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THE REACTION OF ACETYLCHOLINESTERASE WITH PHOSPHYLATED* OXIMES

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Abstract—The reaction of acetylcholinesterase (EC. 3.1.1.7) with phosphylated oximes has been studied. The enzyme is irreversibly inhibited, and the kinetics of the inhibition reaction is shown to be consistent with the formation of a reversible complex which subsequently breaks down to give a phosphylated enzyme. The affinity constant (K) and the first order phosphylation constant k_2 for the inhibition of acetylcholinesterase by 2-, 3-, and 4-O-(isopropyl ethylphosphono)-acetylpyridine oxime (MPA-ES) and 2-, 3-, and 4-O-(isopropyl ethylphosphono)-acetyl-1-methylpyridinium iodide oxime (MPAM-ES) have been determined. For the quaternary compounds, which have approximately the same affinity for the enzyme the difference in inhibitor power is almost entirely due to differences in phosphylation potency. For the tertiary compounds both affinity and phosphylation potency determine the relative rates of inhibition.

IN TREATMENT of anticholinesterase poisoning compounds such as pyridineoximes are generally used. These function by removing the phosphor group from the inactivated enzyme thus restoring enzyme activity.

From the work of Hackley *et al.*[2, 3, 4] it is known that oximes may also react directly with organophosphorus compounds giving rise to phosphylated oximes which are themselves highly toxic. For example,[4] the highly toxic anticholinesterase isopropyl methylphosphonofluoridate (Sarin) is converted under physiological conditions of pH and temperature by a model treatment compound, 4-formyl-1-methylpyridinium iodide oxime (4-PAM) into an equally potent and toxic anticholinesterase, *O*-(isopropyl methylphosphono)-4-formyl-1-methylpyridinium iodide oxime. From comparisons of reactivation and ageing rates it was concluded that the Sarin and phosphylated oxime inhibited enzyme were identical.[5]

In order to investigate the stability of phosphylated oximes, a series of phosphylated pyridine and pyridinium ketoximes have been prepared in this laboratory and their decomposition in alkali studied.[6] It was the purpose of the present study to investigate the reaction of these phosphylated oximes with acetylcholinesterase.

It was suggested by Aldridge[7] that the irreversible inhibition of acetylcholinesterase by organophosphorus compounds could be represented by the scheme

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (EI) \overset{k_2}{\rightarrow} EI \qquad (1)$$

where E is acetylcholinesterase, I is an organophosphorus inhibitor, (EI) is a reversible complex and EI is the irreversibly phosphylated enzyme. After application of

* The term phosphylation is used in this paper to cover both phosphonylation and phosphorylation.[1]

the steady-state treatment to this scheme one obtains, in the case where the inhibitor is in large excess, for the velocity of enzyme disappearance[8, 9]

$$-\frac{dE}{dt} = \frac{k_2(E)(I)}{K + (I)} \tag{2}$$

where $K = (k_{-1} + k_2)/k_{-1}$. Although the presence of a reversible complex was suggested by Aldridge,[7] no kinetic evidence for such a complex has been available until recently.[10] This treatment is based on the integrated form of equation (2)

$$\frac{t(I)}{2{,}3 \log E_o/E_t} = \frac{(I)}{k_2} + \frac{K}{k_2} \tag{3}$$

where E_o is the activity of the uninhibited enzyme and E_t is the activity remaining after incubation of the enzyme for a length of time t with a fixed concentration of inhibitor. According to equation (3) the plot of $t(I)/2{,}3$ (log E_o/E_t) against (I) should be linear with intercept on the ordinate axis equal to K/k_2 and intercept on the abscissa equal $-K$. If no reversible complex is formed $t(I)/2{,}3$ (log E_o/E_t) would be constant and equal the reciprocal second order rate constant for the bimolecular reaction between enzyme and inhibitor. In order to get conclusive evidence for a reversible complex it is necessary to use inhibitor concentrations of the order of K since if $K >$ (I) equation (2) will be indistinguishable from a second order rate equation.

Since the compounds used in this study are characterized by low phosphylation rates and relatively high affinity, as measured by K, they should be particularly well suited to the present treatment. Also, since the only variation in structure is in the oxime part of the molecule, an attempt has been made to relate the relative rates of inhibition to the Hammett substituent constants for the 2-, 3-, and 4-pyridyl and -pyridinium groups.

MATERIALS AND METHODS

Enzyme. Acetylcholinesterase from bovine erythrocytes was obtained from Sigma Chemical Co. Enzyme solution containing 100 units/ml (one unit will hydrolyse 10·1 μg acetylcholine/min at pH 8 and 37°) was prepared by dissolving the appropriate amount in 2 mM-phosphat buffer pH 7·6.

Substrate. Acetylcholine iodide was obtained from Fluka A.G. 3×10^{-3} M, prepared by dissolving the appropriate amount in water, was used throughout.

Phosphylated oximes. All the phosphylated oximes were prepared in this laboratory. For details the paper by Blanch and Andersen[6] should be consulted. Inhibitor solution were prepared by dissolving the phosphylated oxime in 2 mM-phosphate buffer pH 7·6.

Esterase activity measurements. The kinetic measurements were made by continuous titration, using a Radiometer Titrator. The reaction vessel was surrounded by a jacket for constant water-temperature circulation and mounted on a magnetic stirring apparatus. The titrant, 0·02 N-NaOH, was delivered automatically from a 0·5 ml syringe. A stream of nitrogen was directed over the liquid in the reaction vessel to keep the interior free of carbon dioxide.

Measurement of inhibition rates. The measurement of inhibition rates and determination of the affinity constants K and phosphylation constants k_2 was done according to the procedure of Main.[10] Enzyme solution (0·5 ml) and inhibitor solution (0·5 ml) were incubated for a measured time at 20° and pH 7·6 after which the reaction was stopped by adding the enzyme-inhibitor mixture (0·5 ml) to the substrate solution (25 ml) in the reaction vessel. The residual activity was then measured by titration at pH 7·6 and 20°. The activity was calculated from the first 5 min part of the titration curve. From the measured activity was subtracted a non-enzymic hydrolysis blank.

RESULTS AND DISCUSSION

Typical plots for one of the compounds, 4-MPA-ES, are presented in Figs. 1 and 2. In Fig. 1 the curves obtained by plotting log E_t against t at various concentrations of inhibitor are shown. Here E_t refers to the rate of acetylcholine hydrolysis by the

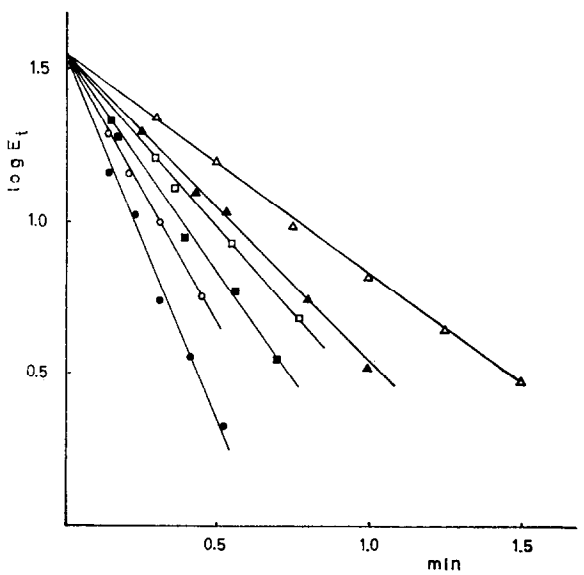


FIG. 1. Plots of log E_t against time for the reaction between 4-MPA-ES and acetylcholinesterase in 2 mM-phosphatbuffer pH 7·6 and 20° at various concentrations of inhibitor. E_t refers to the rate of acetylcholine hydrolysis by the acetylcholinesterase remaining uninhibited after reacting with 4-MPA-ES for time t. The inhibitor concentrations were △ $4{\cdot}45 \times 10^{-3}$ M; ▲ $7{\cdot}12 \times 10^{-3}$ M; □ $8{\cdot}9 \times 10^{-3}$ M; ■ $12{\cdot}5 \times 10^{-3}$ M; ○ $20{\cdot}0 \times 10^{-3}$ M; ● $25{\cdot}3 \times 10^{-3}$ M.

acetylcholinesterase remaining uninhibited after reacting with inhibitor, at 20° in 2 mM-phosphate buffer pH 7·6, for a length of time t. The slopes of these lines gave the value of $(\log E_o/E_t)/t$ which were used to construct the graph of $t(\mathrm{I})/2{\cdot}3\ (\log E_o/E_t)$ against (I) according to equation (3) (Fig. 2). A linear plot with significant slope is obtained indicating that the kinetics of the inhibition reaction is consistent with scheme (1). Similar linear plots were obtained for the other compounds. The kinetic constants obtained from these plots are given in Table 1. Each plot was based on the inhibition rates from at least seven different inhibitor concentrations, the error in the K and k_2 values being of the order of ± 5 per cent.

If K is assumed equal to k_{-1}/k_1 (i.e. if $k_{-1} \gg k_2$) it will be a true equilibrium constant and hence a measure of the affinity of the inhibitor for the enzyme. The first order rate constant k_2 will reflect the phosphylation potency of the inhibitor.[9, 10] According to this interpretation the irreversible inhibition of acetylcholinesterase by organophosphorus compounds will depend on the affinity of the inhibitor as well as the rate

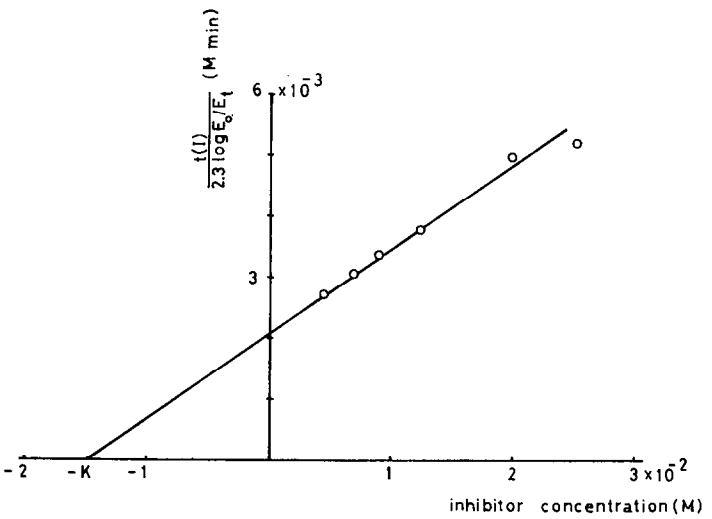


FIG. 2. The 4-MPA-ES concentrations (I) plotted against $t(I)/(\log E_o/E_t)$. The values of $(\log E_o/E_t)/t$ are obtained from the slopes of the lines in Fig. 1. The intercept in the x-axis is $-K$ and on the y-axis $1/k_t$. The slope give the value of $1/k_2$.

of phosphylation. The overall rate of formation of the irreversibly inactivated enzyme may be expressed by the bimolecular velocity constant $k_i = k_2/K$.[9]

It is immediately apparent from Table 1 that the quarternary compounds have much greater affinity for the enzyme than have the corresponding tertiary ones. This is what should be anticipated from the knowledge of the active center which contain a negatively charged 'anionic' site.[12] The difference in free energy of binding between the charged and the corresponding uncharged compounds is in the range 2–4 kcal/mole, which could very well reflect a coulombic attraction.

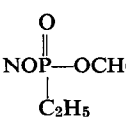
The results obtained for 2-MPAM-ES, the inhibitor with the highest inhibition rate, may be compared with the results obtained by Main and Iverson[10] for the reaction of diisopropyl phosphorofluoridate (DFP) and acetylcholinesterase. For DFP K was $1{\cdot}03 \times 10^{-3}$ M and k_2 was 26·8 min^{-1} at 20° and pH 7·6. It is evident that the phosphylated oxime has greater affinity for the enzyme than has DFP. For this reason the bimolecular velocity constant k_i of 2×10^5 M^{-1} min^{-1} was higher than the comparable value for DFP which was $2{\cdot}6 \times 10^4$ M^{-1} min^{-1} although the phosphylation rate of DFP is about twice that of 2-MPAM-ES. In this sense 2-MPAM-ES inhibited acetylcholinesterase about 10 times more rapidly than did DFP.

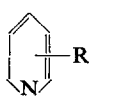
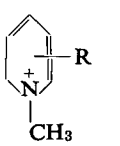
It is evident that there are quite large variations in inhibition rates, as measured by k_t, for the six phosphylated oximes. For the three quarternary compounds, which are bound to approximately the same extent (K values similar) the variation in inhibition

rates is almost entirely due to differences in phosphylation potency. For the tertiary compounds variation in both affinity and phosphylation is responsible for differences in inhibition rates. It should also be noted that there is apparently no correlation between affinity and phosphylation potency. In order to compare the inhibition reaction with the reactivity of the phosphylated oximes, the second order rate constants

TABLE 1. AFFINITY CONSTANTS K (MOLE $\times$ 1^{-1}) AND PHOSPHYLATION CONSTANTS k_2 (MIN^{-1}) FOR THE REACTION OF PHOSPHYLATED OXIMES WITH ACETYLCHOLINESTERASE IN 2 mM-PHOSPHATBUFFER pH 7·6 AND 20°.

The bimolecular velocity constant k_i ($1 \times mole^{-1} \times min^{-1}$) was calculated from the relationship $k_i = k_2/K$. The Hammett substituent constants σ for the pyridyl and pyridinium groups* and the second order rate constants k_{OH} ($1 \times mole^{-1} \times min^{-1}$) for alkaline hydrolysis at 20°† are given in the last two columns.



		K(mole $\times$ 1^{-1})	k_2(min^{-1})	k_i($1 \times mole^{-1} \times min^{-1}$)	σ	k_{OH}($1 \times mole^{-1} \times min^{-1}$)
	2—	$2{\cdot}1 \times 10^{-3}$	0·27	$1{\cdot}3 \times 10^{2}$	0·71	0·84
	3—	$1{\cdot}5 \times 10^{-3}$	5·3	$3{\cdot}6 \times 10^{3}$	0·55	0·67
MPA-ES	4—	$1{\cdot}5 \times 10^{-2}$	7·2	$4{\cdot}8 \times 10^{2}$	0·94	1·08
	2—	$5{\cdot}1 \times 10^{-5}$	10·2	$2{\cdot}0 \times 10^{5}$	3·11	27·1
	3—	$5{\cdot}5 \times 10^{-5}$	0·077	$1{\cdot}4 \times 10^{3}$	2·10	—
	4—	$2{\cdot}2 \times 10^{-5}$	1·5	$6{\cdot}8 \times 10^{4}$	2·57	11·9
MPAM-ES						

* Taken from ref. 11.
† Taken from ref. 6.

for the alkaline hydrolysis (taken from Blanch and Anderson[6]) together with the Hammett substituent constants for the pyridyl and pyridinium groups (taken from Blanch[11]) are included in Table 1. It is immediately apparent that the influence of the pyridyl and pyridinium groups on inhibition rate constants k_i and k_2 does not show Hammett-type free energy relationship. This is in contrast to alkaline hydrolysis where such a relationship has been shown to exist.[6] If, however, the quarternary and tertiary compounds are considered separately, there appears to be, at least qualitatively, a correlation for the quarternary compounds but not for the tertiary ones. Since the quarternary compounds have approximately the same affinity for the enzyme, this could mean that the relative rates of inhibition is governed primarily by k_2 which should be subject to the polar effects of the pyridinium groups, the steric effects being roughly equal. For the tertiary compounds the relative rates of inhibition are apparently more influenced by binding and proper orientation. The number of data is, however, insufficient to justify firm conclusions.

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