was found to be a very potent inhibitor of acetylcholinesterase, with a bimolecular rate constant of $1.5 \times 10^6 M^{-1} \cdot \text{min}^{-1}$ (pH 6.8, 0.1M-phosphate).

On the basis of the experimental results described earlier, it can be postulated that eel acetylcholinesterase is phosphorylated by compound (IV) predominantly at the diethylphospho moiety, where the cyclic phospho moiety serves as the leaving group. In fact, the bimolecular rate constant is in good agreement with the predicted value calculated from the corresponding Brönsted curve, constructed for diethyl phosphates (Ashani *et al.*, 1973). Although the range of concentration of the inhibitor was as low as 0.1–1 μM , where the amount of the reversible complex EI is assumed to be negligible, these findings may help to rule out any unusual binding properties of a molecule containing 1,3,2-dioxaphosphorinane 2-oxide ring to acetylcholinesterase.

The values of K_i obtained for compound (III) by both methods (eqns. 1 and 3) are in good agreement. Although K_i evaluated for compound (III) by eqn. (1) differs somewhat from the value obtained by the other method, it still may be accepted as an indication that rapid formation of the reversible complex occurs before the phosphorylation of the enzyme. We note that K_m for acetylthiocholine was found to be practically the same for both compounds and in the range found for this substrate in a different set of experiments where this constant was evaluated without the presence of inhibitor by using a double-

reciprocal plot (Table 3). This in turn might indicate a common site, presumably the active site, on which both the substrate and the inhibitor compete.

It is worthwhile emphasizing here that the Hunter-Downs (1945) plot for the specific experimental conditions described in this work assume that the inhibitor forms a reversible complex with the enzyme that does not react further! The activity in this case is monitored immediately upon addition of inhibitor to the free enzyme. The second approach (eqns. 1 and 2) is based on a rapid dissociation of a complex (EI) already present in the reaction mixture. Therefore, the close agreement between the results generated from the two different methods may be accepted as a good indication for a very fast formation of equilibrium before phosphorylation.

The re-activability of inhibited acetylcholinesterase in the presence of various nucleophiles may illuminate the nature of the phospho-enzyme linkage. Among many nucleophiles known to affect the rate of the re-activation of phosphorylated acetylcholinesterase 2-pyridine aldoxime methiodide is one of the most powerful re-activators. This oxime was designed specifically to attack the phosphorus atom at the phospho-enzyme linkage and to displace the phospho moiety, where the enzyme is actually the leaving group. Structural requirements are very important, as can be judged from the poor reactivity of 3-pyridine aldoxime methiodide, about 10^6 times less active than 2-pyridine aldoxime methiodide in terms of the bimolecular rate constant for the re-activation of diethylphospho-acetylcholinesterase (Wilson & Froede, 1971). The effect of 2-pyridine aldoxime methiodide on the apparent rate of the spontaneous re-activation of acetylcholinesterase inhibited by compounds (I), (II) and (III) is shown in Fig. 5. First-order plots were obtained provided that the inhibited enzyme was extensively diluted into the recovery solution. The most important observation in this respect is that the re-activations in the presence of the 2-aldoxime were accelerated significantly. In addition, the re-activation rate constants were found to be practically very similar for all three inhibitors (Table 2). Contrastingly, 3-pyridine aldoxime methiodide tetramethylammonium chloride and Tris (<2mM) did not affect significantly the rate of the recovery of the various inhibited enzymes after incubation with compounds (I), (II) and (III). The acceleration of the spontaneous re-activation by 2-pyridine aldoxime methiodide strongly indicates that the restoration of enzyme activity is due to hydrolysis of a covalent intermediate rather than dissociation of a complex. A high concentration of 2-pyridine aldoxime methiodide (1mM) did not accelerate aging, as was found in the case of spontaneous hydrolysis of diphenylphospho-acetylcholinesterase (Maglothian *et al.*, 1975).

In the present study we could show that the pH-

dependence for the rates of the spontaneous recovery of eel enzyme inhibited by compounds (I), (II) and (III) results in the same bell-shaped curve. Such a pH profile is usually interpreted in terms of participation of at least two ionizable groups in the catalytic removal of the phospho moiety and was reported before in studies where the spontaneous hydrolysis was measured for dimethylphospho-enzyme (Wilson *et al.*, 1958), di-(2-chloroethyl)phospho-enzyme (Reiner & Aldridge, 1967), *p*-cyanophenylmethylphospho-enzyme (Hovanek & Lieske, 1972) and diphenylphospho-enzyme (Maglothin *et al.*, 1975).

Noteworthy here is our observation that 'aging' (10–25%) of the inhibited enzyme took place under the experimental conditions where the concentration of compound (II) was 5×10^{-5} M, or even in several cases where compound (I) was used as the inhibitor. Enzymes that could not be re-activated completely did show a significant slowing in their reactivation rate, either spontaneously or in the presence of 10^{-4} M-2-pyridine aldoxime methiodide. In three reproducible experiments, diethylphospho-enzyme incubated with 4×10^{-5} M-compound (II) could be re-activated completely in the presence of 2-pyridine aldoxime methiodide. On the basis of these results we may suggest that at high concentrations of compound (I) or (II) the enzyme is 'damaged' mainly at the active site. However when we compared the kinetic properties of enzyme recovered after inhibition with various cyclic derivatives no significant results were obtained to indicate any unusual changes in the regenerated enzyme (Table 3) though a partial regeneration did result with slowing the re-activation rate. Perhaps an overexposure of the enzyme (concentration and time) to compound (I) or (II) results in non-specific phosphorylation at several sites on the enzyme, and these may subsequently affect the enzyme activity, especially with regard to the rate of the spontaneous re-activation. Nevertheless it is important to note that at low concentrations of compound (II) the inhibited enzyme behaves exactly as phosphorylated acetylcholinesterase obtained both by compounds (I) and (III), and no aging was observed.

That conventional aging (dealkylation of phosphorylated enzyme) does not occur we learn from various experiments where the degree of the re-activated enzyme with or without 2-pyridine aldoxime methiodide was always identical and irrespective of the inhibitor used to phosphorylate the enzyme.

In two different trials, the enzyme was inhibited with compound (IV) (0.4μ M) and subsequently incubated with compound (I) or (III) under experimental conditions enough to provide an almost complete inhibition of the enzyme even without the presence of compound (IV). The inhibited enzyme was diluted into 2-pyridine aldoxime methiodide solution and the re-activation profile was found to be

identical with that of acetylcholinesterase inhibited by paraoxon, diethyl phosphorofluoridate or compound (IV). These results were reproduced where tetraethyl pyrophosphate or diethyl phosphorofluoridate were used to form the diethylphospho-enzyme. These experiments support the assumption that the inhibition of the enzyme by compounds (I) and (III) is directed at the active site of acetylcholinesterase, under the proper experimental conditions.

The results presented in the present work enable us to substantiate the hypothesis of formation of an unstable covalent phospho-enzyme intermediate as an explanation for the slow recovery of the inhibited acetylcholinesterase by either compound (I), (II) or (III). Consequently, eqns. (1) and (2) are relevant for the purpose of separating the individual constant for the various steps depicted in the reaction under 'Inhibition of acetylcholinesterase by compounds (I) and (III)'. This mechanism is suggested on the basis of the following observations.

(a) Three different inhibitors produced the same inhibited enzyme in terms of stability to hydrolysis and pH-rate profile. The same results were obtained from direct (extensive dilution) or indirect measurements.

(b) It was shown that the mixed anhydride (IV) behaves exactly as would be predicted from a typical diethyl phosphate inhibitor, thus minimizing the possibility that the cyclic moiety is responsible for unusual affinity. In more direct experiments we could show that reacting enzyme with cyclic acid and the corresponding methyl ester did not produce any unexpected complex as could be judged from dilution experiments where enzyme activity recovered immediately.

(c) The close agreement between K_i values derived from two different experimental approaches supports the view of rapid formation of the reversible inhibitor-enzyme complex.

(d) The specific acceleration of the spontaneous re-activation by 2-pyridine aldoxime methiodide (and not by 3-pyridine aldoxime methiodide) strongly supports the view of covalent phospho intermediate displaced by specific site-directed nucleophiles.

(e) The active site is essential for interaction between the enzyme and the various dioxaphosphorinanes used in this work.

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