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THE MECHANISM OF INHIBITION OF ESTERASES BY ORGANO-PHOSPHORUS COMPOUNDS*

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A review is given of the present state of knowledge of the chemical nature of the reaction of esterases with organophosporus compounds. The phosphorylation process is known to be analogous to the normal hydrolysis of substrates. Reactivation by nucleophilic reagents and the nature of the conversion from reactivatable to the irreversible type of inhibition is discussed.

Organo-phosphorus compounds are powerful inhibitors of enzymes showing carboxylic esterase activity and their study has done much to demonstrate the importance of an »active centre« in enzymes. Work which I shall describe is now being carried out in several laboratories to try to define this »active centre« in chemical terms.

Inhibition of esterases by organo-phosphorus compounds

In Table 1 is a complete list of all the enzymes which are powerfully inhibited by organo-phosphorus compounds. All of these enzymes possess carboxylic esterase activity for the proteases will hydrolyse esters of amino acids (16, 17). However not all enzymes possessing carboxylic esterase activity are inhibited (12, 18, 19, 20). An explanation of these differences is not known at present but will be discussed later.

The work of Nachmansohn & Wilson & coworkers (21) has indicated that the hydrolysis of acetylcholine may be represented as shown in Fig. 1. In this process the initial reaction is the formation of a complex

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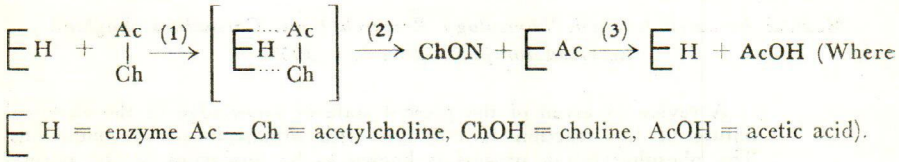
Table 1. Enzymes inhibited by organo-phosphorus compounds

Enzyme	*References*
Chymotrypsin	1, 2, 3, 4
Trypsin	5, 3, 6
Cholinesterase (true and pseudo)	7, 8, 9
Liver esterase	10
Milk lipase	10
Acetylesterase (Orange and Wheat)	11
B-esterase	12
Cholesterol esterase	13
Protcinasc (bacterial) . . .	14
Plasmin	15

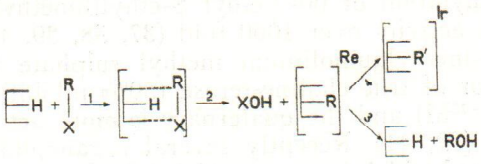


E H = enzyme Ac — Ch = acetylcholine, ChOH = choline, AcOH = acetic acid).

Fig. 1. Mechanism of hydrolysis of acetylcholine by cholinesterase

between enzyme and acetylcholine followed by the formation of the acetylated enzyme (reactions 1 and 2) and the liberation of choline. Reaction 3, the hydrolysis of the acetylated enzyme to give back cholinesterase and acetic acid is relatively fast. The inhibition of cholinesterase by organo-phosphorus compounds would fit into such a scheme if the phosphorylated enzyme were stable unlike the acetylated enzyme which is unstable. There is no doubt now that the phosphorus is especially tightly bound to enzymes. After inhibition of chymotrypsin, pseudocholinesterase and trypsin by di-*iso*propylphosphoro fluoridate the phosphorus cannot be removed by dialysis, or treatment with trichloroacetic acid (22) and inhibited chymotrypsin can be repeatedly recrystallised without losing any phosphorus (2). When chymotrypsin or trypsin are inhibited by tetraethylpyrophosphate or by diethyl *p*-nitrophenylphosphate one molecule of acid is liberated for each molecule of enzyme inhibited (23, 4, 6) and one molecule of phosphorus is bound to every molecule of enzyme (2, 3, 5, 24, 25). The effect of temperature on the rate of the inhibitory reaction indicates that the apparent energy of activation is 10–11 K.cal/mole for true cholinesterase and diethyl *p*-nitrophenylphosphate (26) and 14–15 K. cal/mole for pseudo cholinesterase and NN′N″N‴ tetra-*iso*-propylpyrophosphoro-tetramide (27). When true cholinesterase is inhibited by dimethyl *p*-nitrophenyl phosphate an

inhibited enzyme is produced which spontaneously reactivates following first order kinetics and having a half-life at 37° and pH 7.6 of approximately 1.5 hr. The energy of activation of this process was 14–15 K.cal/mole (26). The rate of return of enzyme activity is identical after inhibition by dimethyl-*p*-nitrophenyl phosphate, dimethyl phosphorofluoridate, tetramethyl pyrophosphate and 00 dimethyl-S-*p*-nitrophenyl phosphorothiolate (28). Treatment of rat pseudo cholinesterase with a similar series of diethyl phosphates produces an unstable enzyme and a similar examination has been made (28, 29). It is clear therefore from a great variety of evidence upon many different inhibitors and enzymes that the inhibitory process appears to be a phosphorylation of the enzyme, the phosphorus being linked by a covalent bond to the enzyme. The fact that the inhibition of enzyme is easily prevented by the presence of substrate indicates that the inhibitor combines at least initially at the active centre (30, 31, 32, 33).



(Where E H = enzyme, R — X = organophosphorus inhibitor, with X the labile group, Re = reactivatible, Ir = irreversible.)

Fig. 2. Mechanism of inhibition of cholinesterase by organo-phosphorus compounds.

A consequence of this view is that the organo-phosphorus compounds are in fact regarded as substrates by the enzymes and that when the process reaches a certain stage the normal enzymic process is stopped. It should therefore be possible to show that there are structural similarities between organo-phosphorus inhibitors and normal substrates. Much of this work has been carried out with cholinesterase because the relationship between enzymic activity and structure of substrates is much simpler than with the proteolytic enzymes (34). A consideration of the process shown in Figure 1 indicates that the final rate of hydrolysis may be governed by the rates of any of the three reactions illustrated – the affinity of the substrate for the enzyme and the formation and hydrolysis of the acylated enzyme. With organo-phosphorus compounds the final stage, reaction 3, the hydrolysis of the phosphorylated enzyme is in most cases very slow so that only the two reactions 1 and 2 are operative.

It must not be forgotten that the very high potency of organophosphorus compounds is dependent upon the fact that the one molecule of inhibitor reacts with one active centre.

The scheme illustrated provides for two sites in the enzyme active centre – an anionic site to bind the quaternary nitrogen atom and an esteratic site to which the carbonyl oxygen is attached. On these grounds

it would be expected that variation of the alkoxy groups attached to the phosphorus would influence the inhibitory power. In fact changes in the alkoxy groups of organo-phosporus compounds from 00 dimethyl to 00 di-*iso* propyl alter their inhibitory power in different directions against true and pseudo cholinesterase (9). Similarly lengthening the acyl groups of choline esters changes their rates of hydrolysis by true and pseudo cholinesterase and there is a good parallelism between substrate and inhibitor specificities of these enzymes (9). It would seem therefore that the esteratic site is important in determining the reaction of organo-phosphorus compounds with cholinesterase. Much success has been obtained in the preparation of active inhibitors containing positively charged groups which simulate the quaternary nitrogen of acetylcholine. The phosphostigmines, m-(dialkoxyphosphinyloxy)-NNN trimethylanilinium methyl sulphates are highly active inhibitors of cholinesterase (35, 36) and the quaternary compounds are much more active than the tertiary compounds. Methylation of 00-diethyl S-ethylthioethyl phosphorothiolate increases its activity over 1000 fold (37, 38, 39, 40). 3-(Diethoxyphosphinyloxy-1-methylquinolinium methyl sulphate is an extremely powerful inhibitor of true cholinesterase (50% inhibition at 37° for 20 min. by 1.5×10^{-10}M) and the quaternary is more active than the tertiary compound (32, 41). Recently several organophosphoryl cholines have been prepared which have very high inhibitory power against true cholinesterase and are very toxic (42, 43, 47). There is no doubt therefore that positively charged groups in suitable positions to organo-phosphorus compounds produce compounds with extremely high inhibitory power and toxicity.

One point I think is relevant here is whether phosphorus is essential to produce an inhibition similar to that described above. A variety of organic acid fluorides inhibit cholinesterase e. g. NN dimethylcarbamyl fluoride, chloromethane-sulphonyl fluoride and toluene-p-sulphonyl-fluoride (44). Earlier kinetic studies on the inhibition of cholinesterase by 2-(NN-dimethyl carbamoyloxy)-5-phenyl trimethylammonium bromide were interpreted as indicating that the inhibition passed through a stage analagous to the phosphorylated enzyme i. e. the dimethylcarbamoyl enzyme which is readily hydrolysed to the original enzyme (45). NN-Dimethylcarbamoyl fluoride produces a similarly unstable inhibited enzyme whereas NN-diethylcarbamyl fluoride produces a stable inhibited enzyme and behaves similarly to organo-phosphorus inhibitors (46). However later work by the same authors has thrown some doubt on this conclusion with other carbamyl esters (48, 49).

Reactivation of phosphorylated esterase

For many years the inhibition of esterases by organo-phosphorus compounds was considered irreversible. Originally a reversible complex between cholinesterase and di-*iso* propylphosphorofluoridate was claimed

to have been demonstrated (50), but this has now been disproved and shown to be an artefact of the technique used (30, 51). The first intimation that the inhibited enzyme could be reactivated was when Wilson demonstrated that the inhibition of electric eel cholinesterase produced by tetraethylpyrophosphate was slowly reactivated upon storage (52). Later it was shown that dimethylphosphorylated true cholinesterase and diethylphosphorylated pseudo cholinesterase were much more unstable (26, 29). If hydroxylamine is added to cholinesterase and acetylcholine, acethydroxamic acid is formed (54) the hydroxylamine acting as an acceptor for the acetyl groups from the acetylated enzyme. In a similar way the spontaneous reactivation of the diethyl phorphorylated enzyme is greatly increased by the addition of hydroxylamine and by choline (52). These observations, the demonstration that hydroxylamine also reacts with the parent inhibitors (54) and the appreciation that the parent inhibitor and inhibited enzyme are chemically similar has greatly aided the search for new compounds. Much work has been carried out on the reactivity of hydroxamic acids and oximes with organo-phosphorus inhibitors (55, 56, 57, 58) and in general it has been possible to make predictions of possible reactivating compounds. However, the phosphorylated unlike the parent inhibitor possesses an anionic site. This will be free while the esteratic site is rendered inactive by the attached dialkylphosphoryl group. Just as a positively charged atom in the right position in an inhibitor molecule has been shown to produce more powerful inhibitors also a nucleophilic reagent such as a hydroxamic acid or an oxime with a positively charged group suitably situated gives a much more powerful reactivator. Trimethylamine will slow down the reactivation by nicotinhydroxamic acid methiodide and pyridine-2-aldoxine methiodide (59, 60) tetramethyl or tetrathyl ammonium ions prevent reactivation by hydroxylamine (61) and acetylcholine slows down reactivation by pyridine-2-aldoxime methiodide (62). All of these observations are entirely consistent with attachment of the reactivator at the anionic site of the cholinesterase (63, 64, 65). This does not mean that all reactivators must possess a positively charged group, for a large number which are active possess no positive charge (66). In a similar way uncharged esters such as triacetin are hydrolysed by cholinesterase (67), and the uncharged compound such as di-iso-propyl phosporofluoridate is highly inhibitory to cholinesterase.

At the moment the best treatment of poisoning by organo-phosphorus compounds is pyridine-2-aldoxime methiodide with atropine (68). Other ranges of compounds have recently been synthesised and although some are more effective *in vitro* than pyridine-2-aldoxine their utility for the treatment of organo-phosphorus poisoning has not been adequately tested (69, 70).

It is clear that the inhibitory and reactivation processes after organophosphorus compounds are all consistent with the enzyme treating the inhibitor as a substrate. However, with the advent of reactivators one

way has been found in which hydrolysis of acetylcholine and the inhibition and reactivation of the inhibited cholinesterase differ. Being a catalyst the original enzyme activity is always obtained after the enzyme has been through the cycle of acetylation and de-acetylation. However the original enzyme activity is not always obtained when inhibited cholinesterase is treated with reactivators. The degree of reactivation obtained with di-*iso*-propylphosphorylated cholinesterase is dependent upon the time and temperature of incubation of the inhibited enzyme before treatment with the reactivator. (71, 72). This change in inhibited true cholinesterase occurs after 00-dimethyl, 00-di-*iso*-propyl, 0-*iso*-propylmethyl but is much slower after 00-diethyl. The difference between the two forms of cholinesterase is at the moment absolute – one form may be reactivated whereas the other cannot. As soon as this observation was made the change from the reactivatible to to the irreversible stage was equated with a movement of the phosphorus atom from histidine to serine but it was difficult to see why this process did not occur with inhibited chymotrypsin. It has now been clearly demonstrated that chymotrypsin inhibited by 0-*iso*-propyl methylphosphoro fluoridate does not alter upon incubation and is always reactivatible with oximes and hydroxamic acids (87). One interesting possibility is that the change in inhibited cholinesterase involves a loss of one of the alkoxy groups so that a mono-alkyl phosphorylated enzyme results. It has now been shown that after ageing mono-*iso*-propyl phosphate may be isolated from pseudo cholinesterase inhibited by di-*iso*-propyl phosphorofluoridate (72). When the rate of conversion of reactivatible to irreversibly inhibited enzyme was determined using pyridine 2-aldoxime methiodide as reactivator, the amount of mono-*iso*-propyl phosphate on the enzyme equalled the amount of irreversibly inhibited enzyme.

So far the reactivation of inhibited cholinesterase has been satisfactorily demonstrated only after treatment with dialkoxy phosphoryl or alkoxyalkyl phosphoryl inhibitors. Reactivators cannot be obtained after octamethylpyrophosphoramide (Schradan), ethyl-NN-dimethyl phosphoroamido cyanidate (Tabun), or the phosphorylcholines (73, 74, 75). On chemical grounds this is expected but there is of course the possibility of a rapid change from the reactivatible to the irreversible form of the inhibited enzyme.

Degradation of the phosphorylated esterase

Since the inhibition of esterases by organo-phosphorus compounds may be explained in terms of the normal mechanism whereby the normal substrates of the enzymes are catalysed, inhibitors containing radioactive phosphorus may be used to locate the site of the »active centre« in the enzyme molecule. The concept of an »active centre« in enzymes is dependent of course upon indirect evidence but the phosphorylated esterases have allowed the possibility of determining the chemical structure

around the phosphorus atom and therefore the active centre. You will all be aware of the classical work of Sanger (76) upon the chemical structure of insulin. The magnitude of the problem of attacking inhibited chymotrypsin by similar methods will be appreciated; the molecular weight of insulin is approximately 6,000 and chymotrypsin around 40,000. In many laboratories at the present time much work is being carried out using chemical and enzymic methods of degradation. When inhibited esterases are degraded the phosphorus is always found attached to serine. This has now been shown with chymotrypsin, true and pseudo cholinesterase, liver ali-esterase, trypsin and red cell ali-esterase. In view of the lack of reactivity of serine and the stability of dialkylphosphoryl serine it has been generally believed that serine is not the primary site of attachment of the phosphorus. On several grounds histidine has been in favour as the primary site of attachment (25). However results are accumulating showing that around the phosphorus atom in a variety of inhibited esterases there is a common amino acid sequence of glycine-aspartic-serine-glycine (77). Although serine itself does not react with organo-phosphorus inhibitors it becomes reactive after it has been condensed to an oxazoline ring structure (78) and a mechanism has been proposed whereby such a ring structure formed from aspartylserine could account for inhibition by organo-phosphorus compounds and the normal mechanism of hydrolysis by esterases. Of great interest is the finding that when phosphoglucomutase is treated with glucose-6-phosphate containing radioactive phosphorus a phosphorylated enzyme is formed and upon degradation, this labelled enzyme also yields a phosphopeptide containing aspartic acid, serine, glycine and glutamic acid (79). The importance of serine in phosphorylated proteins both natural and artificial (80) is rapidly becoming apparent.

Esterases not inhibited by organo-phosphorus compounds

Several organo-phosphorus compounds are enzymically hydrolysed amongst which are diethyl-*p*-nitrophenylphosphate (81), di-*iso*-propyl phosphorofluoridate, (82) and tetraethyl pyrophosphate (83). In one instance, A-esterase of rabbit plasma the enzyme which hydrolyses diethyl -*p*-nitrophenyl phosphate also hydrolyses *p*-nitrophenyl acetate (81, 18). This enzyme, like the esterases inhibited by organophosphorus compounds cannot distinguish between carboxyl and phosphate esters. This however has only been demonstrated in one case. It was claimed that the enzyme hydrolysing di-*iso*-propylphosphorofluoridate also hydrolyses acet-amino acids (84) but this has now been shown to be untrue (85). There are three instances now of esterases which are not inhibited by organo-phosphorus inhibitors and which also cause no detectable hydrolysis of the inhibitor (20, 85, 86).

Conclusion

The mechanism of inhibition of esterases by organo-phosphorus appears to be identical with the normal hydrolytic processes of these enzymes. In one instance change of inhibited cholinesterase from a reactivatible to an irreversible stage has been shown to be due to the loss of one group to produce a mono-alkylphosphorylated enzyme which is stable and unaffected by any of the reactivators so far studied. The reactivators are not generally applicable to all inhibited cholinesterases and depend on the particular groups attached to the phosphorus atom. It is however reassuring that these compounds have been a direct result of the academic work carried out upon the mechanism of the hydrolysis of substrates and of the inhibition by organophosphorus compounds of the cholinesterases. The studies on the structure of chymotrypsin and trypsin and the relationship between the »active centre« and the rest of the protein structure will lead to a much clearer insight into the detailed chemical basis of catalysis by esterases.

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Sadržaj

MEHANIZAM INHIBICIJE ESTERAZA ORGANOFOSFORNIM SPOJEVIMA

Prikazano je današnje poznavanje kemijske prirode reakcije esteraza s organofosfornim spojevima. Poznato je, da je proces fosforiliranja analogan normalnoj hidrolizi supstrata. Reaktivacija nukleofilnim reagensima i priroda konverzije inhibiranog enzima iz stanja, u kojem se još može reaktivirati u ireverzibilni oblik, također su raspravljane u ovom prikazu.

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