that occur in the reaction but further information is needed to establish a definite mechanism and to identify the reaction intermediates.

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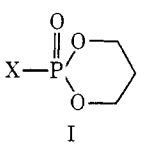
Inhibition of Cholinesterase by 1,3,2-Dioxaphosphorinane 2-Oxide Derivatives†

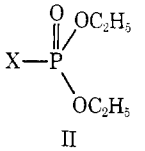
Yacov Ashani, S. L. Snyder, and Irwin B. Wilson*

ABSTRACT: Eel acetylcholinesterase, previously inhibited with 2-chloro-1,3,2-dioxaphosphorinane 2-oxide, was found to reactivate spontaneously with $t_{1/2} \cong 12$ min at pH 7.0, in marked contrast to enzyme inhibited with diethyl phosphoryl derivatives. The spontaneous recovery rate was taken into account in formulating a scheme for the inhibition of the enzyme by the 2-fluoro and 2-*p*-nitrophenoxy derivatives. Inhibition by the chloridate needed a special treatment due to its high hydrolysis rate in aqueous solutions. 2-Fluoro- and 2-*p*-nitrophenoxy-1,3,2-dioxaphosphorinane 2-oxides are poorer inhibitors than the open-chain compounds, whereas the 2-chloro derivative has almost the same activity as *O,O*-diethyl phosphorochloridate. A two-step reaction is suggested for the inhibition of acetylcholinesterase by the ring and open-chain phosphates.

Fukuto and Metcalf (1965) described the preparation and hydrolysis of 1,3,2-dioxaphosphorinane 2-oxides (I) in which X equals Cl, F, and *p*-nitrophenoxy. The anticholinesterase activities of the three compounds were measured



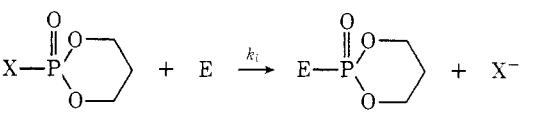
I



II

in terms of I_{50} values using fly head cholinesterase, and these values were compared with the I_{50} value of the open-chain analog *O,O*-diethyl phosphoro-*p*-nitrophenolate (paraoxon) II, X = *p*-nitrophenoxy.

They found that even though the rates of the reactions of the 2-fluoro- and 2-*p*-nitrophenoxy-1,3,2-dioxaphosphorinane 2-oxides with hydroxide ion are comparable to the corresponding open-chain compounds (see also Kahan and Kirby, 1970), the anticholinesterase activities of the cyclic compounds were relatively very poor. Since the reaction of the inhibitor and the enzyme is "formally" a second-order nucleophilic substitution reaction analagous to the reaction of the



inhibitor with hydroxide ion, it seemed interesting to reinvestigate this problem in somewhat greater detail.

We did make a surprising observation. We found that the phosphorylated enzyme (electric eel) (2-enzymo-1,3,2-dioxaphosphorinane 2-oxide) hydrolyzes rather rapidly in water ($t_{1/2} \approx 12$ min) to restore active enzyme in marked contrast to the open-chain *O,O*-diethylphosphoryl-enzyme. This

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phenomenon contributes to the poor anticholinesterase activity of the ring compounds.

We have measured k_i by using a scheme which takes into account the hydrolysis of the inhibited enzyme. The chloro compounds (cyclic and open chain) present a problem because they hydrolyze rapidly in water. However, by suitable technique the measurements can be made (Ashani *et al.*, 1972), and we have also been able to evaluate k_i for these compounds.

Methods

Materials. 2-CHLORO-1,3,2-DIOXAPHOSPHORINANE 2-OXIDE (I, X = Cl). 2-Chloro-1,3,2-dioxaphosphorinane 2-oxide was prepared from 1,3-propanediol and $POCl_3$ by the method of Lanham (1959). This compound was purified by preparing a saturated solution in anhydrous ether and allowing it to stand in the cold (4°) overnight. The large white crystals which had deposited were collected by filtration, and the recrystallization process was repeated. Product obtained in this manner had a mp of 44–45° (lit. 39°) (Lanham, 1959). *Anal.* Calcd for $C_3H_6ClO_3P$: C, 23.00; H, 3.87; Cl, 22.66. Found: C, 23.46; H, 4.22; Cl, 22.39.

2-FLUORO-1,3,2-DIOXAPHOSPHORINANE 2-OXIDE (I, X = F). An attempt to synthesize this compound by the procedure described by Fukuto and Metcalf (1965) resulted in a product containing a considerable amount of chloride (17.4%). Therefore, the conditions were modified slightly. A mixture of 12.0 g of 2-chloro-1,3,2-dioxaphosphorinane 2-oxide and 14.4 g of NaF in 150 ml of dry benzene was gently refluxed for 24 hr. The reaction mixture was filtered through Celite, and the benzene removed under reduced pressure. The crude product was then distilled with a conventional head saving only the middle fraction, bp 123° (2.2 mm), for redistillation. The second distillation over a Vigreaux column (~11-cm head) gave a middle fraction, bp 120° (1.5 mm), n_D^{26} 1.4072, which was found to contain only trace amounts of chloride (0.13%). *Anal.* Calcd for $C_3H_6FO_3P$: C, 25.71; H, 4.33; F, 13.57. Found: C, 25.97; H, 4.64; F, 13.64. The implication of this small amount of Cl will be discussed under Inhibition of the Enzyme.

2-*p*-NITROPHENOXY-1,3-2-DIOXAPHOSPHORINANE 2-OXIDE (I, X = pNP). This ester was synthesized by the procedure of Fukuto and Metcalf (1965). The product used in the present study was recrystallized four times from benzene, mp 100–101° (lit. 99–101°). *Anal.* Calcd For $C_9H_{10}NO_6P$: C, 41.68; H, 3.90. Found: C, 41.59; H, 3.97. All elemental analyses were conducted by Huffman Laboratories, Wheatridge, Colo. Melting points were measured with a Thomas-Hoover capillary melting point apparatus and are uncorrected.

Enzymes. Acetylcholinesterase from the electric organ of *Electrophorus electricus* (Worthington, specific activity 1 μM unit/1 μg) and butyrylcholinesterase from horse serum (Worthington, specific activity 4 μM unit/1 mg) were used. The stock solution was made in phosphate buffer (pH 7.0, μ = 0.1) containing 0.1% gelatine.

Enzyme Assay. The residual activity of the enzyme during the inhibition studies was measured by the Ellman procedure (Ellman *et al.*, 1961), at pH 7.0 in phosphate buffer, μ = 0.1. When butyrylcholinesterase was assayed, butyrylthiocholine (Sigma) was used as substrate, with a final concentration of 5.0×10^{-4} M. The rate of formation of the corresponding thiophenol was monitored at 412 nm, using a Zeiss PM Q II spectrophotometer attached to log converter, and recorded on Varian G-4000 recorder, scale 0–0.1 and 0.06–0.08 slit width. The enzyme was usually assayed for a period that did not exceed 1 min, and excellent straight lines were obtained in spite of the high rate of the spontaneous recovery ($t_{1/2} \cong 12$ min).

Inhibition of the Enzyme. 2-CHLORO-1,3,2-DIOXAPHOSPHORINANE 2-OXIDE. To a 1-ml aliquot of enzyme solution (~5×10^{-10} M) in phosphate buffer (pH 7.0, μ = 0.1, 25°) was added 5–15 μl of stock solution of the inhibitor in acetone ((1–10) $\times 10^{-5}$ M). At time intervals of 0.5–1 min, 10 μl of the inhibition mixture was diluted into 3 ml of phosphate buffer (pH 7.0, μ = 0.1) and assayed as described before.

2-FLUORO-1,3,2-DIOXAPHOSPHORINANE 2-OXIDE. The inhibition of the enzyme with this compound was carried out as described for the chloro derivative. However a preincubation of 30 min in phosphate buffer (pH 7.0, μ = 0.1) prior to the addition to the enzyme was needed to allow time for the hydrolysis of the trace amount of chloro compound present as an impurity. The $t_{1/2}$ value for the fluoro compound under these conditions is about 500 min.

2-(*p*-NITROPHENOXY)-1,3,2-DIOXAPHOSPHORINANE 2-OXIDE. This compound was preincubated for 60 min at pH 7.0 (50% acetone–phosphate buffer, μ = 0.1) prior to the addition to the enzyme. The spontaneous hydrolysis under these conditions is negligible. At concentrations higher than 8×10^{-3} M in the inhibition media precipitation occurred after ~20 min, probably due to the insolubility of the inhibitor at this concentration.

Direct Measurement of the Spontaneous Recovery of the Inhibited Enzyme. The recovery of the phosphorylated cholinesterase was measured directly by the following procedure. The enzyme was inhibited with 2-chloro-1,3,2-dioxaphosphorinane 2-oxide (electric eel cholinesterase inhibited with 2×10^{-6} M and horse serum cholinesterase with 2.0×10^{-4} M) for a period of 1–3 min. Under these conditions (phosphate μ = 0.1, pH 7.0, 25°) 95–98% of the enzyme was inhibited.

The inhibited enzyme was then diluted 500-fold into the recovery medium. At suitable time intervals, 25 μl was diluted into 3 ml of phosphate buffer and assayed for 1 min as described above. Phosphorochloridates have been used previously to obtain inhibited enzyme for recovery studies (Hovanec and Lieske, 1972).

Results

Inhibition of Cholinesterase. In preliminary work we found that the phosphorylated enzyme (enzyme inhibited by 2-chloro-1,3,2-dioxaphosphorinane 2-oxide) recovered quite rapidly ($t_{1/2} \cong 12$ min). As a result, the basic approach to the kinetic treatment in the inhibition studies is similar to that of carbamates (Wilson *et al.*, 1960). However, the inhibition of the enzyme with the 2-chloro compound involves an additional complication in that this compound hydrolyzes rapidly under the conditions of inhibition. Therefore, we shall discuss the 2-chloro separately from the 2-fluoro and 2-*p*-nitrophenoxy compounds.

Inhibition with 2-Chloro-1,3,2-dioxaphosphorinane 2-Oxide. The inhibition of the enzyme with this compound is expected to proceed according to the following scheme

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E \cdot I \xrightarrow{k'} E' \xrightarrow{k_s} E \qquad (1)$$

$$\downarrow k_h$$

products

where E is the free enzyme, I the inhibitor, k_h the hydrolysis rate constant of the inhibitor I, E·I the reversible Michaelis complex, k' the phosphorylation rate constant, E′ the phosphoryl-enzyme, and k_s the rate constant for the spontaneous recovery. Since this compound was found to be a highly potent inhibitor, its concentration in the inhibition studies was less than 5×10^{-7} M, which is believed to be well below K_I. Therefore eq 1 is simplified to

$$\begin{array}{ccccc} E + I & \xrightarrow{k_i} & E' & \xrightarrow{k_s} & E \\ & \searrow^{k_h} & & & \\ & \text{hydrolytic products} & & & \end{array} \qquad (2)$$

where $k_i = k'/K_I$. The rate of enzyme inhibition is given by

$$-\frac{d[E_t]}{dt} = k_i[E_t][I_t] - k_s[E_t'] \qquad (3)$$

eq 3. The concentration of phosphorylated enzyme is

$$[E_t'] = [E_0] - [E_t] \qquad (4)$$

$$[I_t] = [I_0]e^{-k_h t} \qquad (5)$$

where $[E_0]$ is the total concentration of the enzyme (the concentration of enzyme at $t = 0$) and $[I_0]$ is the initial concentration of the inhibitor. The subscript, t, is used to indicate the concentration at time t. Equation 5 is correct only if $[I_0] \gg [E_0]$. Substituting eq 4 and 5 into eq 3 we obtained

$$-\frac{d[E_t]}{dt} = [E_t](k_i[I_0]e^{-k_h t} + k_s) - [E_0]k_s \qquad (6)$$

A solution of eq 6 was derived by Dr. J. T. Hynes, of this department

$$\frac{[E_t]}{[E_0]} = \left[1 - M\left(1, 1 - \frac{k_s}{k_h}, \frac{k_i[I_0]}{k_h}\right)\right] \times e^{[-k_s t - (k_i I_0/k_h)(1 - e^{-k_h t})]} + M\left(1, 1 - \frac{k_s}{k_h}, \frac{k_i[I_0]}{k_h}e^{-k_h t}\right) \qquad (7)$$

The shape of eq 7 is given by the solid line in Figure 1. The three unknowns k_i, k_h, and k_s can be evaluated by curve fitting, where M is the Kummer function and is a function of three arguments as indicated. Tabulations of this function are available (Slater, 1960).

However, k_s can be determined separately by diluting inhibited enzyme and observing the rate of recovery of enzyme activity (see Methods). Also, k_h can be determined independently (Ashani *et al.*, 1972). The approximate value of k_i can then be obtained from the minimum of the curve (Figure 1)

$$-\frac{d[E_t]}{dt} = 0 \qquad (8)$$

Thus, from eq 6

$$k_i = \frac{k_s\left[\left(\frac{[E_t]}{[E_0]}\right)_{min}^{-1} - 1\right]e^{k_h t_{min}}}{[I_0]} \qquad (9)$$

$([E]_t/[E_0])_{min}$ and t_{min} are taken from the curve, $[E_t]/[E_0]$ *vs.* t.

It is evident from the experimental points of Figure 1 that $([E_t]/[E_0])_{min}$ can be obtained with reasonable accuracy but t_{min} will be only approximate. However there is some help here in that the relative error in k_i arising from an error in t_{min} is only about half as great as the relative error in t_{min}. The value obtained for k_i in the particular experiment shown in Figure 1 was 5.5×10^5 M^{-1} min^{-1} using $k_h = 0.156$ min^{-1} (to be published) and $k_s = 0.060$ min^{-1}. The experimental points are in reasonable agreement with the theoretical curve which indicates that the scheme is essentially correct. There are some difficulties in getting a really good match between theory and experiment for at least two reasons. (1) We do not recover all the enzyme at $t \to \infty$ but only about 93%. (2) The functions are tabulated on a relatively wide grid, *i.e.*, at 0.1 intervals. We have to make two interpolations. It turns out that for certain combinations of values these interpolation errors become very significant for eq 7 and the calculated value of $[E_t]/[E_0]$ behaves erratically. For this reason we intentionally used slightly different values for k_h and k_i in constructing the theoretical curve.

Inhibition by 2-Fluoro-1,3,2-dioxaphosphorinane 2-Oxide and 2-(p-Nitrophenoxy)-1,3,2-dioxaphosphorinane 2-Oxide. Since the hydrolysis rate of these two substrates is very small under the inhibition conditions eq 1 is simplified to

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k'} E' \xrightarrow{k_s} E \qquad (10)$$

Because these compounds are poor inhibitors high concentrations had to be used and $[I_0]$ was comparable to K_I.

The scheme indicates that a steady-state level of [E′] will be approached. The experimental technique involves extensive dilution of the enzyme solution in measuring enzyme activity. Therefore $E + E{\cdot}I = \mathcal{E}$ is actually measured. A similar situation was described and solved for carbamates (Wilson *et al.*, 1960). Two kinetic equations, the approach to the steady state (11) and the steady state (12), were used in this study to evaluate the various kinetic parameters

$$\ln\left[\frac{\mathcal{E}_t}{E_0} - \frac{E_t'}{E_0}\left(\frac{\mathcal{E}}{E'}\right)_{ss}\right] = -\left[\frac{k'}{\left(1 + \frac{K_I}{I}\right)} + k_s\right]t \qquad (11)$$

and

$$\left(\frac{\mathcal{E}}{E'}\right)_{ss} = \frac{k_s}{k'}\left(1 + \frac{K_I}{I}\right) \qquad (12)$$

where $\mathcal{E}_t$ is the measured activity of the enzyme at time t, E_0 the initial activity, and E_t' is given by $E_0 - \mathcal{E}_t$. Subscript ss denotes steady-state value. The second-order rate constant k_i is given by k'/K_I.

By plotting $(\mathcal{E}/E')_{ss}$ *vs.* I^{-1} (Figure 2) a straight line is obtained. The intercept is k_s/k' and the slope is $(k_s/k')K_I$. Thus K_I can be evaluated. To separate the individual rates from the ratio k_s/k' eq 11 was used for the approach to the steady state.

The slopes of curves for different [I] give

$$k_{obsd} = \frac{k'}{\left(1 + \frac{K_I}{[I]}\right)} + k_s \qquad (13)$$

and a further plot of k_{obsd} *vs.* $(1 + (K_I/[I]))$ separates k' and

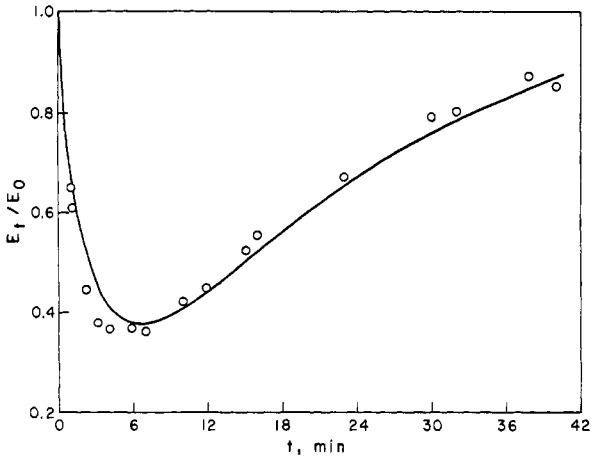


FIGURE 1: The inhibition curve for eel cholinesterase in the presence of 2-chloro-1,3,2-dioxaphosphorinane 2-oxide (5×10^{-1} M). The solid line was constructed theoretically using $k_i = 6.65 \times 10^5$ M^{-1} min^{-1}, $k_h = 0.17$ min^{-1}, $k_s = 0.06$ min^{-1}. The open circles represent experimental points from which k_i was calculated to be 5.4×10^5 M^{-1} min^{-1} using $k_h = 0.156$ min^{-1} and $k_s = 0.060$ min^{-1} (see eq 9).

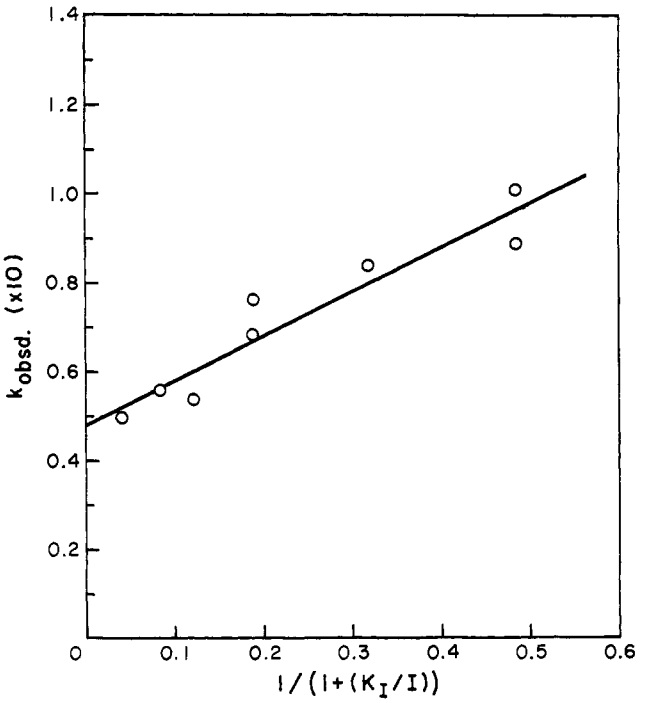


FIGURE 3: The evaluation of k' and k_s according to eq 13 (inhibitor: 2-fluoro-1,3,2-dioxaphosphorinane).

k_s (Figure 3). The value of k_s so obtained (0.049 min^{-1}) was somewhat lower than the value obtained directly (0.06 min^{-1}). We observed that a staight line could be obtained only after preincubation of the fluoridate at pH 7.0 for 30 min (Figure 4). We explain this finding by the fact that the elemental analysis of 2-fluoro-1,3,2-dioxaphosphorinane 2-oxide revealed a small amount of Cl (0.13%) that might be accounted for by 0.5% of the highly active 2-chloro-1,3,2-dioxaphosphorinane 2-oxide. The half-time for hydrolysis of the chloro compound is about 4 min. (This phenomenon did not appear to be im-

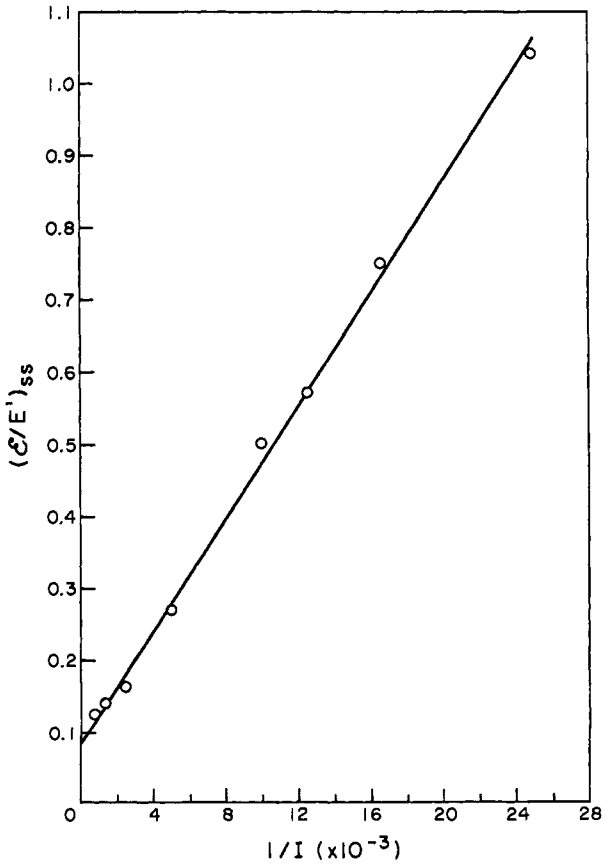


FIGURE 2: Plot of $(\epsilon/E)_{ss}$ *vs.* 1/[I] for the inhibition of eel cholinesterase with 2-fluoro-1,3,2-dioxaphosphorinane 2-oxide in accordance with eq 12.

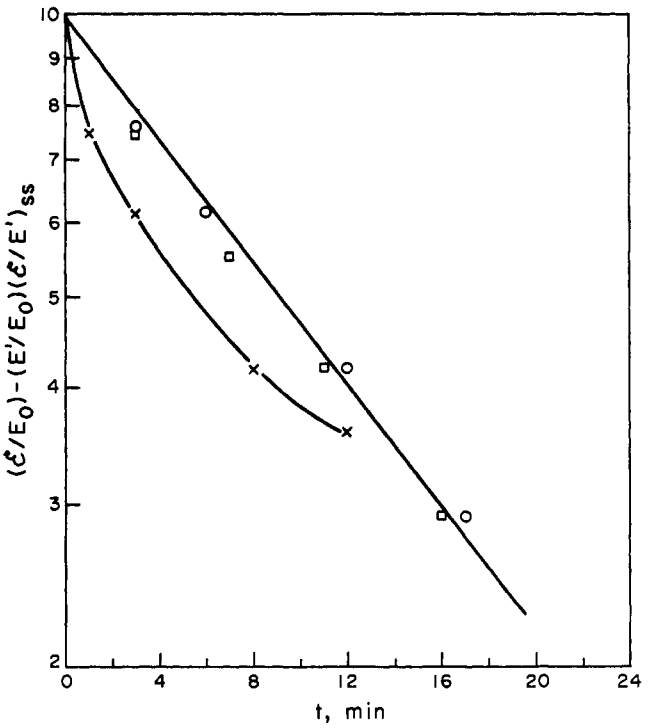
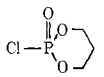
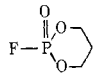
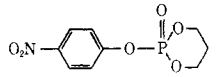


FIGURE 4: The inhibition curve of eel cholinesterase inhibited by 2-fluoro-1,3,2-dioxaphosphorinane 2-oxide (3×10^{-4} M), as a function of preincubation time at pH 7.0 (see also Results section). (X) 5-min preincubation; (□) 20 min; (●) 40 min.

TABLE I: Summary of Kinetic Parameters for the Inhibition of Acetylcholinesterase by 1,3,2-Dioxaphosphorinane 2-Oxides (T = 25°, pH 7.0, μ = 0.1 (Phosphate Buffer))

Inhibitor	Inhibitor Concn (M)	K_I (M)	k' (min^{-1})	k_i (min^{-1} M^{-1})	k_s (min^{-1})
	(2.5–5) × 10^{-7}			5.4 × 10^{5}[a]	0.060[b]
	(0.02–1.0) × 10^{-3}	4.25 × 10^{-4}[c]	0.54[d]	1.27 × 10^{3}[e]	0.049[d]
	(2.0–8.0) × 10^{-3}	9.15 × 10^{-3}[c]	0.038[f]	4.15[e]	

[a] Calculated from eq 9. [b] Calculated from direct measurements. [c] Calculated from eq 12. [d] Calculated from eq 12 and 13. [e] Calculated from dividing the intercept of eq 13, k_s, by the slope of eq 12, $(k_s/k')K_I$. [f] Calculated from eq 12 using k_s = 0.055, the average of k_s from *b* and *d*.

portant in the case of the *p*-nitrophenoxy compound.) Horse serumc holinesterase was found to be nonhomogeneous toward phosphorylation with 2-fluoro-1,3,2-dioxaphosphorinane 2-oxides. A similar phenomenon was observed by Main (1969). We also found nonhomogeneous behavior in the spontaneous recovery studies. Table I summarizes the kinetic parameters which are involved in the inhibition schemes.

Spontaneous Recovery. The spontaneous recovery was treated as a first-order reaction according to the equation

$$\ln \frac{E_0}{E_0 - E_t} = -k_s t \qquad (14)$$

where E_0 is the maximum activity obtained and E_t the activity of the enzyme at t, during the recovery. It is important to mention that only 90–94% of the initial activity of the enzyme was recovered during the spontaneous reactivation studies. It was observed that when the enzyme was treated with high concentrations of the inhibitor for relatively long periods, less and less recovery of the enzyme was obtained. Straight lines were obtained with eel enzyme.

Discussion

In contrast to the corresponding open-chain compounds (II), the 1,3,2-dioxaphosphorinane 2-oxides (I) studied here inhibit acetylcholinesterase at a rate which is highly dependent upon the particular leaving group. In this respect the behavior of the cyclic compounds resembles the poorer inhibitors of the open-chain series (Figure 5). The second-order rate constants for the fluoro and *p*-nitrophenoxy derivatives are much lower for the cyclic compounds than for the open-chain compounds. On the other hand the chloro compounds are of comparable activity.

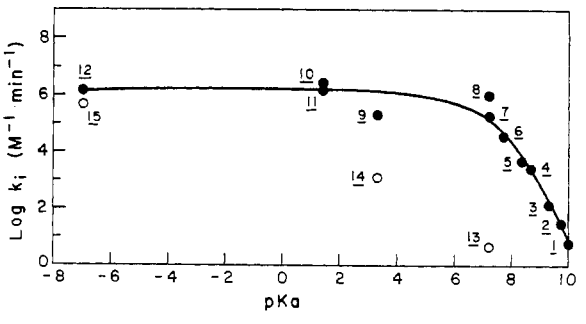
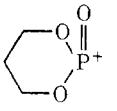


FIGURE 5: Linear free energy plot for the eel cholinesterase inhibition rate constant *vs.* the pK_a of the leaving group. The points 1–11 represent the diethyl phosphate series (Ashani *et al.*, 1972). The 1,3,2-dioxaphosphorinane 2-oxides are represented by 13, 14, and 15 and correspond to the *p*-nitrophenoxy, fluoridate, and chloridate, respectively.

An SN1 mechanism, *i.e.*, formation of phosphorylium ion



in a unimolecular mechanism is not likely to be the reason for the high activity of the chloridate toward acetylcholinesterase for the following reasons. (1) We were not able to detect any significant contribution of a SN1 pathway to the hydrolysis of the chloridate at pH 7.0 (to be published). (2) The bimolecular rate constants for the inhibition of eel cholinesterase are the same in the presence of NaCl or $NaNO_3$ (0.5 N). A general approach, that seems to us the best rationalization at the moment, for the results presented in this paper is as follows: the curve for the open-chain compounds (Figure 5) can reasonably be assumed to reflect the occurence of two successive steps in the phosphorylation of the enzyme (Ashani *et al.*, 1972). One step (A) is highly dependent on the pK_a of the leaving group whereas the other step (B) is either independent of the pK_a of the leaving group, or only slightly dependent upon it. If the ring structure in 1,3,2-dioxaphosphorinane 2-oxides should slow down step A, then the plateau region of Figure 5, which is so prominent for the open-chain compound, would not be reached until much lower pK_a values. Thus, in the case of the ring compounds, the *p*-nitrophenoxy and fluoro derivatives are not in the plateau but the chloro compound is.

The idea that the ring structure might impose steric difficulties is given some support by the high rate of the hydrolysis of the inhibited enzyme (spontaneous reactivation). It would appear that a sterically unfavorable situation exists in the phosphorylated enzyme derivative. The results obtained so far do not suggest any specific formulation for the nature of step A or B, nor do they suggest why step A might be slower

in the ring compounds. It may be that pseudorotation (Westheimer, 1968) plays some part in these reactions.

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Nuclear Magnetic Resonance and Infrared Identification of the N_1–H and the N_3–H Groups of Pseudouridine†

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ABSTRACT: From infrared and nuclear magnetic resonance (nmr) measurements on 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine in $CDCl_3$ solution, the tautomeric keto form is established for the two oxygen functions of the uracil ring. In nmr, the two NH signals of the ribose-substituted pseudouridine were identified on the basis of spin decoupling, the N_1–H signal being found at slightly higher fields and being coupled (J = 5.0 Hz) to C_6–H. The chemical shift of both NH groups is concentration dependent. Upon mixing with 5′-*O*-acetyl-2′,3′-*O*-isopropylidenadenosine the two NH resonances of 5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine are displaced downfield but one (N_3–H) more than the other. In the infrared, the monomeric stretching absorption of N_1–H of the pseudouridine derivative was identified at 3420 cm^{-1}, that of N_3–H at 3390 cm^{-1}. This assignment is based on comparison with N_1- and N_3-substituted uracil derivatives. The molar absorption coefficient of N_1–H (ϵ 314) is twice as large as that of N_3–H (ϵ 154), indicating a greater change in the dipole moment of the molecule caused by the N_1–H stretching vibration. Both NH groups are involved in strong self-association of pseudouridine by hydrogen bonds.

In the structure of tRNAs there are several odd nucleosides, and a search for their function remains one of the more interesting problems in nucleic acid biochemistry. Pseudouridine is one of these. It is present in the loops of the cloverleaf model of tRNA, in tyrosine tRNA it is present in the anticodon (Madison *et al.*, 1966), and very often it is found as a terminal $\psi \cdot A$[1] pair in double-stranded regions at the beginning of a loop. We have recently reinterpreted the ultraviolet (uv) spectrum of 5′-ψMP and established the anti conformation of this nucleotide by nuclear magnetic resonance (nmr) spectroscopy (Dugaiczyk, 1970). The anti conformation of the parent nucleoside has been independently established by Hruska *et al.* (1970).

The most characteristic and odd situation in pseudouridine arises from the two unsubstituted NH functions, both of which have a potential for a biochemical role. These two free NH groups have never been directly observed, let alone distinguished, although their presence was correctly inferred from uv spectroscopy and from the fact that pseudouridine is an isomer of uridine that can be N-substituted by methyls in two different positions (Scannell *et al.*, 1959). It would be highly desirable to be able to observe these two NH groups if one attempted a search for their biochemical function. In the present work we show that they can be experimentally observed and distinguished by nmr and infrared (ir) spectroscopy. Although the present results are obtained on a pseudouridine that has been substituted in the ribose moiety, the conclusions about the parent nucleoside appear to be a logical extension of the conclusions about the derivative.

Materials and Methods

5′-*O*-acetyl-2′,3′-*O*-isopropylidenepseudouridine was obtained as described earlier (Dugaiczyk, 1970). The same derivatives of uridine and adenosine were purchased from Sigma Chemical Co., St Louis. $CDCl_3$ (99.8%, NMR Specialties Inc., New Kensington) contains some exchangeable deuterium, which is evident in ir studies by the appearance of peaks around 3600 and 3675 cm^{-1}. For quantitative ir measurements the chloroform-*d* was used immediately after

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[1] Abbreviations used are: ψ, pseudouridine; A, adenosine; 5′-ψMP, pseudouridine 5′-monophosphate.