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THE INHIBITION OF CHOLINESTERASE BY DIETHYL PHOSPHOROCHLORIDATE

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SUMMARY

A method is described which enables the measurement of the rate constant for the inhibition of enzymes by covalent inhibitors that hydrolyze rapidly. The method(s) also yields the rate constant for the hydrolysis of the inhibitor. It was found that diethyl phosphorochloridate, depending on the buffer composition, hydrolyzes with a first-order rate constant of 1.0 min^{-1} in phosphate buffer ($I = 0.1$) at pH 7.0 and 25 °C and inhibits acetylcholinesterase (eel) (acetylcholine hydrolase, EC 3.1.1.7) with a second-order rate constant of $1.5 \cdot 10^6$ $M^{-1} \cdot min^{-1}$. Cholinesterase from bovine erythrocytes and horse serum were also studied. In any case, the phosphorochloridate was not found to be much more potent as an inhibitor than the corresponding phosphorofluoridate, even though the rate of hydrolysis of the latter is 2000 times slower.

INTRODUCTION

The inhibition of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and other esterases by organophosphate esters, as illustrated by diethyl phosphate esters, follows the reaction:

$$(C_2H_5O)_2\overset{\overset{O}{\|}}{P}\text{–X} + \text{E} \xrightarrow{k_i} (C_2H_5O)_2\overset{\overset{O}{\|}}{P}\text{–E} + \text{X}^-$$

where X is usually a good leaving group such as fluoride, *p*-nitrophenolate, thiocholine, *etc.* This reaction is intrinsically reversible[1] but is unidirectional under the usual conditions of measurements. Similarly, the diethyl phosphoryl enzyme derivatives slowly hydrolyze to yield active enzyme, but this reaction is usually too slow to affect measurements significantly[2]. Thus far, studies of the inhibition of cholinesterase have been made with several series of inhibitors containing a wide variety of leaving groups. However, organophosphates with Cl^- as leaving group were not included in those studies of organophosphate anticholinesterases. The lack of accurate data regarding the rate of phosphorylation of cholinesterases by phosphochloridates is

mainly because these compounds hydrolyze rapidly in water under the normal conditions of enzyme inhibition studies.

Detailed kinetic studies on the solvolysis rates of series of phosphorochloridates were reported by Dostrovsky and Halmann[3] as well as by others[4,5]. In those studies, three different techniques were used to follow the rate of hydrolysis, namely: (a) titration of the liberated Cl^-, (b) titration of the liberated acid, (c) increase in conductivity. Since in most cases the rate was too high to make measurements in 100% water solution, acetone–water or ethanol–water solutions were used as the solvolysis media, and the temperature was kept relatively low, to reduce the reaction rates.

We present in this paper kinetic studies with diethyl phosphorochloridate involving an enzyme technique, which enabled us to measure the inhibition rate constants with the enzyme (k_i) and also the rate of the hydrolysis in water (k_h). Diethyl phosphorochloridate was chosen for this study, since many members of this series were studied with respect to stability (k_h) and anticholinesterase activity (k_i).

Purdie and Heggie[6] derived kinetic equations for the inhibition of bovine erythrocyte acetylcholinesterase in the presence of *N*,*N*-dimethyl-2-phenylaziridinium ion, taking into account the hydrolysis of the inhibitor ($t_{1/2} \cong 150$ min, pH 8.0). The appropriate scheme for the inhibition with diethyl phosphorochloridate is:

$$\begin{array}{l} E + I \underset{K_I}{\rightleftharpoons} E \cdot I \xrightarrow{k'_i} E' + X^- \\ \quad k_h \downarrow \\ \quad \text{diethyl phosphate}^- + X^- + 2H^+ \text{ (hydrolytic products of I)} \end{array} \qquad (1)$$

where in this study I is diethyl phosphorochloridate, X^- is chloride, E is the enzyme, $E \cdot I$ is a reversible Michaelis complex formed between inhibitor and enzyme, and E' is the inhibited enzyme, *i.e.* the diethyl phosphoryl enzyme derivative. In most cases the concentration of I will be much lower than K_i and the scheme will simplify to:

$$\begin{array}{l} E + I \xrightarrow{k_i} E' + X^- \\ \quad k_h \downarrow \\ \quad \text{diethyl phosphate}^- + X^- + 2H^+ \end{array} \qquad (2)$$

where k_i is the second-order rate constant for inhibition. The second-order rate constant is given by: $k_i = k'_i/K_i$.

The concentration of the inhibitor during the inhibition reaction is given by Eqn 3 and the mathematical solution for Scheme 2 is expressed in Eqn 4.

$$I_t = I_0 e^{-k_h t} \qquad (3)$$

$$\ln \frac{E_t}{E_0} = I_0 \frac{k_i}{k_h} \left(e^{-k_h t} - 1 \right) \qquad (4)$$

where E_0 is the activity of the enzyme at zero time, E_t is the activity of the enzyme as a function of t, I_0 is the concentration of inhibitor at zero time and I_t is the concentration of inhibitor at time t. The general form of this function is shown in Fig. 1. As time increases a limiting value is approached:

$$\ln \frac{E_\infty}{E_0} = -I_0 \frac{k_i}{k_h} \qquad (5)$$

Thus by using different values of I_0, or even a single value, it is easy to obtain the ratio k_i/k_h. These rate constants can be separated in two ways:

(1) The constant k_h can be evaluated directly by an entirely independent method.

(2) The constant k_h can be evaluated using the same general enzymic technique:

(2a) Shoot a small volume of an acetone solution of the inhibitor into the standard buffer. The rate of hydrolysis of the inhibitor can be measured as follows. At appropriate time intervals thereafter, say every 10 s, assay for inhibitor by injecting an aliquot of this solution into the enzyme solution as described above. Use Eqn 5 to evaluate the concentration of inhibitor at the time it was injected into the enzyme solution. Thus the I_0 value of Eqn 5 becomes a function of the time of prior hydrolysis, I. This value of I as determined from Eqn 5 is used to evaluate k_h by Eqn 3.

(2b) The value of k_h can be determined from even a single curve of Fig. 1. Eqn 4 can be rearranged and worked to give

$$\ln\left(\ln\frac{E_t}{E_0} - \ln\frac{E_\infty}{E_0}\right) = \ln\ln\frac{E_0}{E_\infty} - k_h t \qquad (6)$$

from which k_h can be evaluated by plotting the left hand side of Eqn 6 *versus* time t.

METHODS

Materials

Diethyl phosphorochloridate (Aldrich) was redistilled with a Vigreaux column (20 cm) b_{10} 67° (lit.[7] b_{6-7} 64°). Diethyl phosphorofluoridate was prepared according to Saunders and Stacey[8]. Stock solution from both phosphates were prepared in acetone (Mallinckrodt, spectroscopic grade). Acetylcholinesterase from the electric organ of *Electrophorus Electricus* (Worthington, spec. act. 1000 units/mg). Acetylcholinesterase from bovine erythrocytes (Sigma, spec. act. 2.6 units/mg). Butyrylcholinesterase from horse serum (Worthington, spec. act. 4 units/mg). Stock solution of enzymes (approx. $5\cdot10^{-8}$ M) were made in phosphate buffer ($I = 0.1$), pH 7.00) containing 0.1% gelatin. *p*-Nitrophenol (Baker), stock solution made in acetone.

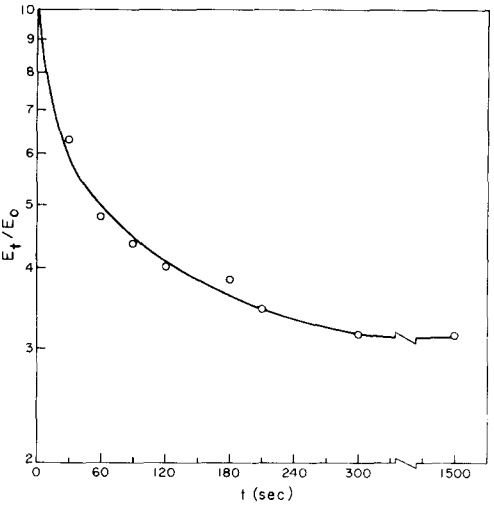
Enzyme assay

The residual activity of the enzyme during the inhibition studies was measured as described by Ellman *et al.*[9]. Butyryl thiocholine ($5\cdot10^{-4}$ M) was used for the butyrylcholinesterase assay. The change in absorbance was recorded at 412 nm using the Zeiss PMQ II spectrophotometer equipped with a log converter and a Varian recorder G-4000, scale 0–0.1 and slit width 0.06–0.08 mm.

Inhibition of the enzyme

At zero time 5–15 μl of diethyl phosphorochloridate (or fluoridate) in acetone ($0.08\cdot10^{-4}$–$1.5\cdot10^{-4}$ M) were added to 1 ml of enzyme solution (approx. $5\cdot10^{-10}$ M) in the appropriate buffer composition at pH 7.0. At time intervals of 10–20 s, 10 μl of the inhibition mixture were diluted into 3 ml of phosphate buffer, pH 8.00 ($I = 0.1$) and the residual activity of the enzyme assayed as described above, by the Ellman procedure. A typical plot of $\ln E_t/E_0$ *vs* time t is presented in Fig. 1 for diethyl phosphorochloridate. The ratio k_i/k_h was determined from Eqn 5 and k_h from Eqn 6.

The value of k_h was also determined in another set of experiments using Eqn 3



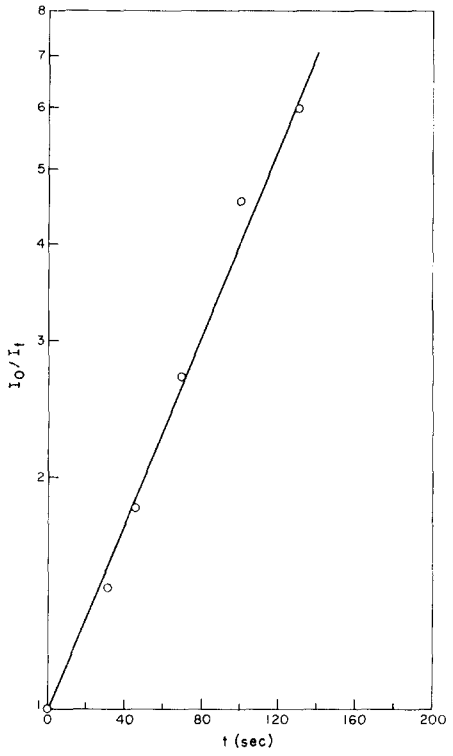


Fig. 1. A semilogarithmic plot of percent inhibition of eel acetylcholinesterase by diethyl phosphorochloridate *versus* time (experimental curve). The conditions of measurement were pH 7.0, 25 °C in a medium phosphate (I = 0.1) + gelatin 0.01%.

Fig. 2. The evaluation of k_h, using Method 2a in accordance with Eqn 3. Buffer composition: see Fig. 1.

and Method 2a. The inhibitor in acetone ($0.7 \cdot 10^{-2}$–$1.5 \cdot 10^{-2}$ M) was diluted 100× into a suitable buffer. At different time intervals (10–20 s) 10 μl were mixed with 1 ml enzyme solution ($5 \cdot 10^{-10}$ M) in the same buffer composition and pH. The limit values, $(k_i/k_h) \cdot I_0(t)$, (for explanation see introduction) were measured after 10–15 min of incubation as described above. Since k_i/k_h is known (see above) (I_0 is the initial concentration of the inhibitor), $I_0(t)$ can be calculated for each different time period of hydrolysis in the buffer without enzyme. The plot of ln I_0/I_t *vs* time t (in accordance with Eqn 3 provides a straight line with the slope k_h (see Fig. 2).

Direct measurement of the rate of hydrolysis (k_h)

During the hydrolysis of $(C_2H_5O)_2P(O)Cl$, two equivalents of acid are released. Therefore the rate of hydrolysis can be monitored directly by spectrophotometry at 400 nm using *p*-nitrophenolate anion as an indicator since proton release during the hydrolysis diminishes the concentration of the *p*-nitrophenolate by converting it to *p*-nitrophenol. (The absorbance of *p*-nitrophenol is negligible under the experimental conditions.) Thus, to 3 ml of *p*-nitrophenol ($0.3 \cdot 10^{-4}$–$1.0 \cdot 10^{-4}$ M, 26 °C) in the appropriate buffer composition (final dilution contains 1% acetone), were added 10–40

μl of the inhibitor in acetone ($1.5 \cdot 10^{-2}$–$7.0 \cdot 10^{-2}$ M). The rate of disappearance of the *p*-nitrophenolate was monitored by Cary 16 at 400 nm, recorded on a Varian recorder G-2000 using 0–0.1 scale and 0.06–0.08 mm slit width. pH measurements were taken at the end of each run using a Radiometer pH meter TTTlC attached with scale expander PH630 Ta. The method was calibrated with benzoic acid in acetone.

RESULTS

Enzyme inhibition

We made 15 runs in which enzyme activity was measured as a function of time following the injection of inhibitor into the enzyme solution using acetylcholinesterase from electric eel. According to Eqns 4 and 5, a plot of ln E_t/E_0 *vs* time t should approach a constant value depending upon the value of I_0. Typical results are shown in Fig. 1. It can be seen that after 5 min practically all the inhibitor is hydrolyzed, leaving behind an inhibited fraction of the enzyme which has reached a constant value. The data of Fig. 1 are plotted in accordance with Eqn 6 in Fig. 3 where

$$\ln\left(\ln\frac{E_t}{E_0} - \ln\frac{E_\infty}{E_0}\right)$$

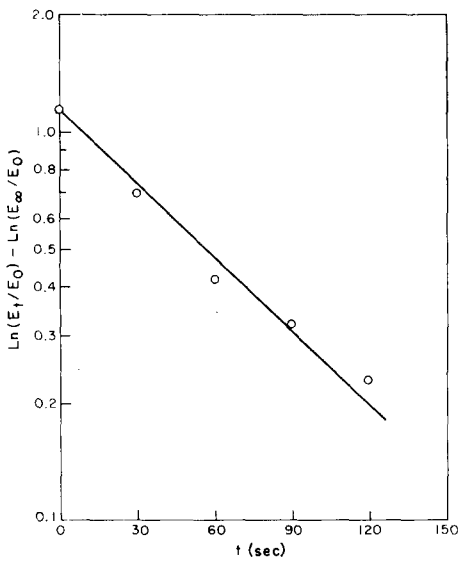


Fig. 3. The evaluation of k_h from the plot of ln(ln E_t/E_0 – E_∞/E_0) *vs* time t (Method 2b). (Best straight line according to Method 2b.) Same data as in Fig. 1.

is plotted *vs* time t. The slope of this plot yields k_h. The value of k_i was calculated from the following expression (Method 2b):

$$k_i = \frac{\left[k_h \cdot \ln\frac{E_\infty}{E_0}\right]}{I_0}$$

derived from Eqn 5 where ln E_∞/E_0 is the limit value for each separate run. The

TABLE I

MEASUREMENT OF k_h AND k_i FOR ACETYLCHOLINESTERASE (EEL) FROM ENZYME INHIBITION

Method 2b. The pH was 7.0 at 25 °C.

Run No.	*Diethyl phosphorochloridate concn (μM)*	*Buffer (I)*	k_h *(min^{-1})*	$k_i \times 10^{-6}$ *($l \cdot mole^{-1} \cdot min^{-1}$)*
1–5	0.80–1.25	Phosphate (0.1)	1.03 ± 0.03	1.54 ± 0.04
6–9	0.90–1.65	Phosphate (0.05) $NaNO_3$ (0.05)	0.82 ± 0.02	1.09 ± 0.05
10–15	0.78–1.65	Phosphate (0.05) NaCl (0.05)	0.82 ± 0.03	1.22 ± 0.05

results obtained by this technique are summarized in Table I for three different buffer compositions (pH 7.0) at 25 °C.

Fig. 2 shows the plot of $\ln I_0/I_t$ *vs* time t from which k_h was evaluated using Method 2a. The value of k_h obtained by this method is in good agreement with the value obtained by the method (Method 2b) illustrated in Fig. 3.

The values of k_h determined directly using the indicator method are given in Table II for the three buffers used in the enzyme inhibition study. These values are in good agreement with the values obtained from the enzyme inhibition studies. Thus the enzyme method gives correct values for k_h and we may have confidence in the k_i values for enzyme inhibition.

TABLE II

DIRECT MEASUREMENT OF k_h USING *p*-NITROPHENOL AS AN INDICATOR, pH 7.00, 26 °C

During the course of the run the pH dropped on the order of 0.03 unit.

Run No.	*Buffer (I)*	*Diethyl phosphorochloridate concn (mM)*	*p-Nitrophenol concn* $\times 10^4$ *(M)*	k_h *(min^{-1})*
1–11	Phosphate (0.1)	0.23–0.95	0.33–1.00	0.98 ± 0.02
12–16	Phosphate (0.05) $NaNO_3$ (0.05)	0.23	0.50–1.00	0.86 ± 0.03
17–21	Phosphate (0.05) NaCl (0.05)	0.23	0.50–1.00	0.82 ± 0.04

TABLE III

SECOND-ORDER RATE CONSTANTS FOR THE REACTION OF DIETHYL PHOSPHOROCHLORIDATE AND FLUORIDATE WITH DIFFERENT SOURCES OF CHOLINESTERASE

The substrate used for the assay of eel and erythrocyte enzyme was acetylthiocholine, $5 \cdot 10^{-4}$ M. The substrate used for the assay of horse serum enzyme was butyrylthiocholine, $5 \cdot 10^{-4}$ M. The medium used for phosphorylation contained Na_2HPO_4–KH_2PO_4 buffer (I = 0.05, pH 7.0) and NaCl (I = 0.05). Temperature, 25 °C.

Enzyme source	k_i *($l \cdot mole^{-1} \cdot min^{-1}$)*	
	$(C_2H_5O)_2P(O)Cl$	$(C_2H_5O)_2P(O)F$
Electric eel	$1.2 \cdot 10^6$	$2.2 \cdot 10^5$
Bovine erythrocyte	$1.2 \cdot 10^6$	$1.0 \cdot 10^5$
Horse serum	$7.0 \cdot 10^6$	$6.6 \cdot 10^6$

Finally, Table III summarizes some of the inhibition rate constants for the reaction of diethyl phosphorochloridate and diethyl phosphorofluoridate with different cholinesterases. The rate constants for inhibition by diethyl phosphorofluoridate (which hydrolyzes so slowly that its hydrolysis may be neglected) were calculated using the following equation:

$$\ln \frac{E_t}{E_0} = -k_i I_0 t$$

where I_0 is the concentration of the inhibitor, k_i the second-order rate constant, E_0 the initial activity of the enzyme and E_t the activity at time t.

DISCUSSION

Even though diethyl phosphorochloridate hydrolyzes with a half life of less than 1 min, it is possible to get accurate values of the rate constant for the inhibition of cholinesterase. The method also gives the value of the rate constant for the hydrolysis of phosphorochloridate.

Although the phosphorochloridate hydrolyzes 4000 times faster than the phosphorofluoridate, it inhibits butyrylcholinesterase (horse serum) only 10% faster and red cell and eel acetylcholinesterase only 5 and 12 times faster, respectively. Since the reaction of the enzyme with these inhibitors is a nucleophilic substitution reaction analogous to the hydrolytic reaction, it seems surprising that the phosphorochloridate is not considerably more potent relative to the phosphorofluoridate.

However these results are consistent with previous observations of a levelling effect when the logarithm of k_i is plotted as a function of the pK_a of the conjugate acid of the leaving group[10]. Our present results extend the range by 8 pK_a units (Fig. 4) and make the phenomenon quite distinct. The pK_a range is very large, circa 17 pK_a

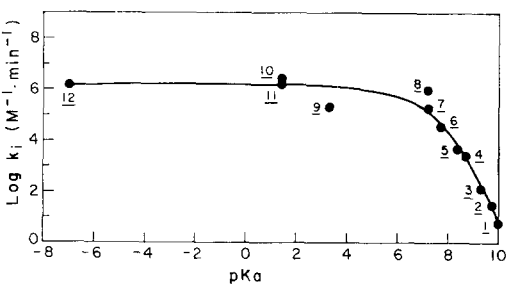


Fig. 4. Brønsted plot for the inhibition of eel cholinesterase *vs* the pK_a of the leaving group of $(C_2H_5O)_2P(O)X$. The numbers refer to various diethyl phosphates with the following leaving group (X): 1, phenol; 2, 8-hydroxyquinoline; 3, *p*-chlorophenol; 4, 6-hydroxyquinoline; 5, *m*-nitrophenol; 6, 3-hydroxyquinoline; 7, 8, *p*-nitrophenol; 9, fluoride; 10, 11, diethyl phosphate; 12, chloride. The data for 1, 3, 5, 8 and 10 are from ref. 13. 2, 4, 6, 7 and 11 were taken from ref. 10.

units, which tends to bring out curvature in Brønsted plots. Nonetheless these compounds react with hydroxide ion at rates that provide a linear Brønsted plot (to be published). Thus, the non linearity of the logarithm of the rates of inhibition *vs* pK_a appears to be a consequence of the special events which attend the reaction of the

enzyme with the inhibitor. Such a curved plot suggests a change in mechanism or a change in the rate controlling step of a multistep process[11].

The presence of two enzyme conformations, only one of which can react with the inhibitor, can also account for the break in the Brønsted plot. However this possibility is not likely since we found that the reaction of the enzyme with diethyl phosphorochloridate is a second-order reaction.

The effect of buffer composition on the rate of hydrolysis of the chloridate is worth some comment. Substitution of chloride for nitrate (sodium salts) does not diminish the rates (k_h). This suggests that an S_N1 pathway is probably not the major mechanism of hydrolysis. This also minimizes the possibility that the enzyme is inhibited by phosphorylium ion, $(RO)_2\overset{O}{\overset{\|}{P}}^+$. (Such a mechanism might also explain the break in the Brønsted plot.) The increased rate in the presence of phosphate is consistent with observations made with other phosphate esters[12] and is probably a general base catalysis.

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