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REACTIVATION AND AGING OF DIPHENYL PHOSPHORYL ACETYLCHOLINESTERASE

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Summary

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is readily inhibited by 10^{-5} M diphenylphosphorochloridate even though the inhibitor hydrolyzes in a few seconds. The fluoridate is a much weaker inhibitor. The inhibited enzyme, diphenyl phosphoryl enzyme spontaneously recovers only about 50% of its activity with a half time of about 17 min at pH 7.0 and 6 min at pH 8.0. The fact that only 50% of the original activity returns is due to aging.

The rates of reactivation and aging can be very greatly increased by a few percent of an organic solvent. Depending on the solvent even 1% may increase the rates by a factor of 5 or 6. The highest increase in rate was 70-fold. Quaternary NH_4^+ also increases the rates. Organic solvents and NH_4^+ also accelerate the reactivation of the much more stable diethyl phosphoryl enzyme derivative.

Introduction

Organophosphate and phosphonate esters that contain a good leaving group, X, are potent inhibitors of acetylcholinesterase. These compounds react at the active site with the enzymic nucleophile, the hydroxyl group of serine, in a reaction analagous to the normal acylation of the enzyme, E, that occurs with esters of carboxylic acids:

$$(RO)_2P(O)X + E \underset{k_r}{\overset{k_i}{\rightleftharpoons}} \underset{E'}{(RO)_2P(O)E} + X^- \qquad (1)$$

This reaction is reversible and it is possible to reactivate the inhibited enzyme,

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E′, using a variety of nucleophiles. Since water can serve as a nucleophile, the inhibited enzyme reactivates spontaneously, in a reaction analagous to the normal deacylation reaction to produce active enzyme. The inhibited enzyme, E′, may also react with water in a different manner to release an alcohol instead of the enzyme:

$$\begin{array}{l}(RO)_2P(O)E + H_2O \xrightarrow[\text{spontaneous reactivation}]{k_r} (RO)_2P(O)OH + E \\ \quad\Big|\ E' \\ k_a\ \Big\downarrow\ \text{aging} \\ (RO)(OH)P(O)E + ROH\end{array} \tag{2}$$

This second mode of reaction is called aging and produces an enzyme derivative that is still inactive and is not readily reactivated with nucleophiles.

The spontaneous reactivation and also the aging of the diphenyl phosphoryl enzyme is quite rapid [1]. In further studying this reaction we found that the rates of these processes are very greatly increased by the addition of small quantities of organic solvents. Therefore, we also studied the effect of organic solvents on the diethyl phosphoryl enzyme with similar results. We here report on these and related studies with the two enzyme derivatives.

We used diphenylphosphorochloridate to produce the diphenyl phosphoryl enzyme according to the scheme

$$\begin{array}{l}E + I \xrightarrow{k_i} E' + X \\ \quad\Big|\ + H_2O \\ \quad\Big\downarrow\ k_h \\ \text{diphenylphosphate} + X^- + 2H^+\end{array} \tag{3}$$

where I is the inhibitor and E′ is diphenyl phosphoryl enzyme. The solution for scheme 3 is

$$\ln\frac{[E]}{[E^0]} = [I_0]\frac{k_i}{k_h}\left(e^{-k}h^t - 1\right) \tag{4}$$

where $[E^0]$ is the initial concentration of enzyme and $[I_0]$ the initial concentration of inhibitor. When the inhibitor has hydrolyzed completely the activity that remains, E_0, is given by

$$\ln\frac{[E_0]}{[E^0]} = -[I_0]\frac{k_i}{k_h} \tag{5}$$

Ashani et al. [2]. We use the symbol E_0 rather than the more logical E_∞ because the activity that remains will be the zero time activity for the recovery and aging processes that now take place, Fig. 1. The constant k_h may be measured by any of several means [2] allowing the evaluation of k_i. This technique was applied to a number of compounds containing P-Cl bonds [1]. Inhibition with P-Cl bonds are convenient for the study of reactivation and aging since excess inhibitor is rapidly removed by hydrolysis [3] on a time scale that is usually short compared to the recovery processes. The solution for

the rate of recovery is

$$\ln \frac{[E_\infty] - [E_0]}{[E_\infty] - [E]} = (k_a + k_r)t$$

and (6)

$$\frac{k_a}{k_r} = \frac{[E^0] - [E_\infty]}{[E_\infty] - [E_0]}$$

where E_∞ is the final activity after the recovery process has been completed [3,4]. In applying the rate equation we used the Guggenheim method [5].

Experimental

Materials. Eel acetylcholinesterase was purchased from Worthington Biochemical Corp. as the lyophilized preparation ECHP 1JA, specific activity 1076 units per mg. This is an 11-S enzyme form [6,7]. We also used, where stated, 18-S and 14-S forms obtained from frozen tissue [8] and the aggregated form obtained from them at low ionic strength [8—10]. Diphenylphosphorochloridate (Aldrich) was double distilled prior to use, first by passage through a falling-film still at 132°C (refluxing xylene), 1.0 mm Hg, then by fractionation on a 15 cm Vigreaux column at 1.2 mm Hg. The middle (major) fraction $b_{1.2}$ = 148—149°C (lit. $b_{3.3}$ = 217—220°C) was saved for inhibition studies. Solvents were spectroscopic quality or reagent grade. When used as the solvent for inhibitor stock solutions, acetonitrile was distilled from anhydrous $MgSO_4$. Acetylthiocholine iodide was prepared by the reaction of *S*-(2-(dimethylamino)ethyl)thioacetate (Eastman) with methyl iodide in ether and was recrystallized from ethanol. Flaxedil (gallamine triethiodide) was a gift from American Cyanimid Co., Lederle Laboratories Division, Pearl River, N.Y. Water was double distilled, the second time from a quartz still. All other materials were reagent grade from various sources.

Methods. A typical reaction profile for the inhibition of acetylcholinesterase by diphenylphosphorochloridate and subsequent recovery of the phosphorylated enzyme is shown in Fig. 1. Since the half time for hydrolysis of the inhibitor is about 2 s, the inhibition reaction has essentially ceased after 10—15 s. The inhibition procedure was to add a few microliters of a stock solution of the inhibitor in an aprotic solvent, usually acetonitrile, to 1 ml of buffered enzyme solution which was stirred rapidly for 10—15 s. Desired conditions for the study of recovery were established either by addition of required components or by dilution with the desired medium. With increasing time, the enzyme activity does not return to the pre-inhibition (100%) value, but rather to some intermediate activity as a consequence of the aging process.

Enzyme assays. Enzyme activity was determined spectrophotometrically by the method of Ellman et al. [11]. The typical assay medium was $1.0 \cdot 10^{-3}$ M acetylthiocholine iodide, $3.3 \cdot 10^{-4}$ M 5,5′-dithio-bis(2-nitrobenzoic acid) in I = 0.1 M sodium phosphate buffer, pH 7.0, 25°C.

Determination of the ratio k_i/k_h for the inhibition of acetylcholinesterase by diphenylphosphorochloridate. Solutions of diphenylphosphorochloridate

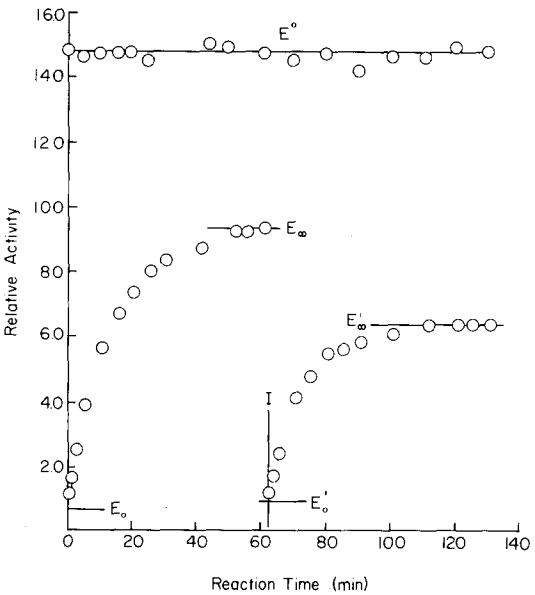

Fig. 1. Reaction profile for two successive inhibitions of the same acetylcholinesterase sample by diphenyl phosphorochloridate I = 0.10, pH 7.0, 25°C. The initial inhibitor concentration was $1.0 \cdot 10^{-5}$ M for both determinations, but the concentration of acetonitrile was different, 1.0% for the first recovery and 2.0% for the second. For the first process, $k_a + k_r = 0.079$ min^{-1} and $k_a/k_r = 0.63$, for the second, $k_a + k_r = 0.098$ min^{-1} and $k_a/k_r = 0.59$. The differences in rate may be explained by the different concentrations of acetonitrile present.

were prepared ranging in concentration from $2 \cdot 10^{-3}$ to $1 \cdot 10^{-2}$ M in dry acetonitrile. At zero time, 2.0 μl of inhibitor were added to 1.0 ml of approx. 10^{-8} M enzyme solution in I = 0.05 phosphate, I = 0.05 NaCl, pH 7.0, 25°C, and the mixture was stirred rapidly for 10—15 s. At 0.5-min intervals 50-μl aliquots were withdrawn and assayed for residual enzyme activity. The activity immediately after inhibition (t = O) was determined by extrapolation of the activity versus time plot to t = O order to correct for the small increase in activity arising from spontaneous reactivation during the time prior to the first assay. The entire procedure was repeated with 0.8% acetonitrile and 1.0% 2-butanone in the same buffer system.

Determination of k_h for diphenylphosphorochloridate. 2 μl of 10^{-2} M inhibitor in acetonitrile were added to 1.0 ml I = 0.1 phosphate buffer, pH 7.0, 25°C at t = O. At times varying from 1 to 5 s later, a 10-μl aliquot of 10^{-6} M acetylcholinesterase in buffer was added, and samples were withdrawn at 0.3-min intervals for a period of 2 min for assay. The concentration of inhibitor remaining (i.e. not hydrolyzed) at the time of addition of enzyme was determined from the degree of inhibition obtained according to Eqn 3.

Rate of solvolysis of diphenylphosphorochloridate in 80% ethanol. Dostrovsky and Halmann [12] have determined the rate of solvolysis of diphenylphosphorochloridate in 80% ethanol by chemical means. This rate was determined in the following manner using the enzyme to measure the phosphorochloridate. At zero time, 10 μl of 0.1 M diphenylphosphorochloridate in

acetonitrile were added to 1.0 ml 80% ethanol, 25°C. 10-μl aliquots were withdrawn at times varying from 0.6 to 6 min and assayed for inhibitory activity by addition to 1.0 ml 10^{-8} M acetylcholinesterase in $I = 0.1$ phosphate buffer, pH 7.0, 25°C.

pH dependence of the individual rate constants for reactivation and aging. Enzyme was inhibited by the addition of 2 μl 10^{-2} M diphenylphosphorochloridate in acetonitrile to 1.0 ml 10^{-8} M acetylcholinesterase in $I = 0.1$ phosphate buffer, pH 7.0, 25°C. After vortexing for 10—15 s, 0.8 ml was removed and added to 7.2 ml of the appropriate recovery buffer. The initial pH and ionic strength of the recovery buffer were such that the addition of the inhibited enzyme mixture resulted in the desired final conditions. Small differences in the ionic strength of the buffer due to pH changes were adjusted with NaCl. The buffer was sodium phosphate for the pH range 6—8 and sodium borate for the range 8—10.5. At pH 8.0, changing the buffer from phosphate to borate had no effect upon the recovery process. Likewise, changes in buffer concentration had no effect so long as pH and total ionic strength were held constant.

Screening studies of possible effectors for the recovery of diphenyl phosphoryl acetylcholinesterase. The enzyme was inhibited as described in the above paragraph at ionic strength 0.01—0.10. Effector substances were added immediately either as concentrated stock solution or neat (liquids). The final volume was generally 1.0 ml. Aliquots were assayed at various times by the Ellman method [11].

Inhibition of the enzyme by diethylphosphorofluoridate. Diethyl phosphoryl enzyme was produced by the addition of one volume of freshly prepared diethylphosphorofluoridate (10^{-5} M in $I = 0.1$ sodium phosphate buffer, pH 7.0) solution to four volumes of an approx. 10^{-6} M solution of the enzyme in the same buffer. The solution was allowed to stand for 1 h at room temperature prior to use. The degree of inhibition was >99%.

Effects of some organic solvents upon the recovery of diethyl phosphoryl acetylcholinesterase. Reactivation of the diethyl phosphoryl enzyme was begun by the addition of 10 μl of the inhibited enzyme solution to 1.0 ml $I = 0.1$ sodium phosphate buffer, pH 7.0, 25°C, containing no additional solvent and containing various organic solvents, 1% *n*-butanol, 2% *n*-butanol, 10% acetone or 10% Me_2SO. 50-μl aliquots were withdrawn for assay. Since the spontaneous recovery and aging of diethyl phosphoryl enzyme are very slow processes, we measured the intial rates of recovery.

Effect of 10% dimethylsulfoxide upon the rate of reactivation of diethyl phosphoryl acetylcholinesterase by 2-pyridinealdoxime methiodide. The reactivation solutions consisted of 1.0 ml Me_2SO plus 9.0 ml buffer containing sufficient 2-pyridinealdoxime methiodide to yield final concentrations of $2 \cdot 10^{-4}$, $1 \cdot 10^{-4}$, $6.7 \cdot 10^{-5}$ and $5 \cdot 10^{-5}$ M. Reactivation was initiated by the addition of 10 μl of the inhibited enzyme solution. 0.25-ml aliquots were withdrawn for enzyme assays.

Effect of 10% dimethylsulfoxide upon the rate of reactivation of diethyl phosphoryl acetylcholinesterase by F^-. Reactivation was initiated by the addition of 10 μl of the phosphorylated enzyme solution to 100 or 10 ml of the reactivation buffer containing 10^{-2} M NaF. In a parallel experiment, the buffer also contained 10% dimethylsulfoxide. 0.25-ml aliquots were used for assay.

Results

The inhibition of eel acetylcholinesterase by diphenylphosphorochloridate

The first-order plot for the inhibition of eel acetylcholinesterase by diphenylphosphorochloridate is shown in Fig. 2; the data are plotted according to Eqn 5. Since organic solvents are necessarily introduced into the inhibition mixture along with the inhibitor, the ratio k_i/k_h was determined in the presence of 0.2% acetonitrile ($k_i/k_h = 4.3 \cdot 10^5\ M^{-1}$), 1.0% acetonitrile ($k_i/k_h = 4.6 \cdot 10^5 M^{-1}$ and 0.2% acetonitrile plus 1.0% 2-butanone ($k_i/k_h = 3.5 \cdot 10^4\ M^{-1}$). The latter solvent was chosen for investigation because of the large acceleration of recovery rates for the phosphoryl enzyme obtained in its presence (see later). The value of k_i/k_h appears independent of acetonitrile concentration in this range of concentration, so that the value $4.3 \cdot 10^5\ M^{-1}$ may be assumed to be the ratio one would obtain in buffer free of organic solvents. The presence of 1.0% 2-butanone causes k_i/k_h to decrease by a factor of 12. It is unlikely that this small quantity of inhibitor could increase k_h, since organic solvents at higher concentration decrease the rate of hydrolysis of the inhibitor [12]. The dramatic effect of the 2-butanone must therefore be to decrease k_i, the rate of inhibition of the enzyme. Other workers have observed that the presence of organic solvent can decrease the rate of covalent inhibition of acetylcholinesterase by organophosphates [13,14].

The rate of hydrolysis of diphenylphosphorochloridate was determined by the method of Wins and Wilson [1]. From the rate of loss of anticholinesterase activity of the inhibitor in $I = 0.10$ phosphate buffer, pH 7.0, 25°C, it was determined that $k_h = 0.27\ s^{-1}$, or $16\ min^{-1}$. Wins and Wilson [1] reported k_h =

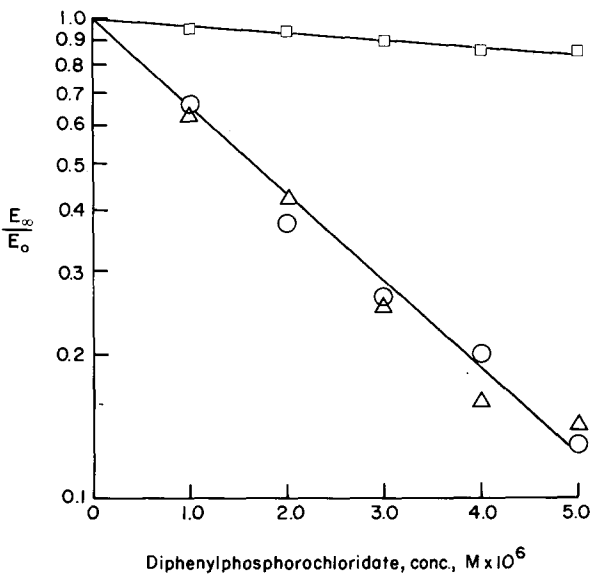

Fig. 2. First-order plot for the inhibition of eel acetylcholinesterase by diphenylphosphorochloridate (Eqn 3), $I = 0.10$, pH 7.0, 25°C. The various symbols represent different concentrations organic solvents present in the inhibition mixture: ○, 0.2% acetonitrile; △, 1.0% acetonitrile; □, 0.2% acetonitrile, 1.0% 2-butanone.

0.34 s^{-1}. A sample of $1.0 \cdot 10^{-5}$ M diphenylphosphorochloridate allowed to hydrolyze for 1.0 min in buffer showed no anticholinesterase activity, indicating that significant amounts of stable phosphorylating agents are not present in the diphenylphosphorochloridate preparation and are not formed in water.

The value of k_i calculated from the above results is $7.1 \cdot 10^6$ $M^{-1} \cdot min^{-1}$. Wins and Wilson [1] report $k_i = 1.9 \cdot 10^7$ $M^{-1} \cdot min^{-1}$. The reason for the discrepancy is not known.

In order to establish that the anticholinesterase activity of the diphenylphosphorochloridate preparation used in these studies was attributable to the material comprising the bulk of the sample and not to some impurity present, the rate of loss of inhibitory activity in 80% ethanol was determined. The rate of solvolysis of diphenylphosphorochloridate determined in this manner (k_h = 0.34 min^{-1}) is in good agreement with the value (k_h = 0.38 min^{-1}) obtained by Dostrovsky and Halmann [12] by monitoring changes in the conductivity of the solvolysis mixture. The 60 MHz proton magnetic resonance spectrum of the inhibitor prepared as described in Experimental was consistent with the structure of the desired compound; likewise, the infrared absorption spectrum agreed with a published spectrum.

The spontaneous recovery of diphenyl phosphoryl acetylcholinesterase

It is possible to conceive of several explanations alternative to aging to account for the partial recovery of diphenyl phosphoryl acetylcholinesterase as shown in Fig. 1. Perhaps there is a less potent but more stable inhibitor present which is carried over into the recovery medium, causing phosphorylation to continue at a slower rate coincident with spontaneous reactivation. The value E_∞ would thus represent a "steady-state" level at which phosphorylation and reactivation are in balance. As mentioned earlier, however, after 1-min exposure to the recovery buffer, diphenylphosphorochloridate shows no inhibitory activity towards the enzyme. Moreover, the inhibitor is homogeneous with respect to its phosphorylating ability and behavior of the phosphoryl enzyme it generates. The recovery kinetics are the same for diphenyl phosphoryl enzyme produced from inhibitor subjected to either 0.6 or 6.0 min prior exposure to 80% ethanol.

The enzyme likewise appears homogeneous with respect to the recovery process. Rates of reactivation and aging are independent of the initial degree of inhibition. In addition, enzyme which has been inhibited and is allowed to recover behaves the same as fresh enzyme with respect to a second inhibition.

It might be argued that reaction with the inhibitor yields two inhibition products, only one of which can be reactivated. In this conception non-reactivatable enzyme is produced from active enzyme by the action of the inhibitor, rather than by reaction of the diphenyl phosphoryl enzyme with water. Evidence against this possibility is the observation that the extent of recovery of enzyme inhibited under standard conditions is dependent upon the treatment of the sample subsequent to inhibition. For example, more recovery occurs in the presence of acetone and less at high pH in low ionic strength buffer.

Figs 3 and 4 show the pH dependence for the rates of spontaneous recovery and aging at ionic strengths 0.10 and 0.01 M. At the higher ionic strength the curves for recovery and aging are almost superimposable with a pH opti-

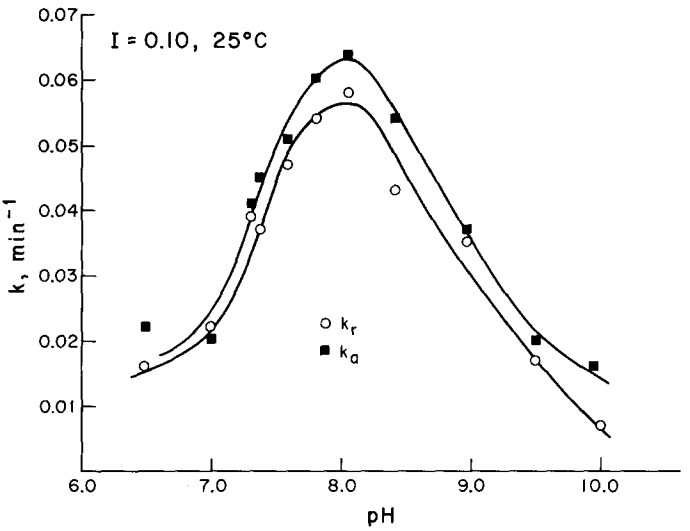

Fig. 3. The pH rate profile for the recovery of diphenyl phosphoryl acetylcholinesterase at I = 0.10, 25°C, 0.02% acetonitrile. Symbols represent k_r (○) and k_a (■).

mum of 8.1 and are shifted toward more acidic pH with respect to the curves at lower ionic strength. This acidic shift accompanied by little change in maximum rate that occurs with increasing ionic strength is similar to previous observations of the recovery of the dimethyl phosphoryl enzyme [15]. At the lower ionic strength the curves are again superimposable but only up to pH 9. Above pH 9.0 aging is much greater than recovery. At this ionic strength the pH optima are 9.5 for aging and 8.9 for reactivation.

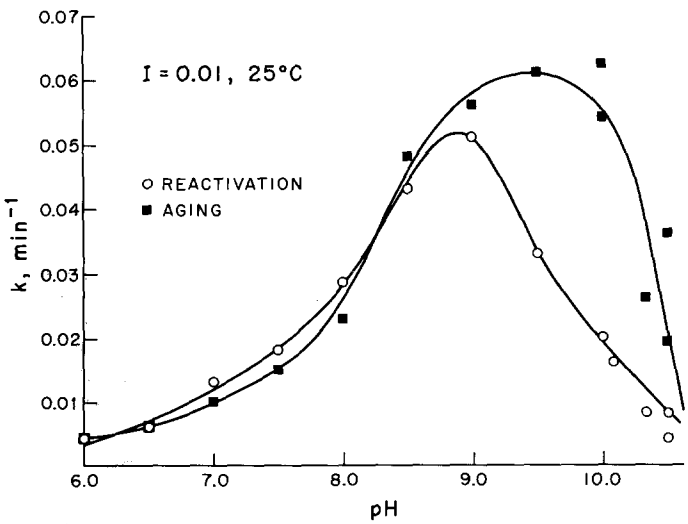

Fig. 4. The pH vs rate profile for the recovery of diphenyl phosphoryl acetylcholinesterase at I = 0.01, 25°C, 0.02% acetonitrile. Symbols represent spontaneous reactivation, k_r (○) and aging, k_a (■).

Reactivation and aging of diphenyl phosphoryl acetylcholinesterase in the presence of potential effector substances

In order to further characterize the reactions of diphenyl phosphoryl acetylcholinesterase the effects upon k_a and k_r of certain compounds known to interact with other phosphoryl cholinesterase derivatives were examined. Various nucleophilic reagents are known to greatly increase the rate of reactivation of various phosphorylated cholinesterases. F^- appears to be a fairly non-specific reagent in this respect, since it has not been demonstrated to bind significantly to the inhibited enzyme and is a good nucleophile toward phosphate and phosphonate esters. For example, in 0.1 M NaF the rate of reactivation of diethyl phosphoryl acetylcholinesterase is 5000 times greater than in its absence. Yet the diphenyl phosphoryl enzyme appears almost inert towards F^-, since little change in the rate of reactivation or aging is observed in 0.1 M NaF. The rate of reactivation does increase by about 50% in 0.5 M F^- as compared to 0.5 M Cl^-. This observation corresponds to a rate constant of about $2 \cdot 10^{-2}$ $M^{-1} \cdot min^{-1}$.

The reactivator 2-pyridinealdoxime methiodide is an especially potent nucleophile toward a number of phosphoryl enzyme derivatives, but in contrast to fluoride, has a high affinity for the enzyme active site because of molecular complementarity. The rate of dephosphorylation of diethyl phosphoryl enzyme is 10 000 times faster in $1.0 \cdot 10^{-4}$ M 2-pyridinealdoxime methiodide than in its absence. At this concentration the inhibited enzyme is half saturated with 2-pyridinealdoxime methiodide. This reactivator gives only a small increase in the rate of reactivation of diphenyl phosphoryl enzyme (Table I). The rate of aging was also increased although 2-pyridinealdoxime methiodide causes little aging of other phosphoryl enzyme derivatives.

Some substituted NH_4^+ are observed to slow the spontaneous reactivation of diethyl phosphoryl enzyme [16]. Compounds of this type have been

TABLE I

THE EFFECT OF VARIOUS SUBSTITUTED QUATERNARY NH_4^+ UPON THE RATES FOR REACTIVATION (k_r) AND AGING (k_a) OF DIPHENYL PHOSPHORYL ACETYLCHOLINESTERASE AT pH 7.0, 25°C

Effector	Concentration (M × 10^3)	k_r (min^{-1})	k_a (min^{-1})
I = 0.10			
Flaxedil	4.0	0.067	0.036
	2.0	0.064	0.038
	0.2	0.039	0.027
Tetraethylammonium ion	10	0.039	0.026
	1	0.040	0.040
2-Pyridinealdoxine methiodide	10	0.023	0.032
	1	0.030	0.028
None		0.024	0.018
I = 0.01			
Flaxedil	2	0.074	0.031
Tetraethylammonium ion	1	0.020	0.017
2-Pyridinealdoxine methiodide	10	0.026	0.043
None		0.012	0.015

demonstrated to affect the rates of other similar enzymic processes, such as methanesulfonylation [17], carbamylation [18] and decarbamylation [19-21] and deacetylation [22]. We find that some quaternary NH_4^+ accelerate both reactivation and aging of diphenyl phosphoryl acetylcholinesterase (Table I). The phenomenon appears to display saturation kinetics. The accelerations observed in the presence of 2-pyridinealdoxime methiodide probably better correlate with its quaternary ammonium structure than with its nucleophilic reactivity. The rate increases here observed for ammonium compounds are largest with flaxedil, especially at $I = 0.1$ (Table I). This compound has been shown to accelerate the decarbamylation of dimethylcarbamyl enzyme, presumably by an allosteric mechanism [20].

Since the presence of organic solvents in the recovery medium cannot be completely eliminated, their effect upon the aging and reactivation rates was also examined. Surprisingly large rate enhancements were obtained in the presence of a few percent of a number of common solvents, as shown in Table II.

The concentration dependence of these solvent effects were more completely investigated for acetonitrile (up to 12%), acetone (10%) and dimethylsulfoxide (18%). With dimethylsulfoxide and acetone the rate of reactivation

TABLE II

RATES OF REACTIVATION (k_r) AND AGING (k_a) FOR DIPHENYL PHOSPHORYL ACETYLCHOLINESTERASE IN THE PRESENCE OF VARIOUS ORGANIC SOLVENTS $I = 0.10$, pH 7.0, 25°C

Solvent	k_r (solvent min^{-1})	k_a (solvent min^{-1})
None	0.024	0.019
(A) 1.0%, v/v		
Dimethylsulfoxide	0.16	0.11
2-Butanone	0.14	0.055
Acetone	0.076	0.027
Pyridine	0.049	0.051
Acetonitrile	0.047	0.030
1,4-Dioxane	0.025	0.023
Ethylene glycol	0.027	0.019
Propylene carbonate	0.026	0.011
Phenol	(denatures active enzyme rapidly)	
(B) 5.0%, v/v		
2-Butanone	0.80	0.35
Dimethylsulfoxide	0.60	0.250
n-Butanol	0.68	0.35
tert-Butanol	0.34	0.19
2-Butanol	0.30	0.21
n-Propanol	0.33	0.26
2-Chloroethanol	0.14	0.16
Acetone	0.23	0.045
Acetonitrile	0.12	0.095
Dimethylformamide	0.13	0.083
Ethanol	0.12	0.088
Methanol	0.064	0.060
(C) 10%, v/v		
Dimethylsulfoxide	0.85	0.22
Acetone	0.52	0.15
Acetonitrile	0.15	0.15

TABLE III

Nucleophile	Organic solvent	K_R (M)	k_r° (min^{-1})	k_r ($M^{-1} \cdot min^{-1}$)
2-Pyridinealdoxine methiodide	none	$1 \cdot 10^{-4}$	4	$4 \cdot 10^4$
	10% Me_2SO	$2 \cdot 10^{-4}$	0.6	$3 \cdot 10^3$
Fluoride	none	—	—	8.3
	10% Me_2SO	—	—	2.8

increased linearly with increasing solvent concentration up to the point where the rate of denaturation of enzyme controls became significant with respect to the rate of recovery. The apparent rate of aging increased at an accelerated rate as the concentration of Me_2SO and acetone increased. With acetonitrile the aging rate was linear but recovery began to decline above 10%. In 18% Me_2SO the rate of recovery was 1.6 min^{-1}, which is 67 times greater than in buffer alone. The acceleration of recovery and aging obtained at a given solvent concentration increased sharply with molecular weight for a given class of solvent. This effect is illustrated by the homologous series of the normal aliphatic alcohols. This trend is also indicated by the greater effect obtained with butanone over acetone (Table II). Branching appears to decrease the accelerating potential, as seen in the comparison of *n*-butanol with 2- and *tert*-butanol.

We made a few measurements with 18-S + 14-S enzyme and the aggregated form. The rate of recovery was about 25% faster than the 11-S enzyme, aging was the same for 18-S + 14-S as for 11-S enzyme, but aging of the aggregate was 3—4 times faster. Recovery and aging were accelerated by 1% Me_2SO.

To determine whether other phosphorylated enzyme derivatives behave similarly with respect to recovery in the presence of organic solvents, we did some experiments with the diethyl phosphoryl enzyme which recovers only very slowly with a half time of about 2 days. In these experiments we measured recoveries only over a few hours and used only three organic solvents.

Organic solvents increase the rate of recovery of the diethyl phosphoryl enzyme (Table IV), but the observed rates and the rate enhancement factors are different for the diethyl phosphoryl enzyme than for the diphenyl phos-

TABLE IV

THE EFFECTS OF THREE ORGANIC SOLVENTS UPON THE REACTIVATION OF DIETHYL PHOSPHORYL ACETYLCHOLINESTERASE

I = 0.10, pH 7.0, 25°C.

Organic solvent	k_r ($min^{-1} \times 10^4$)	$\frac{k_r \text{ (solvent)}}{k_r \text{ (water)}}$
None	1.6	
1% *n*-butanol	5.5	3.4
2% *n*-butanol	8.5	5.3
10% acetone	1.7	1.1
10% Me_2SO	27	17

phoryl enzyme (cf. Table II). Thus, 10% Me_2SO yields a 35-fold increase in the rate of recovery for the diphenyl phosphoryl enzyme, but only a 17-fold increase for the diethyl phosphoryl enzyme. The differences with acetone are striking; 10% acetone increases the recovery rate for the diphenyl phosphoryl enzyme 22-fold, but has little effect upon the rate of recovery of the diethyl phosphoryl enzyme. Aging of the diethyl phosphoryl enzyme is very slow and therefore quite difficult to measure.

The presence of 10% Me_2SO in the reactivation buffer considerably reduces the rates of reactivation of diethyl phosphoryl enzyme by the nucleophiles 2-pyridinealdoxime methiodide and F^-. Our observations of the apparent pseudo first-order rate constant at different concentrations of 2-pyridinealdoxime methiodide were plotted in double reciprocal form to obtain both the dissociation constant, K_R, for binding with the diethyl phosphoryl enzyme and the turnover number k_r^o. The second-order rate constant, k_r, is given by k_r^o/K_R. The results for 2-pyridinealdoxime methiodide and F^- are summarized in Table III.

Discussion

The rate constant for the inhibition of acetylcholinesterase with diphenylphosphorochloridate is very large which indicates that reaction occurs with the active site nucleophile. To make sure that only the active site of acetylcholinesterase is phosphorylated by the highly reactive phosphorochloridates, it is desirable to minimize the amount of inhibitor by limiting the extent of inhibition to a level short of 100%. This limitation also allows one to monitor the activity of inhibitor stock solutions. The concentration of diphenylphosphorochloridate required is quite low, in the range 10^{-5} to 10^{-6} M. We found a linear dependence of $\ln[E_\infty]/[E_0]$ upon [I] which indicates that it is not necessary at these concentrations to take into account the possible formation of a reversible enzyme-inhibitor complex prior to phosphorylation.

It would be desirable to demonstrate that the recovery of diphenyl phosphoryl acetylcholinesterase does not vary when alternative inhibitors are used to produce this enzyme species. We accordingly used diphenylphosphorofluoridate. This compound is reported to be very stable with respect to hydrolysis and to exhibit negligible miotic action, indicating that it is a poor anticholinesterase agent [23]. The concentrations which may be employed for inhibition studies are limited by the low solubility of the compound. We found that after pre-treatment with buffer for 1 min to hydrolyze any residual phosphorochloridate present, 10^{-4} M diphenylphosphorofluoridate inhibited the enzyme at an initial rate of 0.04 min^{-1}. This rate decreased with increasing inhibition time as expected since partial recovery by spontaneous reactivation makes the rate of phosphorylation appear low. From the initial rate, the k_i for this reaction is about $4 \cdot 10^2$ $M^{-1} \cdot min^{-1}$. Since the rate for the spontaneous recovery of the diphenyl phosphoryl enzyme is also about 0.04 min^{-1}, this compound was not suitable for use in the study of reactivation and aging. However, it is interesting that this compound is such a poor inhibitor, diethylphosphorofluoridate inhibits 500 times faster, even though the two phosphorochloridates are comparable inhibitors. From the rate of inhibition of the enzyme by diphenylphosphorofluoridate and its reactivation by fluoride, the equilibrium constant is

estimated to be about $1.7 \cdot 10^4$. This value is about the same as for the reaction of diethylphosphorofluoridate with the enzyme.

It was mentioned earlier that phosphorous chlorides such as the phosphorochloridates and phosphonochloridates are in some instances superior to the more stable inhibitors for use in the study of reactivation and aging of phosphorylated cholinesterases, since it is not necessary for the experimenter to remove excess inhibitor by dilution or other means. To render it possible for inhibition and recovery to be treated as discrete processes, the inhibitor must react rapidly with the enzyme and be removed before recovery has progressed to any appreciable extent. If recovery is rapid, the chloridate may be the only suitable inhibitor. The inhibitor must contain no active impurities. These conditions appear to be satisfied by diphenylphosphorochloridate.

The pH rate profiles we obtain for the spontaneous reactivation and aging of diphenyl phosphoryl acetylcholinesterase (Figs 3 and 4) bear a striking resemblance to pH rate profiles obtained for other deacylation reactions of this enzyme such as the spontaneous reactivation of dimethyl phosphoryl enzyme [15], the reactivation and aging of *p*-cyanophenyl methylphosphonyl enzyme [3], reactivation of *N*-methyl and *N*-dimethyl carbamyl enzyme and di(2-chloroethyl)phosphoryl enzyme [24], reactivation of carbamyl enzyme [25], and deacetylation [26]. All these profiles are bell-shaped with pH optima between 7 and 10, suggesting the participation of (at least) two ionizable groups in the catalytic mechanisms. The acylated residue for all these processes is assumed to be serine.

The effect on reactivation of increasing the ionic strength is to decrease the apparent pK_a values of the two ionizing groups by a similar amount and slide the pH profile to lower pH values. This shift has been observed previously with the dimethyl phosphoryl enzyme [15]. Although the direction of the shift corresponds to positively charged acids, the magnitude is too large for a simple model of ionization in a neutral environment, but could be accounted for if the acidic groups were located in a region containing anionic groups. The effect on aging is grossly similar, in that in both cases the maximum rate does not change and one pK_a changes by the same amount. However, the second acidic group clearly behaves in a different manner which suggests that it may be a different group or finds itself in a different environment in spontaneous reactivation and aging. Since the maximum velocity is the same in different ionic strengths, the simplest assumption is that no primary salt effect is involved and that only a secondary salt effect occurs as discussed above.

One might wonder whether the effect of small concentrations of organic solvents are nothing more than still further shifts of the pH profile, so that the maximum rate of spontaneous reactivation and aging now appear at pH 7.0. This is not the case because the effects on recovery and aging are sometimes quite different and also because in most cases, we have obtained rates that are much greater than the maximum rate obtained by pH changes. We did not do a complete pH profile in the presence of organic solvents, but we did make a few measurements at pH 8.5. In each case rate accelerations still occurred and were quite large, although they were less than at pH 7.0.

At pH 7, the rate of spontaneous reactivation of the diphenyl phosphoryl enzyme is about 100 times as great as that for diethyl phosphoryl enzyme and

20 times as great as that for dimethyl phosphoryl enzyme. Enzyme inhibited by the heterocyclic organophosphate 2-chloro-1,3,2-dioxaphosphorinane-2-oxide reactivates spontaneously with a rate comparable to that obtained for diphenyl phosphoryl enzyme [2]. The large variations in the rates of hydrolysis of the above phosphoryl enzyme derivatives are only partly apparent in the hydrolysis rates for the corresponding fluorides and chlorides [12,27], and may reflect effects caused by interactions of the substituents on phosphorous with the enzyme.

In particular 2-chloro-1,3,2-dioxaphosphorinane-2-oxide and other derivatives react somewhat more slowly with water than *O,O*-diethyl phosphates and much more slowly than *O,O*-diphenyl phosphates, yet the corresponding enzyme derivative reactivates spontaneously much more rapidly than diethyl phosphoryl enzyme and as rapidly as diphenyl phosphoryl enzyme. Of the four aryl methyl phosphonyl derivatives studied by Hovanek and Lieske [3], the *p*-cyanophenyl derivative recovered most rapidly, but at pH 7 reactivation of the diphenyl phosphoryl derivative is 10 times as fast.

Interactions with the protein might explain the relative inertness of diphenyl phosphoryl enzyme towards nucleophiles, F^- and 2-pyridinealdoxime methiodide, which are quite reactive towards other phosphoryl acetylcholinesterases.

The hydrolysis of the phenyl phosphate ester linkage as the molecular event measured as the aging reaction is not directly demonstrated by this work, but rests upon discoveries of others. Lee and Turnbull [28] have correlated aging with the liberation of phenol from diphenyl phosphoryl chymotrypsin. Bender and Wedler [29] have also shown that the rate of release of *p*-nitrophenol corresponds to the rate of aging for di-*p*-nitrophenyl phosphoryl chymotrypsin. Berends et al. [3] have measured the release of isopropanol during the aging of pseudo-cholinesterase inhibited by di-isopropyl phosphorofluoridate.

These studies show that aging results from the hydrolysis of the phosphoryl enzyme derivative, a phospho triester, to form an alcohol or phenol and a phospho diester enzyme derivative. However, there are two kinds of aging which occur by different mechanisms. The aging that occurs fairly rapidly with alkyl substituents and was the first recognized occurs via a carbonium ion mechanism. The evidence for this mechanism is that in contrast to spontaneous reactivation, it occurs more rapidly as the pH is dropped from 8 to 6, thereby suggesting the involvement of an acidic group in the enzyme, and it occurs more rapidly with branched substituents. Finally, the formation of olefin when branched groups are lost would seem to be quite definitive [31]. The second type of aging occurs with good leaving groups, phenols, and like spontaneous reactivation increases in rate as the pH is raised from 6 to 8. This type of aging seems to arise from nucleophilic attack on phosphorus. The aging observed in this work is the second type.

The organic solvents could conceivably act as nucleophiles but for most of them this must be regarded as an extremely remote possibility because, except for pyridine, the solvents are relatively poor nucleophiles toward phosphorus in water at neutral pH and the neat liquids, including the alcohols, are often used to prepare convenient stock solutions of phosphorofluoridates and phosphono-

fluoridates and other organophosphorus inhibitors. In our experience even the chloridates are stable in acetone and acetonitrile and we have already noted that *O,O*-diphenyl phosphorochloridate hydrolyzes slowly in 80% ethanol/water, but rapidly in water. Dostrovsky and Halmann [12] found that the rates of solvolysis of di-isopropyl phosphorochloridate in various alcohols decreased in the order water > methanol > ethanol > *tert*-butanol and increased with water concentration in ethanol/water mixtures. This order is not only the reverse of our findings, but differs especially in that in our work the alcohols are far more effective than water. Thus 0.7 M *tert*-butanol is 13 times more effective than 55 M water. If these alcohols acted as nucleophiles it would be surprising to find ethylene glycol relatively inert. We find that fluoride, which is a very good nucleophile toward phosphorus, is barely active in reactivating diethyl phosphoryl enzyme even at 0.5 M concentration.

If these solvents were acting as nucleophiles we should expect the ratio of their contributions to reactivation and aging to be precisely independent of the concentration of the solvent, but this is not the case although it is true that the ratios do not change by more than a factor of two for the data given in Table II. Finally Bender and Wedler [29] did not find that 4 M methanol served as an effective nucleophile in the aging of di-*p*-nitrophenyl phosphoryl α-chymotrypsin. For all these reasons it seems most unlikely that the solvents, with the exception of pyridine, act as nucleophiles.

The rate accelerations of spontaneous reactivation and aging of diphenyl phosphoryl acetylcholinesterase caused by solvents are very large considering the low solvent concentrations employed in this study. Large effects are observed at solvent concentrations that have very little effect upon the bulk properties of the medium and must derive from differential solvation of the phosphoryl enzyme derivatives in such a way as to favorably stabilize the transition states for both spontaneous reactivation and aging. These two transition states must bear certain similarities as already attested to by the similarities in the pH dependence of aging and spontaneous reactivation.

In a formal sense, spontaneous reactivation and reactivation by nucleophiles are the same process because water may be regarded as just another nucleophile. However, organic solvents diminish the rate of reactivation by nucleophiles so that in this case differential solvation destabilizes the transition state relative to the diethyl phosphoryl enzyme and also relative to the free enzyme, because the rate of inhibition by inhibitors is also diminished.

The rate of hydrolysis of acetylthiocholine is also decreased by organic solvents which, because deacylation is rate determining, would seem to suggest that the reaction of the acetyl enzyme with water is slowed by organic solvents, but we do not actually know this because the rate-determining step may well have shifted in the presence of the solvent.

It is hard to escape the conclusion that despite the formal similarity there is a basic difference in the enzymic mechanism when water is a nucleophile, and when a leaving group is a nucleophile, at least in the case of the organophosphates.

The pH dependence ($I = 0.1$) suggests the involvement of a basic group with a pK_a of about 7.1. This pK_a usually turns up in the pH dependence of various reactions of this enzyme (and also of chymotrypsin) and has been attributed to

the imidazole group of histidine. The role of imidazole in aging and reactivation may be that of a general base or of a nucleophile. Actually the pH dependence does not tell us whether the imidazole group must be in basic or acidic form. The presumed requirement that the more acidic group is unprotonated and the less acidic is protonated gives the same pH dependence in a bell-shaped curve as the reverse proposal that the more acidic group is protonated and the less acidic group is not. Although it is true that the population of active species is very much smaller in the second instance, the active groups would be correspondingly stronger acids and bases. Therefore, a role as a general acid becomes a third possibility for the imidazole group. Perhaps in the first type of aging, the type which occurs more readily in acidic solution, the protonated imidazole serves as a general acid. In the case of the diphenyl and the di-*p*-nitrophenyl phosphoryl chymotrypsin derivatives it was concluded that imidazole acted as nucleophile in aging (second type) [28,29].

It is hard to specify what feature of the organic solvent is responsible for the effects reported here. There is no obvious correlation with dielectric constant or internal pressure. In the alcohol series from methanol to butanol, there is a very obvious increase in the solvent effect when the non-polar portions of the molecule are increased. This same effect is apparent in comparing acetone and butanone. Thus amphiphilic properties seem to be important.

Various organic solvents increase the rate of deacylation of three acyl papains [32] and a correlation was observed between rate acceleration and molecular weight of solvent [33]. Hinkle and Kirsch [32] propose that solvents may bind to the active site and force the acyl group into a more reactive orientation relative to the enzymatic catalytic functions. To explain the greater acceleration of the slowly hydrolyzing acyl papains and the possible inhibition of deacylation with good substrates, they reason that a reorientation is more likely to facilitate a slow reaction than a fast one, because in a fast reaction the orientation is more likely to be close to optimum. This is a general argument that need not hold in any specific case and does not fit our observations of lesser accelerations with diethyl phosphoryl enzyme than with diphenyl phosphoryl enzyme. However, we do find that the maximum velocity of acetylthiocholine hydrolysis is decreased and K_m is increased.

Hovanek and Lieske [3] found a good Hammet correlation for aging of aryl methyl phosphonyl enzyme derivatives and hydrolysis of the corresponding phosphonochloridates, but poor correlation for the reactivation reaction. They suggested that the phenyl group is extended into the solvent during aging, but is associated more tightly with the enzyme during reactivation. Their idea is consistent with a reorientation of groups by differential solvation of the active site, but would seem to imply that greater effects would occur in reactivation than in aging. A reorientation could involve not only the phosphoryl group, but also the protein structure.

The effect of solvents could also be less direct and might cause a change in conformation at the active site by solvation effects at some other region of the protein. A change in conformation is consistent with our observed decrease of the rate at which F^- and 2-pyridinealdoxime methiodide reactivate the diethyl phosphoryl enzyme, but then this change in conformation, which is favorable for reaction with water, must be unfavorable for reaction with other nucleophiles.

Substituted NH_4^+ accelerate a number of reactions of acetylcholinesterase, but this is the first observation of acceleration of dephosphorylation. In contrast to acceleration of decarbamylation and deacetylation which occurs only at low salt concentration, dephosphorylation also occurs at higher salt concentrations. In this respect it is similar to acceleration of sulfonylation and carbamylation. Originally NH_4^+ were thought to influence reactions only by interaction at the anionic subsite of the active site, but this was soon altered to include a second anionic site to accommodate observations with diquaternary compounds [34] and two or three additional sites of interaction have been proposed.

Flaxedil is particularly effective in promoting decarbamylation [20] and is thought to bind at an allosteric site. (+)-Tubocurarine, which is also thought to bind at an allosteric site, inhibits aging of the isopropyl methyl phosphonyl enzyme [35], but this is the first type of aging. If, as seems likely, these substances do bind at an allosteric site, the effects must be mediated by a change in conformation of the protein which stabilizes the transition state. The effects of solvents might also be mediated by a change in conformation of the protein.

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