SESSION I

THE INHIBITION OF ESTERASES BY ORGANOPHOSPHORUS COMPOUNDS AND CARBAMATES

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The Nature of the Reaction of Organophosphorus Compounds and Carbamates with Esterases

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This paper outlines our knowledge of the reaction of organophosphorus compounds and carbamates with esterases, examples of particular aspects of the reaction being confined to cholinesterases, although the general principles discussed apply to all B-esterases. Mathematical expressions are given for the different rate constants, and some of the factors that may affect the response of insect and mammalian cholinesterases to organophosphorus compounds and carbamates are discussed.

It is assumed in this paper that insects are killed by the effects of organophosphorus compounds and carbamates on their acetylcholinesterase; therefore the examples given are confined to this enzyme, but the principles discussed are applicable to B-esterases in general. Aldridge & Johnson (1971) discuss a biological action of organophosphorus compounds that is dependent upon reaction with a protein with esteratic activity; this enzyme is a B-esterase but not a cholinesterase.

Organophosphorus compounds react with cholinesterase to produce a relatively stable phosphorylated enzyme. This reaction is progressive and temperature-dependent. It may not be described as a simple association-dissociation phenomenon

$$\mathrm{EH + I} \overset{K_i}{\rightleftharpoons} \mathrm{EHI} \qquad \text{Eq. 1}$$

but is better formulated by Eq. 2

$$\mathrm{EH + AB} \xrightarrow{k_a} \mathrm{EA + BH} \qquad \text{Eq. 2}$$

where EH is free enzyme, I is a reversible inhibitor, AB is an ester of an organophosphorus acid, EA is the phosphorylated enzyme and BH is the first leaving product. The constant K_i is the dissociation constant for combination of EH with I and k_a is the bimolecular rate constant for the combination of EH with AB. The bimolecular reaction defined by Eq. 2 almost always shows first-order kinetics because the concentration of inhibitor is at least ten times that of enzyme. Inhibitory potency should always be expressed as rate constants qualified by the experimental conditions.

Because the addition of substrate greatly diminishes the rate of reaction of organophosphorus compounds with cholinesterase we conclude that the inhibitor reacts with the catalytic centre. Other evidence has shown that there are resemblances with the structural requirements for good substrates and good inhibitors (Aldridge, 1953a; Burgen & Hobbiger, 1951; Heath & Vandekar, 1957; Fukuto et al., 1955). It was natural therefore to amend Eq. 2 to include an intermediate analogous to a Michaelis complex with substrate.

$$\mathrm{EH + AB} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{EHAB} \xrightarrow{k_{+2}} \mathrm{EA + BH} \qquad \text{Eq. 3}$$

Although such an intermediate was postulated almost 20 years ago (Aldridge & Davison, 1952) it is only recently that evidence for its existence has been available. As for substrates the evidence is indirect and depends upon experimental results indicating that when the unphosphorylated enzyme is all present as the complex, a maximum rate of phosphorylation is obtained—in other words, saturation of the enzyme by inhibitor is demonstrated. Since k_{+2} is usually fairly fast, considerable technical ingenuity is required to obtain reliable results.

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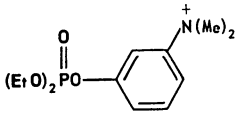
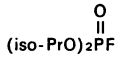
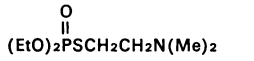
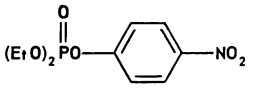
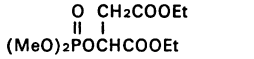
Table 1

The constants K_a and k_{+2} for the reaction of some organophosphorus compounds and acetylcholinesterase

Compound	Temperature (°C)	K_a (M)	k_{+2} (min^{-1})	Reference [a]
	25	1.2×10^{-4}	5.2	1
	5	1.6×10^{-3}	11.9	2
	5	1.8×10^{-4}	126	3
	5	3.6×10^{-4}	42.7	4
	5	2.4×10^{-3}	67.0	4

[a] References: 1, Reiner & Aldridge (1967); 2, Main & Iverson (1966); 3, Chiu & Dauterman (1970); 4, Chiu, Main & Dauterman (1969).

Main and his colleagues have solved these problems and have established the necessary controls. With such experiments, and when the rates of inhibition are determined with the necessarily high concentrations of inhibitors, the K_a and k_{+2} can be evaluated by the following equation.

$$\frac{[AB]}{k} = \frac{[AB]}{k_{+2}} = \frac{K_a}{k_{+2}} \qquad \text{Eq. 4}$$

where k is the first-order rate constant for the rate of formation of phosphorylated enzyme with a given concentration of inhibitor. K_a is a constant that is analogous to the Michaelis constant, K_m, for the hydrolysis of substrate by enzyme; it is the concentration of inhibitor when the rate of phosphorylation of the enzyme is one half of the maximum rate. Some examples of these constants are given in Table 1. It is a matter for argument whether K_a may be regarded as a measure of the affinity of inhibitor for enzyme. Studies of the effect of temperature on both of these constants (Main & Iverson 1966; Reiner & Simeon, unpublished data) give some indication that K_a is an expression of the association–dissociation reaction of cholinesterase and inhibitor (cf. Eq. 3).

In early studies the inhibition of cholinesterase was considered to be irreversible. The analogy of the inhibitor and the substrate interactions indicated the possibility that some phosphorylated enzyme derivatives might be unstable. This is now an established fact and Eq. 3 must be extended.

$$EH + AB \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EHAB \xrightarrow{k_{+2}} BH + EA \xrightarrow{k_{+3}} EH + AOH \qquad \text{Eq. 5}$$

Some examples of the rates of hydrolysis of phosphorylated acetylcholinesterases are given in Table 2.

As discussed earlier in this paper, the addition of substrate slows down the reaction of organophos-

Table 2

Rates of hydrolysis of phosphorylated acetylcholinesterases (pH 7.4–7.8, temperature 37°C)

Enzyme	Groups attached to P	$10^3 \times k_{+3}$ (min^{-1})	Reference [a]
rabbit	$(CH_3O)_2$	8.5	1
human	$(C_2H_5O)_2$	0.2	2
sheep	$(CH_2Cl \cdot CH_2O)_2$	29.5	3
cow		38	4
rat		33	3
guinea-pig		28.5	3
bovine	$(CH_3 \cdot CHCl \cdot CH_2O)_2$	37	4
bovine	$(CH_2Cl \cdot CH_2 \cdot CH_2O)_2$	19	4

[a] References: 1, Aldridge (1953b); 2, Burgen & Hobbiger (1951); 3, Lee (1964); 4, Pickering & Malone (1967).

phorus compounds and cholinesterase. In recent years the quantitative effects of substrate have been examined and formulated; the first work on this subject was by Main & Dauterman (1963), who solved the simple case of the inhibition of cholinesterase by diisopropyl phosphorofluoridate in the presence of phenyl acetate. The rate of phosphorylation, k', was related to the substrate concentration by the following expression.

$$k' = \frac{k_a[AB]}{1 + \frac{[S]}{K_m}} \qquad \text{Eq. 6}$$

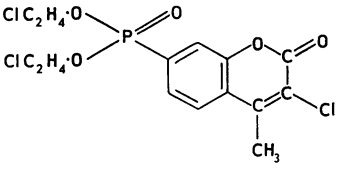
This expression only deals with a rather simple case and must be modified if a reasonable proportion of the enzyme is present either as a Michaelis complex or as another complex or if excess substrate causes inhibition of enzyme activity (Aldridge & Reiner, 1969, 1971).

It is clear that the analogies between the reaction of cholinesterase and organophosphorus compounds or substrate are so numerous that the inhibitory process may be formally regarded as identical with the substrate reactions. It is now accepted that the site of the phosphorus on one serine in the inhibited enzyme indicates that serine is involved in the catalytic mechanism (Oosterbaan, 1967).

The hydrolysis of substrates is influenced by pH and other experimental conditions and the reaction with organophosphorus compounds is likewise affected (Reiner & Aldridge, 1967). The well known inhibition of cholinesterase by high concentrations of acetylcholine (Myers, 1952; Wilson & Bergmann, 1950; Augustinsson, 1948; Shukuya, 1951; Zeller & Bisseger, 1943) has also been shown to have an inhibitor analogy. Haloxon (Fig. 1)

Fig. 1

Structural formula of haloxon (Aldridge & Reiner, 1969)



interacts with acetylcholinesterase in two ways, one to yield a phosphorylated enzyme and the other to give a reversible but catalytically inactive complex (Aldridge & Reiner, 1969). This complex seems to be produced by an interaction of haloxon with the same site to which acetylcholine is attached when it causes " inhibition by excess substrate ".

It may be concluded from all of these many studies that a theoretical framework now exists within which the rates of the various steps in the formation and breakdown of phosphorylated cholinesterase may be evaluated.

For many years it was accepted that carbamates caused reversible inhibition, as indicated by Eq. 1. Although as early as 1951 Goldstein explicitly stated from good experimental evidence that both physostigmine and neostigmine are substrates for cholinesterase and that the enzyme is inhibited owing to their slow rate of hydrolysis it was not until further work in the 1960s that this view was accepted (Wilson, Hatch & Ginsburg, 1960; Wilson, Harrison & Ginsburg, 1961). It is now clear that monomethyl and dimethylcarbamates react with cholinesterase to give carbamylated enzymes that are unstable; the basic reaction is formulated in Eq. 5. Free enzyme is regenerated from monomethyl and dimethylcarbamylated acetylcholinesterase with a half-life of 30 min and 56 min, respectively, at pH 8 and 25°C (Reiner & Aldridge, 1967). Other species of carbamylated acetylcholinesterase are much more

stable (Myers, 1956; Davies, Campbell & Kearns, 1970; Macfarlane, Jewess & Porter, unpublished data). Some values for k_{+2} and K_a are available; for example, Main (1967) showed that for physostigmine k_{+2} and K_a were 10.8 min^{-1} and 3.3 μM and the corresponding values for phenyl methylcarbamate were 7.1 min^{-1} and 26 mM. Values for other carbamates have been published but there are serious discrepancies between the results given in different papers, without any explanation of the reasons for them (O'Brien, Hilton & Gilmour, 1966; O'Brien, 1968). In a recent paper (Davies, Campbell & Kearns, 1970) the values attributed to K_a almost certainly should be assigned to K_i, a constant for a reversible complex similar to that described for haloxon (Aldridge & Reiner, 1969).

It is therefore certain that organophosphorus compounds, carbamates, and also organosulfur compounds (Kitz & Wilson, 1962; Ryan, Ginsburg & Kitz, 1969) react with cholinesterase to produce relatively stable acylated enzymes. The maximum rate of deacylation of cholinesterase in its reaction with acetylcholine must be as high as 295 000 and may be higher (Cohen, Oosterbaan & Warringa, 1955). The corresponding values for all phosphorylated and carbamylated cholinesterases so far examined are lower by a factor of at least 10^5–10^6 (Table 3).

Table 3

Catalytic centre activity for inhibitors or substrates of acetylcholinesterase

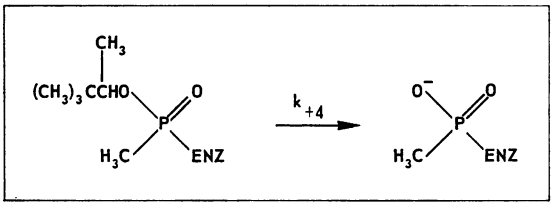
Enzyme derivative	Enzyme	Catalytic centre activity	Temperature (C°)	Reference[a]
$(C_2H_5O)_2P(O)$–	rabbit	~ 0.0005	37	1
$(CH_3O)_2P(O)$–	rabbit	0.0085	37	2
$(CH_2ClCH_2O)_2P(O)$–	bovine	0.0168	25	3
$CH_3NHC(O)$–	bovine	0.0234	25	3
$(CH_3)_2N \cdot C(O)$–	bovine	0.0123	25	3
$NH_2C(O)$–	electric eel	0.35	25	4
$CH_3C(O)$–	bovine	295 000	37	5
$CH_3C(O)$–	electric eel	610 000	25	6

[a] References: 1, Aldridge (1954); 2, Aldridge (1953b); 3, Reiner & Aldridge (1967); 4, Wilson, Harrison & Ginsburg (1961); 5, Cohen, Oosterbaan & Warringa (1955); 6, Kremzner & Wilson (1964).

In the reaction of cholinesterase with organophosphorus compounds a change may take place in the phosphorylated enzyme for which there is no analogy in the enzyme–substrate reactions. This has been called the " aging phenomenon " and was demonstrated when it was found that some phosphorylated cholinesterases could not be reactivated by nucleophilic reagents when they had been stored (Wilson, Ginsburg & Meislich, 1955; Hobbiger, 1955; Jandorf et al., 1955; Davies & Green, 1956). As a result of the work of Cohen and his colleagues in the Netherlands, this change is now known to be due to the loss of an alkyl group from an alkoxy group attached to the phosphorus (Jansz, Brons & Warringa, 1959; Berends et al., 1959). This is a general phenomenon and it occurs at different rates with many different phosphorylated cholinesterases (Coult, Marsh & Read, 1966; Pickering & Malone, 1967; Benschop & Keijer, 1966). For the species illustrated in Fig. 2 the change occurs rapidly,

Fig. 2

Dealkylation of a phosphorylated cholinesterase (k_{+4}=0.115 min^{-1}; half-life at pH 7.4 and 25° C=6 min; bovine erythrocyte enzyme)*



* Data of Coult, Marsh & Read (1966).

with a half-life of 6 min at 25°C. The reactions of organophosphorus compounds with cholinesterase must be extended and Eq. 5 re-written as in Eq. 7, where EA′ is a dealkylated form of the phosphorylated enzyme. Only those phosphorylated enzymes containing a P–O–C bond can react in this way.

The rates of all the steps of the reaction of organophosphorus compounds with esterases will be influenced by the enzyme concerned. This is very clear for such different enzymes as cholinesterase and chymotrypsin (Becker et al., 1963). However, the acetylcholinesterases, which presumably have the same biological function in different species of animal, do differ in their properties—e.g., their substrate patterns. This being so, they will also

$$EH + AB \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EHAB \xrightarrow{k_{+2}} BH + EA \xrightarrow{k_{+3}} EH + AOH \qquad \text{Eq. 7}$$

$$EA \xrightarrow{k_{+4}} EA'$$

react with acylating inhibitors at different rates and the acylated enzymes so obtained will have differing stabilities. The main purpose of this Conference is to evaluate those factors that may be exploited to provide compounds for vector control with maximum toxicity to pests allied with minimum toxicity to man. In general terms we can indicate the direction the constants for the steps of the reaction of inhibitors ought to take to produce maximum safety (Table 4).

Table 4

Relative magnitudes of constants for reaction with acetylcholinesterase required for maximum pesticide action and safety to man

affinity for cholinesterase	K_a (pest) $< K_a$ (man)
conversion of enzyme–inhibitor complex to acylated enzyme	k_{+2} (pest) $> k_{+2}$ (man)
stability of acylated enzyme	k_{+3} (pest) $< k_{+3}$ (man)
aging	k_{+4} (pest) $> k_{+4}$ (man)

Other factors are probably equally important. Destruction of the inhibitor *in vivo* either by hydrolysis or by oxidative processes can be considerable. These reactions probably have less effect on the initial acute inhibition because the rates of inhibition are usually much higher than the rates of destruction. This assertion may well be true for the injection of inhibitors, but in practice the administration of inhibitor is usually slow—for example, when exposure occurs through the skin, the lungs, or the mouth. In this case, and particularly if the compound has to be metabolized to an active form, the rate of breakdown of the inhibitor may be crucial. Recent work at Rothamsted (Lewis, 1969) indicates that a major difference between sensitive and resistant flies is their ability to destroy the inhibitor by a glutathione-dependent reaction. With an organophosphorus compound administered externally the rate of this destruction is obviously sufficient to prevent a lethal inhibition of the acetylcholinesterase in the resistant flies.

Enzymes other than cholinesterase that react with the inhibitor may substantially reduce the concentration of the latter. In contrast, an inhibitor that is specific for cholinesterase relative to other enzymes will not be wasted. Another factor that is very difficult to evaluate is the influence of the concentration of enzyme. For most *in vitro* studies the concentration of cholinesterase is almost always 10^{-10}M or lower, whereas *in vivo* local concentrations may be much higher; this will influence the rate of removal of circulating inhibitor.

In some countries mixtures of inhibitors are sprayed together—for example, a mixture containing a carbamate and an organophosphorus compound. This may have value in that different insects may be removed by one spray. From the human hazard point of view it is difficult to assess the consequences of repeated administration of such mixtures. It is well known that prior administration of physostigmine to animals will protect against a dose of diisopropyl phosphorofluoridate that would be lethal in untreated animals (Koelle, 1946). The complex interplay between the enzyme, a carbamate or an organophosphorus compound, and acetylcholine is difficult to evaluate. However, sufficient is now known to design sensible experiments; possibly the theoretical framework to describe these relationships might be defined.

Sufficient information is now available to examine rationally most of the problems associated with the inhibition of acetylcholinesterase by organophosphorus compounds, carbamates, and other acylating inhibitors. It is to be hoped that by such studies improved and safer pesticides may be developed. As a personal assessment of the factors that will prove more fruitful in achieving this aim, I consider a more intensive study of the stability of acylated cholinesterases and their capacity to age will prove useful.

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DISCUSSION

HELLENBRAND: Are the two sites in the acetylcholinesterase identical?

ALDRIDGE: Dr Reiner and I find it very difficult to envisage that the two sites (1 and 2) are the same or that they overlap. Different concentrations of substrate interfere with the two effects of haloxon—namely, progressive phosphorylation of the enzyme and the immediate reversible combination.

HEILBRONN: Could you enlarge on the relation between the substrate inhibition site on acetylcholinesterase (site 2), which you describe, and Dr Krupka's well known explanation of substrate inhibition?

ALDRIDGE: Dr Reiner has derived the equations if the Krupka explanation of a combination of acetylcholine with the acylated enzyme is correct. She considers that our experimental findings and views are not compatible with this mechanism.

HOLMSTEDT: What relationship, if any, do the two sites have to the well known bell-shaped activity–pS curve for acetylcholinesterase?

ALDRIDGE: The relationship is fairly straightforward. The reaction of inhibitor with the active site (site 1) to form a Michaelis complex is slowed down by concentrations of substrate defined by K_m. Combination with site 2 is competitive with those concentrations of acetylcholine defined by K_{ss}. Thus, the combination of haloxon with site 2 prevents its reaction with site 1 to give a phosphorylated enzyme; likewise, the combination of acetylcholine with site 2 prevents or greatly slows down its reaction with site 1, leading to its hydrolysis.