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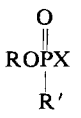
Spontaneous Reactivation of Acetylcholinesterase Inhibited with Para-Substituted Phenyl Methylphosphonochloridates*

Joseph W. Hovanec† and Claire N. Lieske

ABSTRACT: Four para-substituted phenyl methylphosphonochloridates (H, I; CH_3O, II; NO_2, III; and CN, IV) were prepared by the reaction of excess methyl phosphonodichloridate with the appropriate phenol. The rates of hydrolysis of I–IV were determined at pH 4.90 (25°, acetate buffer) and showed excellent correlation with published inductive σ values for para substituents. Spontaneous reactivation of acetylcholinesterase (AChE) inhibited with I, II, and IV exhibited a 3.6 times larger substituent effect, which is interpreted in terms of hydrophobic interactions within the active site. No significant enhancement of the substituent effect was observed in the parallel aging reaction. AChE inhibited with III showed no detectable reactivation. The pH–rate profile for the aging reaction of AChE inhibited with IV is bell shaped implicating participation by two groups within the active site having pK_a's of 6.2 and 9.0. The rate of spontaneous reactivation shows a similar pH profile. Inhibition and spontaneous reactivation results using the bis ester analogs of III and IV support the active site specificity of I–IV.

Organophosphorus compounds of the type



where X is a labile leaving group will irreversibly inhibit AChE[1] by formation of a phosphonate ester bond to the active site serine. Once phosphonylated, the enzyme may react in one of two ways (Cohen and Oosterbaan, 1963; Engelhard *et al.*, 1967): (1) it may reactivate by cleavage of the serine phosphonate bond, or (2) it may age by cleavage of the other phosphonate ester bond. The reactivation is catalyzed by strong nucleophiles such as oximes (Gilbert *et al.*, 1961) and hydroxamic acids (Franchetti *et al.*, 1970) while aging, in the case where R is an alkyl group, has been shown to be acid catalyzed and probably involves a carbonium ion intermediate (Michel *et al.*, 1967).

To date, few *in vitro* studies of the spontaneous (*i.e.*, no external catalytic species other than H_2O, OH^-, and H_3O^+) reactivation and aging of phosphorylated AChE have been done (Wilson *et al.*, 1958; Lee, 1964; Reiner and Aldridge, 1967; Aldridge, 1969). Similarly, little, if anything, is known about the reactivation–aging behavior when R is an aryl group. It is to these areas that the work in this paper is directed.

Experimental Section

Synthesis. The four phosphonochloridates used in this work were all prepared in a similar manner. The appropriate para-substituted phenol was dissolved in toluene and added to a twofold excess of methyl phosphonodichloridate also in toluene. The reaction mixture was stirred at room temperature for several hours and the toluene evaporated under reduced pressure. The addition of a tertiary amine to scavenge the HCl produced during the reaction was found to be unnecessary since aryl phosphonates are not particularly sensitive to acid attack (Bunton *et al.*, 1968). All four were purified by distillation. The physical data for each compound follow.

Phenyl methylphosphonochloridate (I), liquid at room temperature, was distilled at 82° and 0.02 mm. Petrov *et al.* (1961) reported a distillation temperature for this compound of 153–155° at 21 mm. *Anal.* Calcd for $C_7H_8ClO_2P$: C, 44.1; H, 4.2; Cl, 18.6. Found: C, 43.8; H, 3.9; Cl, 18.4. The nuclear magnetic resonance (nmr) spectrum (given in parts per million) showed (60 MHz, $CDCl_3$, Me_4Si external standard) a doublet at δ 2.09 (J = 17 Hz integrating to three protons) and a multiplet centered at δ 7.3 integrating to five protons.

p-Methoxyphenyl methylphosphonochloridate (II), liquid at room temperature, was distilled at 120–125° at 0.06 mm. *Anal.* Calcd for $C_8H_{10}ClO_3P$: C, 43.6; H, 4.6; Cl, 16.1. Found: C, 43.3; H, 4.6; Cl, 16.1. The nmr spectrum (60 MHz, $CDCl_3$, Me_4Si external standard) showed a doublet at δ 2.10 (J =

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[1] Abbreviations used are: AChE, acetylcholinesterase.

17 Hz integrating to three protons), a singlet at δ 3.74 integrating to three protons, and a multiplet centered at δ 7.0 integrating to four protons.

p-Nitrophenyl methylphosphonochloridate (III), solid at room temperature, was distilled at 115° and 0.02 mm. deRoos (1959) reported a distillation temperature of 121° at 0.04 mm. *Anal.* Calcd for $C_7H_7ClNO_4P$: C, 35.7; H, 3.0; Cl, 15.1. Found: C, 36.2; H, 3.1; Cl, 14.7. The nmr spectrum (60 MHz, $CDCl_3$, Me_4Si external standard) showed doublets at δ 2.30 (J = 17 Hz integrating to three protons), δ 7.40 (J = 2 Hz integrating to two protons), and δ 7.54 (J = 2 Hz integrating to two protons).

p-Cyanophenyl methylphosphonochloridate (IV), solid at room temperature, was distilled at 113–121° and 0.03–0.05 mm. *Anal.* Calcd for $C_8H_7ClNO_2P$: C, 44.6; H, 3.3; N, 6.5; P, 14.4. Found: C, 44.2; H, 3.3; N, 6.6; P, 14.5. The nmr spectrum (60 MHz, $CDCl_3$, Me_4Si external standard) showed a doublet at δ 2.18 (J = 17 Hz integrating to three protons) and a multiplet centered at δ 7.4 integrating to four protons.

Bis(*p*-nitrophenyl) methylphosphonate was prepared by the slow addition of sodium *p*-nitrophenolate to a solution of methyl phosphonodichloridate in benzene. After 18-hr stirring at room temperature, the reaction mixture was filtered, washed with water, and dried over sodium sulfate. The benzene was evaporated under reduced pressure and the product recrystallized from acetone–cyclohexane to give yellow crystals melting at 122–123°. Mel'nikov *et al.* (1966) reported a melting point of 119–120°. *Anal.* Calcd for $C_{13}H_{11}N_2O_7P$: C, 46.4; H, 3.3; N, 8.3. Found: C, 46.1; H, 3.3; N, 8.2.

Bis(*p*-cyanophenyl) methylphosphonate was prepared by adding a toluene solution of *p*-cyanophenol to a toluene solution of methyl phosphonodichloridate and diethylaniline. After 18 hr at reflux, the reaction mixture was filtered and washed, in order, with 0.01 N HCl, water, pH 8 boric acid buffer, and water. It was then dried over magnesium sulfate and the toluene removed under reduced pressure. Recrystallization from acetone gave white crystals melting at 119–121°. *Anal.* Calcd for $C_{15}H_{11}N_2O_3P$: C, 60.4; H, 3.7; N, 9.4; P, 10.4. Found: C, 60.2; H, 3.7; N, 9.6; P, 10.3.

Hydrolysis Kinetics for I–IV. Three sodium acetate buffers of pH 4.90 were prepared with total acetate concentrations of 0.05, 0.115, and 0.25 M. The hydrolysis rates of each phosphonochloridate at 25° in these buffers were measured spectrophotometrically at 2675 Å for I, 2875 Å for II, 3100 Å for III, and 2600 Å for IV. In all cases, good pseudo-first-order kinetics were observed for more than 2 half-lives and reproducibility was better than ±5%.

Inhibition–Spontaneous Reactivation. The buffers used in the inhibition–reactivation work were of the zwitterionic variety described by Good *et al.* (1966) and available commercially from several sources. All were 0.10 M in buffer, 0.01 M in Mg^{2+}, and 0.01% in gelatin.

Acetylcholine acetylhydrolase (EC 3.1.1.7) from electric eel was obtained from commercial sources. From this, a stock solution of *ca.* 125 units/ml in 0.225 M KCl and 0.1% gelatin was prepared. (A 400-fold dilution of this stock solution of AChE at pH 7.5 and 25° will hydrolyze phenyl acetate with a V_{max} of about 2×10^{-4} M/min.)

The separation of phosphonylated AChE from excess inhibitor was effected on a column of Sephadex G-25 coarse which had been swelled in the buffer of choice overnight. The column was glass 1.25 cm in diameter and 30 cm long and had a void volume (as determined with Blue Dextran 2000) of 13 ml and a flow rate under gravity feed of *ca.* 400 ml/hr.

For the inhibition–reactivation experiments, 200 μl of AChE stock solution was added to 13 ml of buffer. From this, two controls were prepared by dilution of two 4.00-ml portions to 100.0 ml each using the same buffer. The remaining enzyme solution was treated with 100 μl of a 0.15 M solution of the phosphonochloridate in acetonitrile. A 4.00-ml sample of the inhibited AChE solution was passed through the column and 25 ml of the eluate collected (in *ca.* 4 min). Previous tests established that all the AChE and no inhibitor were present in this fraction. This 25-ml sample was then diluted to 100.0 ml, again with the same buffer, and incubated along with the control solutions at 25°. The zero time for reactivation was taken as the time when the entire 4.00-ml sample of inhibited AChE had passed into the gel bed of the column.

All AChE solutions were assayed for activity spectrophotometrically at 2725 Å using 4×10^{-3} M phenyl acetate as the substrate. The controls were assayed three times each and the six values agreed to within ±2%. The inhibited AChE solution was assayed at appropriate time intervals with the activity expressed as a per cent of the control value. With the exception of II, all reactivations were monitored to completion.

Two basic methods were used to calculate the observed rate of reactivation (k_{obsd}) from the activity *vs.* time data. In most cases, the end point (final activity value) was measured and the rate determined from the usual first-order kinetic equations. In the case of II, a reliable end point could not be determined experimentally due to the length of the reaction. To circumvent this problem, a computer program developed by Hayo and Wilcoxson (1963) was utilized. This program fits the data (V,t) to eq 1. The printout gives the best value for

$$V = V_f[1 - e^{-k_{obsd}(t - t_0)}] \quad (1)$$

the end point (V_f) and the first-order rate constant (k_{obsd}). The only restriction on the program is that V must have a positive value when $t = 0$. This can be accomplished by shifting the t values an equal amount if necessary. The validity of this calculation for our data was checked by using the data from I and comparing the results with the experimental end point and rate constant. Agreement of the two values for k_{obsd} and the computed and experimental end points were within ±2%.[2]

Since the spontaneous reactivation and aging of phosphonylated AChE are parallel first-order reactions, the observed rate of reactivation is actually the sum of the true reactivation and aging rates.[3] Further, the ratio of reactivated to aged AChE is the same as the ratio of their true first-order rates of formation (Frost and Pearson, 1961). These considerations may be expressed mathematically in eq 2 and 3. Thus, from

$$k_{obsd} = k_R + k_A \quad (2)$$

$$\frac{k_R}{k_A} = \frac{\%\ \text{reactivation}}{100 - \%\ \text{reactivation}} \quad (3)$$

the observed rate of reactivation (k_{obsd}) and the per cent re-

[2] In the calculation of first-order kinetic constants from data where no experimental end point is available, it is felt that this program offers a distinct advantage over the more generally accepted method of Guggenheim in that there is no need to space the kinetic parameter at a constant Δt.

[3] If the concentration of aged AChE could be measured as a function of time, the same k_{obsd} would result.

TABLE I: Kinetic Data for the Hydrolysis of Para-Substituted Phenyl Methylphosphonochloridates at pH 4.90 and 25° (Extrapolated to Zero Buffer Concentration).

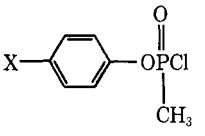
Compound	Para Substituent	k (min^{-1})	$t_{1/2}$ (min)
I	H	0.21	3.30
II	CH_3O	0.15	4.62
III	NO_2	1.26	0.55
IV	CN	0.89	0.77

activation, the true rates of reactivation (k_R) and aging (k_A) may be evaluated.

The value for K_m for both the control and reactivated (from IV) AChE was determined with phenyl acetate at pH 5.77 (0.10 M MES buffer–0.01 M Mg^{2+}–0.01% gelatin). The substrate concentration was varied between 0.81×10^{-4} and 58.4×10^{-4} M. The data were treated according to the method of Lineweaver and Burk (1934).

Results

Four phosphonochloridate inhibitors of AChE having the general formula



(where X = H, I; CH_3O, II; NO_2, III; and CN, IV) were prepared from methyl phosphonodichloridate and the corresponding phenol. The hydrolysis kinetics of these compounds were determined at pH 4.90 and 25° in various concentrations of acetate buffer. The results extrapolated to zero buffer concentration are given in Table I. In all cases, the kinetic data showed good pseudo-first-order kinetics with stoichiometric production of the phosphonic acid. Examination of III at two other pH's showed catalysis by H_2O and OH^- but, as expected (Bruice and Benkovic, 1966), none by H_3O^+.

Although the rates of hydrolysis of these compounds are too large to measure at pH 7, they may still be used effectively as *in vitro* inhibitors of AChE between pH 5 and 9. It was found that except at the upper pH limit, an initial inhibitor concentration of 3×10^{-3} M was sufficient to produce 100% inhibition of AChE.

The presence of a minute amount (<1%) of *p*-nitrophenyl phosphorodichloridate was detected in the mass spectrum of III. This impurity can interfere with reactivation studies since, unlike III, its hydrolysis product, *p*-nitrophenyl phosphorochloridate, is also capable of phosphorylating AChE. This was confirmed by adding III to a pH 7 buffer followed by AChE. This procedure produced completely inhibited enzyme in *ca.* 1 hr even though control experiments with the hydrolysis product of III, *i.e.*, pure *p*-nitrophenyl methylphosphonic acid, showed it to be inert to AChE. As similar evidence of trace impurities was observed in I and IV, gel filtration was used to isolate the phosphonylated enzyme.

The reactivation of the inhibited AChE was monitored by periodic assay with phenyl acetate. The activity of the enzyme showed a first-order increase with time.[4] For AChE inhibited with I–IV, the per cent reactivation, observed rate of reactivation, corresponding $t_{1/2}$ of reactivation, true rate of spontaneous reactivation (k_R), and the true aging rate (k_A) are given in Table II. Note that no spontaneous reactivation was observed with III.

TABLE II: Data on the Spontaneous Reactivation and Aging of AChE Inhibited with Para-Substituted Phenyl Methylphosphonochloridates (0.10 M MOPS, pH 7.55, 25°).[a]

Inhibitor	% Reactivn	k_{obsd} (hr^{-1})	$t_{1/2}$ (hr)	k_R (hr^{-1})	k_A (hr^{-1})
I	83	0.0196	35	0.0163	0.0033
II	94[b]	0.0107	65	0.0101	0.0006
III	0				
IV	21	0.822	0.84	0.173	0.649

[a] See footnote *b* of Table III. [b] Extrapolated value; reactivation was not followed to completion.

TABLE III: Reactivation–Aging Data for AChE Inhibited with *p*-Cyanophenyl Methylphosphonochloridate (Various pH's, 25°).

Buffer	pH	% Reactivn	k_{obsd} (min^{-1})	k_R (min^{-1})	k_A (min^{-1})
MES[a]	5.77	44.7	0.00526	0.00235	0.00291
MES	6.21	32.8	0.00751	0.00246	0.00505
MOPS[b]	6.71	25.1	0.0109	0.0027	0.0082
MOPS	7.08	24.1	0.0117	0.0028	0.0089
MOPS	7.55	20.5	0.0137	0.0028	0.0109
Tricine[c]	8.12	18.7	0.0119	0.0022	0.0097
Tricine	8.64	17.1	0.0109	0.0019	0.0090
Bicine[d]	9.03	10.7	0.0050	0.0005	0.0045

[a] 2-(*N*-Morpholino)ethanesulfonic acid. [b] 2(*N*-Morpholino)propanesulfonic acid. [c] *N*-Tris(hydroxymethyl)methylglycine. [d] *N*,*N*-Bis(2-hydroxyethyl)glycine.

AChE was also inhibited by two phosphonate esters related to III and IV. With the exception of slightly lower inhibitor concentrations, the procedure used was identical with that used with the phosphonochloridates. Bis(*p*-nitrophenyl) methylphosphonate, like III, gave no spontaneous reactivation. Because of its lower reactivity, bis(*p*-cyanophenyl) methylphosphonate inhibited AChE to only 85%. This was sufficient however to obtain an observed rate of spontaneous reactivation of 0.840 hr^{-1} and a return of activity of *ca.* 16%.

The spontaneous reactivation of AChE inhibited with IV was examined at 25° over the pH range of 5.77–9.05. The experimental procedure was identical with that used at pH 7.55 with the exception of different zwitterionic buffers to accommodate the pH changes. The results of this pH–rate profile are summarized in Table III.

[4] All reactivation reactions were monitored for 1.5–2.0 half-lives and all values of k_{obsd} are averages of three or four determinations. Reproducibility was ±5%.

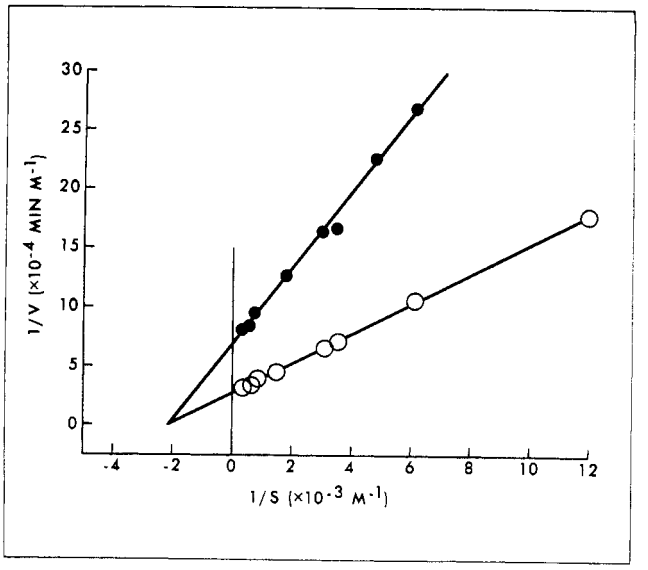


FIGURE 1: Lineweaver–Burk plot for the hydrolysis of phenyl acetate at pH 5.77 and 25° by control AChE (○) and AChE reactivated from IV (●).

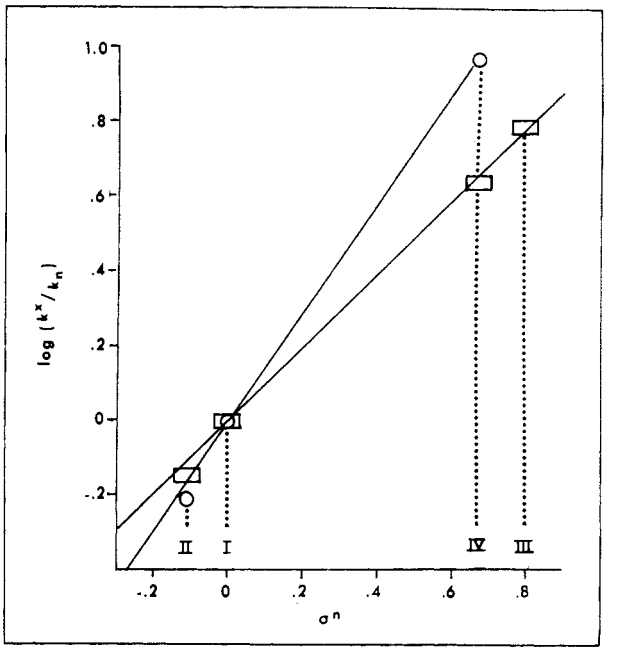


FIGURE 2: Linear free-energy relationships for the rates of hydrolysis of I–IV at pH 4.90 and 25° (□) and the rates of spontaneous reactivation at pH 7.55 and 25° for AChE inhibited with I, II, and IV (○).

The values of K_m for the hydrolysis of phenyl acetate were determined for both control AChE and that which had reactivated from IV. Both determinations were made at 25° in 0.10 M MES buffer of pH 5.77. A value of 4.77×10^{-4} M for the control AChE and 4.69×10^{-4} M for the reactivated AChE was obtained. The Lineweaver and Burk (1934) plots for both determinations are reproduced in Figure 1.

Discussion

The most striking features of the data in Table II are the sensitivity of the observed rate of spontaneous reactivation and the sensitivity of the percent reactivation to the para substituents of the inhibiting moiety. While the percent reactivation decreases with more electron attracting substituents, k_{obsd} increases. Detailed analysis of the two components of k_{obsd}, k_R, and k_A reveals several interesting relationships.

Mechanistically, the spontaneous reactivation reaction reflects hydrolysis of the phosphonyl–serine linkage. As such it is intrinsically related to the hydrolysis of the phosphonochloridates shown in Table I. Both reactions involve the cleavage of a bond which is insulated from resonance interaction with the aromatic ring. Wells (1962) has published a set of σ values (σ^n) derived from reactions not involving resonance interactions. If one uses these inductive values along with the hydrolysis rates of I–IV, the resultant σ^n, ρ plot (Figure 2) yields a slope of 1.0 or perfect correlation. Once the substituents move into the active site of AChE, however, their effect is significantly different as reflected by a slope of 1.56 in the σ^n, ρ plot for the spontaneous reactivation reactions (Figure 2). The para substituents thus exert a 3.6 times greater inductive effect on hydrolysis at phosphorus inside the active site of AChE than in aqueous solution.

While the remoteness of the para substituents in the phosphonochloridates precludes any steric interaction in their hydrolyses, once inside the active site, direct interaction between protein and substituent is quite possible. The linearity of the σ^n, ρ plot, however, indicates that this must be more than a simple steric interaction; it must be dependent on the charge distribution around the substituents which are responsible for their inductive σ values. It is known that changes in the magnitude of inductive substituent values may be caused by changes in the dielectric constant of the reaction medium (Amis, 1966). In the case of the phosphonylated AChE, the critical factor is the dielectric constant of that part of the active site which houses the phenyl group. While studies have been done to probe the hydrophobic regions of the active site of AChE using substrate (Hillman and Mautner, 1970) and phosphorylation (Kabachnik *et al.*, 1970) reactions, this is the first evidence for hydrophobic interactions participating in the dephosphonylation of inhibited AChE.

In contrast with the reactivation reaction, the aging rate reflects cleavage of the phosphonyl–phenolic oxygen bond. One would thus expect the para substituent to influence this reaction rate through both inductive and resonance interaction. Verification of this is afforded by the work of Haake *et al.* (1968). They obtained a ρ of 1.0 for the hydroxide ion catalyzed hydrolysis of para-substituted phenyl diphenylphosphinates using σ values based on the dissociation constants of the phenolic leaving groups. Using the same σ values and the rates of aging from Table II results in a ρ value of 1.1 which strongly supports an analogous interaction in the aging process and indicates complete solvation of the para substituent. This result, coupled with the spontaneous reactivation results, suggests that some conformational change prior to the rate-controlling step(s) of spontaneous reactivation and/or aging alters the microenvironment of the para substituent.

The results of the pH–rate profile for the spontaneous reactivation of AChE inhibited with IV (Table III and Figure 3) show the bell shape observed in numerous enzymatic reactions (Gutfreund, 1965). The analogous plot for the aging reaction (Table III and Figure 4) is also bell shaped. Such curves are indicative of the operation of two ionizable groups in the active site, one dissociated and the other protonated. In Figure 4, for the aging reaction, the groups involved have pK_a's of 6.2 and 9.0 (based on pH at one-half maximum k_A).

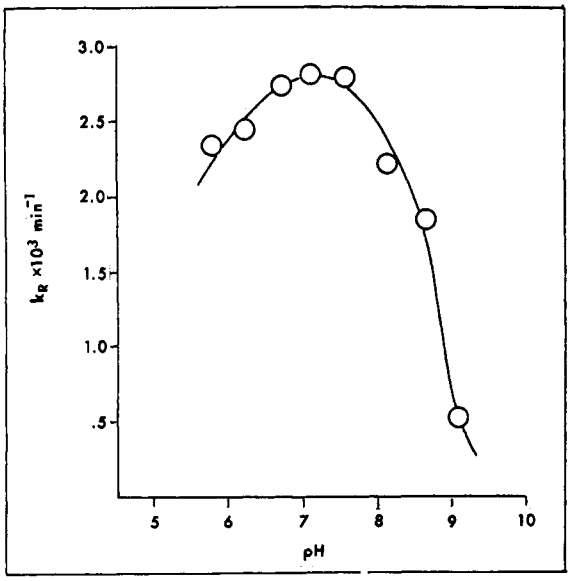


FIGURE 3: pH–rate profile for the spontaneous reactivation at 25° of AChE inhibited with *p*-cyanophenyl methylphosphonochloridate (IV).

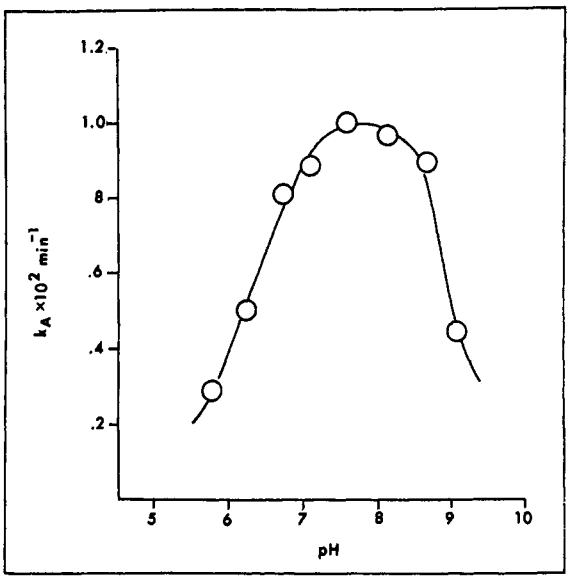


FIGURE 4: pH–rate profile for the aging at 25° of AChE inhibited by *p*-cyanophenyl methylphosphonochloridate (IV).

Studies of substrate hydrolysis by AChE have implicated groups of like pK_a's which may represent histidine (Krupka, 1966a,b) and tyrosine (Bergmann, 1958; Bergmann *et al.*, 1958). A reasonable interpretation of our results involves the operation of the histidine imidazole as a general base while optimal conformation is maintained *via* a hydrogen bond with tyrosine as the donor. Figure 3, for the reactivation reaction, does not permit assignment of a pK_a to the dissociated group. However, it does implicate a protonated species with a pK_a of 8.7, which may or may not be the tyrosine postulated in the aging reaction.

It is recognized that incorporated in these simple interpretations is the presently inaccessible parameter of pH-dependent conformational change (Yakovlev and Agabekyan, 1967). While good methods for studying protein conformation have been developed (Hirs, 1967), no definitive correlations of pH, conformation, and activity for AChE are available. It is known, however, that changes in the microenvironments of amino acids resulting from conformational changes in protein can affect their dissociation constants (Timasheff and Gorbunoff, 1967; Harmsen *et al.*, 1971). A good illustration of this phenomenon is found in the work of Wilson *et al.* (1958). In their results on the spontaneous reactivation of dimethylphosphoryl-AChE, they observed a bell-shaped pH profile, but the pH at k_{max} was shifted by 1 pH unit upon changing the medium from 1.0 to 0.02 M NaCl. Thus, the nonidentity of the pH maxima for aging and reactivation affords supporting evidence for a conformational change preceding one or both of these reactions.

It is not surprising that the aging of AChE inhibited with I, II, and IV is base catalyzed rather than acid catalyzed as observed with alkyl phosphonate inhibitors. This result is consistent with the lability of phenolic leaving groups in nucleophilic displacements and their reluctance to form carbonium ions. Viewing the aging reaction as the hydrolysis of an aryl phosphonate, the k_A values can be compared to the rates of base catalyzed hydrolyses of other aryl phosphonates (Cox and Ramsay, 1964). Such a comparison indicates that the enzyme affords a 300-fold enhancement of the rate of aging. A similar comparison of k_R to the base-catalyzed components of alkyl phosphonate hydrolyses shows a rate enhancement of about 10^6. From these figures, one may conclude that the conformation intrinsic to reactivation is favored over that involved in aging.

During the course of these studies, two possible sources of anomalous results were considered and investigated. First, since the reactivation is measured by changes in the hydrolytic activity toward phenyl acetate, it was necessary to be certain that the enzyme was reactivating to its original form and not to some less active form. Thus, the values for K_m and V_{max} were determined with phenyl acetate using both a control and reactivated sample of AChE. The K_m's were identical within experimental error while the values for V_{max} were in the same ratio as their activities. Since $V_{max} = [E] \times k_{cat}$, this indicates that the AChE from both sources had the same k_{cat} and differed only in concentration.

The second area of consideration was to demonstrate that the highly reactive phosphonochloridates inhibit AChE only at the active site. It is known that the less reactive phosphonofluoridates and phosphonate esters react only in this manner (Cohen and Oosterbaan, 1963), and so bis(*p*-nitrophenyl) methylphosphonate and bis(*p*-cyanophenyl) methylphosphonate were studied. The spontaneous reactivation results obtained with these esters were identical with their phosphonochloridate analogs confirming the active-site specificity of the latter.

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Synthesis of an Adenosine 5′-Monophosphate Analog and Its Use for the Affinity Labeling of the Effector Binding Site of Rabbit Skeletal Muscle Phosphorylase *b*†

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ABSTRACT: The synthesis of 6-(purine 5′-ribonucleotide)-5-(2-nitrobenzoic acid) thioether provided a reagent that will form stable thioether bonds between the 6 position of the purine moiety and aliphatic sulfhydryls. 2-Nitro-4-mercaptobenzoic acid is eliminated during this reaction. The nucleotide reagent, labeled with ^{32}P, was used to activate phosphorylase *b* from rabbit muscle. The activation showed a stoichiometric relation to the amount of nucleotide incorporated into the enzyme. The nucleotide was covalently linked to the protein. We propose that the nucleotide became bound at or near the 5′-AMP binding site of the enzyme.

Affinity labeling of enzymes by substrate analogs which form covalent bonds with amino acid side chains of the protein is an useful and well-established technique in enzymology. Although a large number of nucleotide analogs have been synthesized (Follmann *et al.*, 1967; Holy, 1970) for the purpose of studying enzyme mechanisms, relatively few of them were designed to form covalent bonds with protein side chains (Cuatrecasas, 1970; F. Eckstein, personal communication).

Experiments on side-chain modifications at or near the 5′-AMP binding site in phosphorylase *b* (H. Fasold *et al.*, 1971, unpublished data) suggested the need for a protein-reactive 5′-AMP analog. Previous studies had already suggested the participation of a sulfhydryl group in 5′-AMP binding (Damjanovich and Kleppe, 1967; Batell *et al.*, 1968; Gold, 1968; Kastenschmidt *et al.*, 1968). Therefore, we tried to synthe-

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