Biochimica et Biophysica Acta, 334 (1974) 137–145
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BBA 67103

THE INHIBITION OF ACETYLCHOLINESTERASE BY ORGANOPHOSPHORUS COMPOUNDS CONTAINING A P–Cl BOND

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(Received August 6th, 1973)

SUMMARY

1. The inhibition of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from electrophorus by a number of substituted phosphono- and phosphoro- mono- and dichloridates was studied. Rate constants were determined for both the inhibition of the enzyme and the spontaneous hydrolysis of the inhibitors; the two parameters could be evaluated even when the half time for hydrolysis of the inhibitor was less than 1 s.

2. The second-order rate constants (k_i) for enzyme inhibition were higher than 10^7 $M^{-1} \cdot min^{-1}$ for most of the chloridates used; it was as high as $3 \cdot 10^9$ $M^{-1} \cdot min^{-1}$ for phenyl phosphorodichloridate. No correlation was found between k_i and the rates of hydrolysis of the inhibitors used.

3. Isopropyl methyl phosphonochloridate was only six times more potent as an inhibitor than the corresponding fluoridate (i.e. sarin) although the rate of hydrolysis of the former compound is at least 5000 times higher. In contrast, methyl phosphonodichloridate was considerably more potent than the difluoridate.

4. The rate of phosphorylation of the enzyme by the inhibitors was decreased in the presence of reversible cholinesterase inhibitors such as substituted quaternary ammonium compounds.

5. The enzyme inhibited by dichloridates was not reactivatable. It is an "aged" enzyme.

6. The diphenyl phosphoryl enzyme reactivates spontaneously and rapidly. It also ages rapidly.

INTRODUCTION

Some phosphonate and phosphate esters with good leaving groups are remarkably potent inhibitors of acetylcholinesterase (acetylcholine hydrolase, EC

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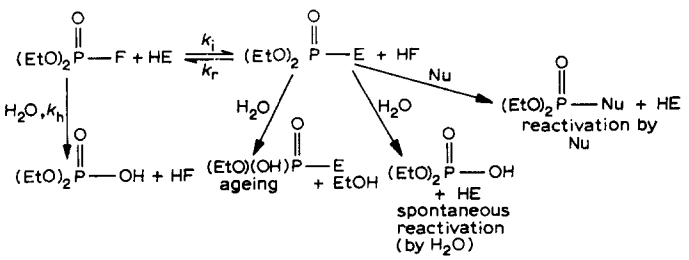
3.1.1.7) and other serine esterases. These inhibitors are of practical interest since some war gases and insecticides are compounds of this type. Some of these compounds even find use in medicine. These substances are also of interest in enzymology because they react extremely rapidly with esterases and appear to react by much the same mechanism as is involved in the hydrolysis of carboxylic esters.

The reaction of these inhibitors with cholinesterase here illustrated with *O,O*-diethyl phosphofluoridate, is intrinsically reversible but is usually carried out under conditions where it is unidirectional. The phosphoryl enzyme is analagous to the acetyl enzyme that is formed during the hydrolysis of carboxylic esters, and is, of course, not capable of hydrolyzing esters. Other pertinent reactions are also shown in the scheme:



where HE is the active enzyme and Nu is a nucleophile, fluoride for example. The reader is referred to three reviews (refs 1, 2 and 16) for discussion of these reactions and references.

The speed of the various reactions depend upon the actual compounds involved. Phosphochloridates and phosphonochloridates hydrolyze very rapidly. Phosphorylated enzyme with a branched alkyl group "ages" rapidly. "Aged" enzyme is inactive and cannot be reactivated. Some inhibited enzymes are spontaneously reactivated fairly rapidly. Reactivation may be very rapid depending upon the alkyl (aryl) groups and upon the nucleophile. *N*-Methyl pyridinium-2-aldoxime (2-PAM, PAM-2) very praidly reactivates some inhibited enzymes such as the *O,O*-diethyl phosphoryl enzyme illustrated above.

The hydrolysis of a fluoro compound is relatively slow and this is typically true for the compounds that are usually investigated as enzyme inhibitors. This is not true of the corresponding chloro compounds, which hydrolyze very rapidly.

Although chloro compounds have been used to produce inhibited enzyme [3], until recently no rate measurements had been made with chloro compounds, probably because these compounds hydrolyze very rapidly. However it is possible to make the measurements with ordinary "slow" techniques provided the half time for hydrolysis is not too short; in this work not less than 0.1 s. The method consists of rapidly introducing a small sample of the inhibitor dissolved in acetonitrile (or other suitable solvent) into a stirred solution of enzyme. After suitable time intervals, starting some seconds later, a small sample of this solution is injected (with the aid of a spring loaded syringe that automatically withdraws the proper sample size) into a filled 3-ml cuvette for enzyme assay [4].

Since the inhibitor hydrolyzes very rapidly, the inhibition reaction is soon over and the final extent of inhibition is given by

$$\ln \frac{E_\infty}{E_0} = -I_0 \frac{k_i}{k_h} \quad (1)$$

Thus we require a knowledge of k_h in order to evaluate k_i. When inhibitor is introduced into buffer solution the concentration remaining after time t is

$$I \text{ (remaining)} = I \text{ (initial)}\, e^{-k_h t} \quad (2)$$

The value of I (remaining) can be obtained by using a large initial concentration and adding enzyme at short time intervals thereafter. Under these circumstances I (remaining) becomes the I_0 for Eqn 1 and can therefore be evaluated from E_∞/E_0.

In this way we have obtained values for k_h and k_i for a number of chlorophosphorous compounds.

METHODS

Materials

Methyl phosphonodichloridate was prepared by reacting PCl_5 with dimethyl methylphosphonate after Monard and Quinchon [5]. Methyl phosphonodifluoridate was prepared from the dichloride [5].

Isopropyl methyl phosphonochloridate and ethyl methyl phosphonochloridate were prepared after Neal and Williams [6].

Phenyl phosphonodichloridate [7], phenyl phosphorodichlorate [8], diphenyl phosphorochloridate [9], chloromethyl phosphorodichloridate [9] and diethyl phosphorochloridate [10] were redistilled commercial products (Aldrich Chem. Co.).

All compounds were again distilled immediately before use.

All stock solutions were made in acetonitrile (Fisher, analytical grade) distilled over $MgSO_4$. All stock solutions were freshly prepared. Methyl phosphonodichloridate which is solid below 33°C, is only sparingly soluble in acetonitrile but a supersaturated solution is easily prepared from the melt.

Acetylcholinesterase from electric organ of *Electrophorus electricus* (Worthington, spec. act. approx. 1000 units/mg) was used. Stock solutions (approx. $2 \cdot 10^{-7}$ M) were made in a medium containing 0.8 M NaCl, phosphate buffer, $I = 0.02$ (pH 7.8) and 0.2% gelatin. This enzyme solution was stable at 0–3 °C for several weeks.

Edrophonium chloride ("tensilon") was a gift from Hoffmann–La Roche Inc., Nutley, N.J.

Inhibition and assay of the enzyme

At zero time, 5–20 μl of the inhibitor in dry acetonitrile were added to 2 ml of enzyme solution (approx. 10^{-9} M) in a medium containing phosphate buffer, $I =$ 0.05 (pH 7.0), 0.05 M NaCl and, in some cases, a reversible cholinesterase inhibitor. The organophosphate was always added to the medium under very rapid magnetic stirring. After 8–10 s, and then after different time intervals (20–60 s), 20 μl of the incubating mixture were diluted into 3 ml of phosphate buffer, $I = 0.1$ (pH 8.00), and

the residual activity of the enzyme assayed as described previously [4], by the Ellman procedure.

Data on the inhibition of the enzyme were plotted in accordance with Eqn 1 [4].

RESULTS

Our results are summarized in Table 1. Almost all the compounds hydrolyze very rapidly and almost all are very potent inhibitors. Except for Compounds 8 and 9 we typically found that the final extent of inhibition was reached at the time of our first measurement (Fig. 1), which would be expected for compounds that hydrolyze so rapidly.

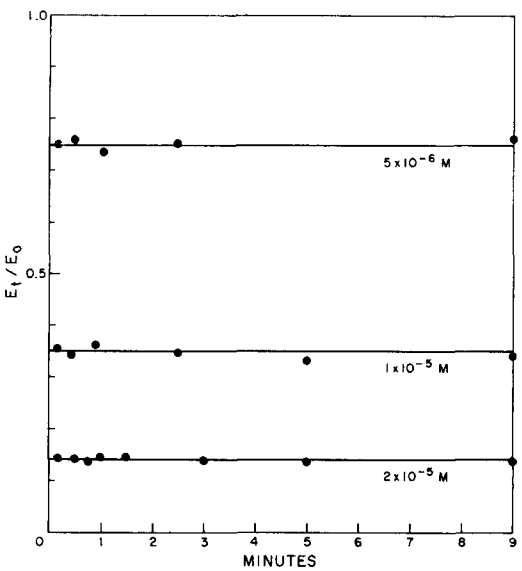


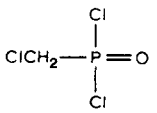
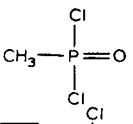
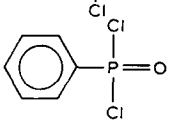
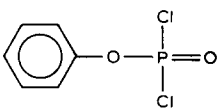
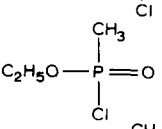
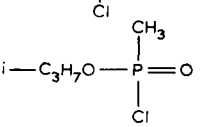
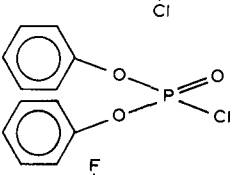
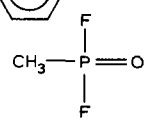
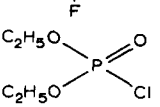
Fig. 1. The final extent of inhibition for methyl phosphonodichloridate. The value of E_t has become E_∞ by the time of our first measurements. Concentrations are the initial concentrations, I_0.

In applying Eqn 1, relatively low concentrations of inhibitor are used but in applying Eqn 2 to obtain k_h rather high concentrations must be used. Under these circumstances the final extent of inhibition was not quite constant but usually increased slowly with time. This phenomenon is easily explained. If the compound should contain a very small amount of a relatively stable inhibitor as an impurity or if a relatively stable secondary inhibitor should be formed when the chloro compound is introduced into water we should have an explanation for this "after inhibition". In retrospect both possibilities, especially the latter, seem so likely that we should have anticipated this behavior. The "after inhibition" was quite severe in the case of Compounds 1 and 2 because we had to use quite high concentrations and we were able to confirm only that the half time for hydrolysis is less than 0.1 s. The high concentrations were required in part because the rate of hydrolysis is so high. In other cases the after inhibition was slow and therefore not troublesome.

TABLE I

RATES OF REACTION OF SEVERAL UNSTABLE ORGANOPHOSPHATES WITH WATER AND WITH ACETYLCHOLINESTERASE

k_h is the first-order rate constant for hydrolysis in a medium containing phosphate buffer ($I = 0.05$) and NaCl ($I = 0.05$) pH 7.0. $t_1/_2$ is the half time of hydrolysis. k_i is the second order-rate constant for the reaction with acetylcholinesterase in the same medium.

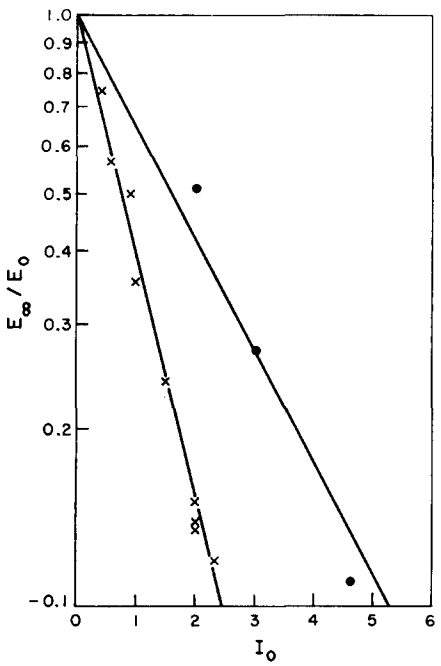
No.	Compound	Structure	$t_1/_2$ (k_c) s	k_h (s^{-1})	k_i ($l \cdot M^{-1} \cdot min^{-1}$)
1	Chloromethylphosphono-dichloridate		0.035	20*	$9 \cdot 10^7$
2	Methylphosphonodi-choridate		0.069	10*	$5.7 \cdot 10^7$
3	Phenylphosphonodi-chloridate		0.13	5.3	$5.5 \cdot 10^7$
4	Phenylphosphorodi-chloridate		0.15	4.6	$3.5 \cdot 10^9$
5	Ethylmethylphosphono chloridate		0.70	1.0	$2.2 \cdot 10^8$
6	Isopropylmethylphos-phonochloridate		1.2	0.60	$3.6 \cdot 10^8$
7	Diphenylphosphoro chloridate		2.0	0.34	$1.9 \cdot 10^7$
8	Methylphosphono-difluoridate		35	0.020	$2.5 \cdot 10^4$
9	Diethylphosphoro-chloridate**		41	0.017	$1.5 \cdot 10^6$

* Data from Hudson and Moss (1964).
** Data from Ashani, Wins and Wilson [4].

Rates of inhibition

All the chloro compounds are very potent inhibitors. Compound 4 is amongst the most potent inhibitor known. It is far more potent in comparison with Compounds 1, 2 and 3 than would be suggested by the relative rates of hydrolysis. The dichloro

compounds should produce an "aged" enzyme since the inhibited enzyme that is immediately formed will contain a P–Cl bond that should rapidly hydrolyze. Enzyme inhibited by Compounds 1 and 2 were treated with the potent reactivator 2-PAM at high concentration ($5 \cdot 10^{-4}$ M) but no reactivation was obtained. This result is anticipated for an "aged" inhibited enzyme. On the other hand enzyme inhibited with Compound 6 was readily reactivated by 2-PAM. This too was anticipated because enzyme so inhibited should be identical with enzyme inhibited by sarin and the later is known to be readily reactivated by 2-PAM. Our inability to reactivate the enzyme inhibited by the dichloro compounds is, as already noted, consistent with the supposition that the dichloro compounds react with the active site of the enzyme but could also be explained by the assumption that another site is involved. This possibility is all the more possible because these compounds are extremely active phosphorylating agents. However we were able to show that reaction is at the active site. First we



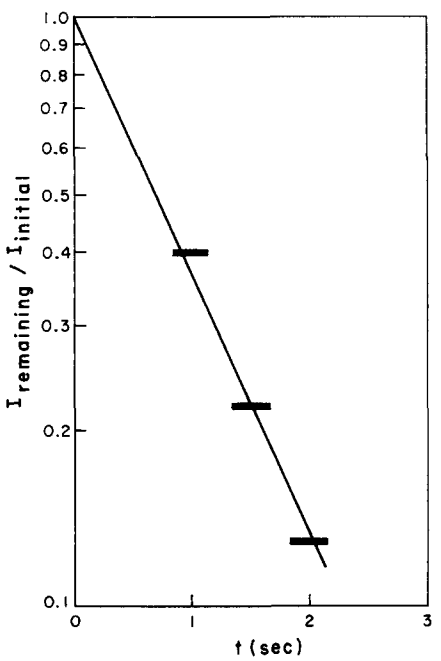


Fig. 2. The final extent of inhibition plotted in accordance with Eqn 1 for methylphosphonodichloridate (×) and ethyl methylphosphonomonochloridate (●).

Fig. 3. Data for ethyl methylphosphonomonochloridate plotted according to Eqn 2 for determining k_h.

found that inhibition of the enzyme by Compound 2 was itself inhibited by the presence of fluoride. Fluoride reversibly inhibits the reaction of the enzyme with diethyl phosphorofluoridate, with carbamates, with methane sulfonates,t and with substrates. Similarly we found that quaternary ammonium ions such as etramethyl ammonium ion, tetraethyl ammonium ion and edrophonium, which are reversible inhibitors, inhibit the inhibition of the enzyme and finally the very poor substrate butyrylcholine inhibits the inhibition of the enzyme. The effect of these ions was

studied in some detail (Fig. 4) and a K_i for fluoride was evaluated. The value obtained was $3.5 \cdot 10^{-4}$ M which is about the same value found from the inhibition of the hydrolysis of substrates and from the other inhibitory effects of fluoride mentioned above. Binding constants were also obtained for the other ions and they were about the same as is found for inhibition of the hydrolysis of acetylthiocholine. The curvature in Fig. 4 (Curves 2 and 4) indicates that tetramethyl ammonium ion and tetraethyl ammonium ion do not completely prevent reaction with the organophosphonate. Fluoride probably binds at the esteratic site and therefore completely prevents reaction. Edrophonium probably binds at both subsites.

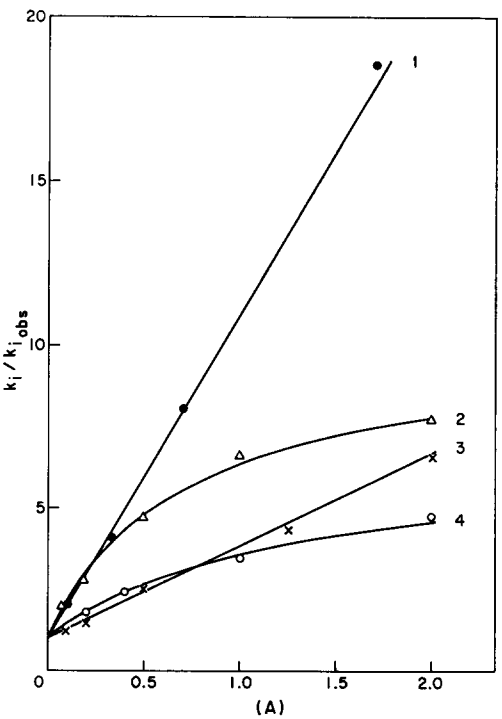


Fig. 4. The effect of F^- (×), tetramethyl ammonium ion (△), tetraethyl ammonium ion (○) and edrophonium (●) in decreasing the rate of inhibition of acetylcholinesterase by methylphosphonodichloridate. The values of the abscissa should be multiplied by 10^{-3}, 10^{-2}, 10^{-3}, and 10^{-6}, respectively. Dissociation constants, K_A, are $3.5 \cdot 10^{-4}$, $6.8 \cdot 10^{-4}$, $1.8 \cdot 10^{-4}$, and $1.0 \cdot 10^{-7}$ M, respectively. The equation used was

$$k_{i\,obs} = k_i \left[1 + \alpha \frac{[A]}{K_A}\right] \Big/ \left[1 + \frac{[A]}{K_A}\right]$$

where α, the factor for the rate constant with saturating modifier concentration, was 0, 0.10, 0.15 and 0, respectively. The modifier concentrations are [A].

The effectiveness of Compound 6 is about six times greater than sarin, $k_i = 6.3 \cdot 10^7$ [11]. This difference is quite small considering the very large difference in the pK_a values of the leaving groups (the pK_a of HCl vs HF; −7 vs 3), and in their rates of hydrolysis.

The *O,O*-diphenyl compound, Compound 7, is rather more active than the *O,O*-diethyl compound, Compound 9. Compound 7 produces an inhibited enzyme that recovers rapidly by reactivation with water. The half time for reactivation is about 12 min. Ageing is also rapid however, and full recovery is not obtained.

Rates of hydrolysis

There are few measurements of the rates of hydrolysis of compounds with a P–Cl bond in 100% water. In this work we have obtained data only for the total rate of hydrolysis without regard to general base catalysis by 0.01 *I* phosphate and without separating the H_2O and OH^- contributions. In the case of diethyl phosphorochloridate there are significant contributions from all three sources but at pH 7.0 the water reaction is the most prominent [4]. Fig. 2 illustrates the kind of experimental results we have obtained using our method.

The values for k_h given in the table for Compounds 1 and 2 are taken from the literature for 20 and 40% dioxane. Since there was little difference in these two solvents it was assumed that the value in 100% water would not be very different. We did confirm that the $t_{1/2}$ value was less than 0.1 s.

The faster rate of hydrolysis of Compound 5 as compared to Compound 9 is a well known phenomenon that is explained by the concept that the unshared electrons of the ether oxygen atoms interact with the d orbitals of phosphorus and thereby suppress the rate of nucleophilic attack on phosphorus. This suppresion of rate does not appear in the comparison of Compounds 3 and 4 possibly because in this case the oxygen electrons tend to occupy molecular orbitals involving the π electrons of the benzene ring. Conjugation of the lone pair electrons of oxygen with the aromatic ring produces an inductive effect which may balance out the small amount of $p\pi$–$d\pi$ overlap with phosphorus. Thus the much greater rate of hydrolysis of Compound 7 compared to Compound 9 arises from a greater inductive effect and less $p\pi$–$d\pi$ overlap.

The dichloro compounds hydrolyze more rapidly than the monochloro compounds as would be expected from the inductive effect of chloride. These rationalizations are discussed by Kirby and Warren [12].

DISCUSSION

The chloro compounds can be used to inhibit enzyme and may be of some use for this exeprimental purpose. An advantage is that the inhibitor is quickly hydrolyzed. Also the chloro compounds are usually the starting materials for more exotic inhibitors. The rapid hydrolysis of inhibitor 7 did uncover the rapid spontaneous recovery of the inhibited enzyme (diphenyl phosphoryl enzyme) and its rapid ageing. Substituted phenyl methyl phosphonochloridates have been used by Hovanec and Lieske [3] to produce a related inhibited acetylcholinesterase (substituted phenyl methylphosphonyl enzyme) and they too observed spontaneous but rather slower reactivation and ageing.

The comparison of the rates of inhibition of Compound 6, "sarin chloride", with the fluoro analogue, sarin, is of some interest. It has been found that in the *O,O*-diethyl phosphoryl series the value of k_i is extremely dependent upon the pK_a of the leaving group for $pK_a \geqslant 7$ [13] but is not very dependent for lower pK_a [14, 4]. Thus the phosphorochloridate ($k_i = 1.2 \cdot 10^6$) is only six times more potent than the

phosphorofluoridate ($k_i = 2.2 \cdot 10^5$) despite the difference of ten pK_a units for the leaving groups. Here too in the case of the chloro and fluoro "sarin" compounds we see a relatively small difference in k_i, suggesting that this series will be similar to the *O,O*-diethyl phosphoryl series. A discussion of possible explanations for this type of Brönsted relationship is given in ref. 15.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health grant NS07156.

REFERENCES

1 Handbuch der Experimentellen Pharmakologie (1963) (Koelle, G. B., ed.), Springer-Verlag, New York
2 Froede, H. C. and Wilson, I. B. (1971) in The Enzymes (Boyer P. D., ed.), Vol. V, p. 87, Academic Press, New York
3 Hovanec, J. W. and Lieske, C. N. (1972) Biochemistry 11, 1051
4 Ashani, Y., Wins, P. and Wilson, I. B. (1972) Biochim. Biophys. Acta 284, 427
5 Monard, C. and Quinchon, J. (1961) Bull. Soc. Chim. Fr. 1084
6 Neale, E. and Williams, L. T. D. (1955) J. Chem. Soc. 2485
7 Gefter, Y. L. (1958) Zh. Obsch. Khim. 28, 1338
8 Korshak, A. M. (1958) Izv. Akad. Nauk SSSR (Khim.) 880
9 Kinnear, A. M. and Perren, E. A. (1952) J. Chem. Soc. 3437
10 Steinberg, G. M. (1950) J. Org. Chem. 15, 637
11 Michel, H. O. (1955) Fed. Proc. 14, 255
12 Kirby, A. J. and Warren, S. G. (1967) The Organic Chemistry of Phosphorus, Mono. 5, Elsevier, Amsterdam
13 Aldridge, W. N. and Davison, A. N. (1952) Biochem. J. 51, 62
14 Kitz, R. J., Ginsburg, S. and Wilson, I. B. (1967) Mol. Pharm. 3, 225
15 Ashani, Y., Snyder, S. L. and Wilson, I. B. (1973) J. Med. Chem. 16, 446
16 Aldridge, W. N. and Reiner, E. (1972) Enzyme Inhibitors as Substrates, Elsevier, Amsterdam