Metabolism of Organophosphorus Insecticides in Relation to Their Antiesterase Activity, Stability, and Residual Properties

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Organophosphates are biologically active, because they inhibit enzyme action through formation of inactive phosphoryl esterases. Stable phosphate precursors are in many cases converted within the organism to reactive antiesterases. Certain but not all dimethylphosphoramides and phosphorothioates require in vivo oxidation to effect their antiesterase action. Other biochemical reactions either increase or decrease the organophosphate antiesterase activity. The stability of the phosphoryl esterase is very important; if this complex is unstable, the enzyme may serve in detoxification, whereas if a phosphoryl cholinesterase is very stable in vivo, a definite chronic toxicity problem may result. A good organophosphate insecticide requires a suitable balance of group specificity, antiesterase activity, and stability—the balance varying with each new economic use for an antiesterase agent.

THE TOXICITY OF CERTAIN NEUTRAL PHOSPHATE ESTERS and their efficiency as inhibitors of physiologically important esterase enzymes are well recognized. Only a few dozen organophosphorus compounds out of the thousands which have been synthesized and tested for biological activity are represented among current-day insecticides, nematocides, nerve gases, and chemotherapeutic agents. Excellent reviews are available on their chemistry (*19, 109, 157, 219*), biochemistry (*9, 17, 117, 149, 150, 221*), pharmacological action to mammals (*34–36, 71, 81, 120, 153, 154, 205*), antiesterase activity (*8, 16, 33*), and development as contact and systemic insecticides (*103, 109, 131, 177, 206, 218, 219, 223*).

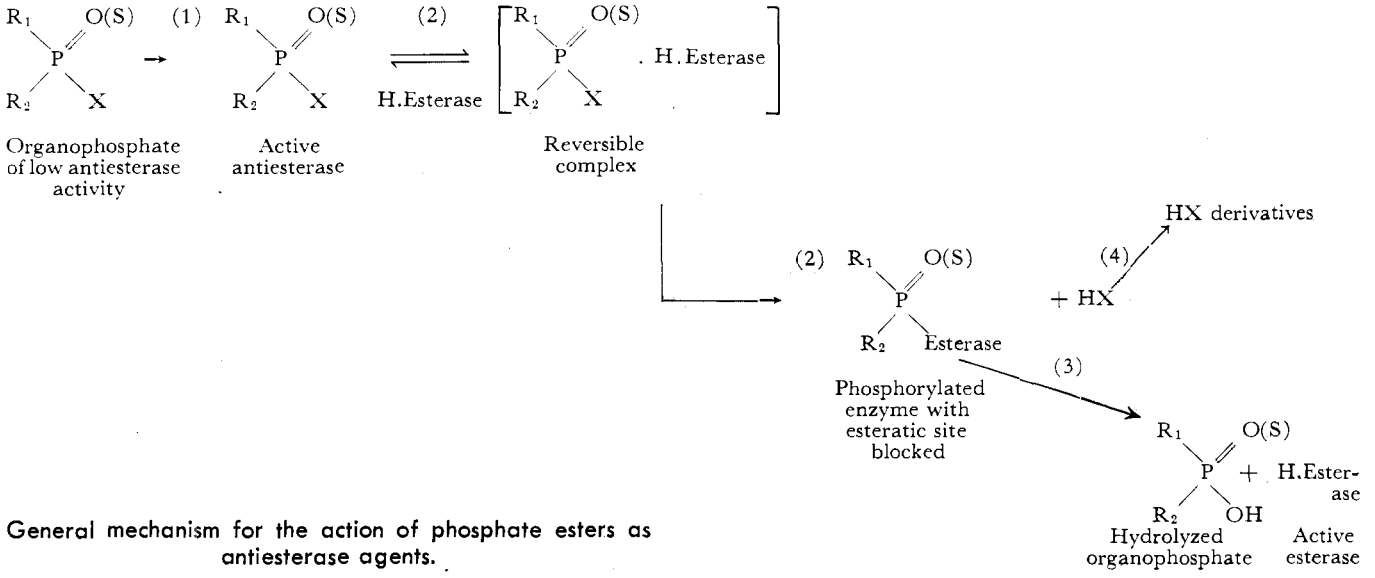
Each new use for an organophosphorus antiesterase agent demands a different balance of reactivity and spatial arrangement of the groupings. Our current knowledge on the exacting nature of these specifications has been derived primarily from large-scale industrial screening programs. When applied to an insect, plant, or mammal, the organophosphate must have sufficient stability and suitable physicochemical properties to be absorbed and translocated to the ultimate site of action. Once in proximity to the physiologically important esterase, the steric relationships and chemical reactivity of the groupings must allow the inhibitor to approach, combine with, and inhibit the enzyme. With the rapid accumulation of knowledge, the time is approaching when "tailor-made" anticholinesterase agents may be available to deal with many of the major insect control problems (*9, 146, 156*).

Relation of Structure to Stability and Residual Properties

Reports of Schrader (*218, 219*) pertaining to the development of organophosphate insecticides have frequently emphasized the need for suitable stability properties. His basic structure for insecticidal activity was $R_1R_2P(O)X$, where R_1 and R_2 are alkyl, alkoxy, or alkylamino groups, and where X is an acid residue making P—X an acid anhydride bond. This anhydride bond was increased in stability as the R groups were changed from alkyl to alkoxy, and finally to alkylamino. Increasing the alkyl or alkoxy chain lengths further increased the hydrolytic stability, as did the use of dialkylamides instead of monoalkylamides. The very stable organophosphate insecticide, schradan (octamethylpyrophosphoramide or tetramethylphosphorodiamidic anhydride) was developed through successive substitutions of dialkylamino groups for the alkoxy group in TEPP (tetraethyl pyrophosphate or diethyl phosphoric anhydride).

Another stabilization approach utilized by Schrader was the substitution of sulfur for oxygen to yield such phosphorothioates as *O,O*-diethyl phosphorothioic anhydride and parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate). Further development of phosphorothioates and phosphorodithioates has led to low volatility insecticides which are more persistent on the surface of plant tissues then certain chlorinated hydrocarbon insecticides currently in use (*132, 210*), or to systemic materials persisting within the plant as effective antiesterase toxicants for over 6 weeks (*48, 133*). Excellent insecticidal activity and desirable stability properties have also resulted where the acid anhydride bond consists of such groupings as chloro- or nitrophenyl phosphates, substituted vinyl phosphates, 2-chloro-1-hydroxyethyl phosphonates and alkylmercaptoalkyl phosphates, phosphorothiolates, -thioates, and -dithioates.

A recent approach for increasing stability is the use of salts of basic organophosphates (*102*), which would presumably effect a toxic action only as a free base. This may be somewhat analogous to the stabilization of nicotine as a salt. Complexing of phosphorothioates with metallic ions has also shown some promise for stabilization (*89*). Attention is not being entirely placed on long residual type phosphorus insecticides. For use on food crops near harvest time it is advantageous to have an insecticide with little persistence to alleviate the danger of toxic residues.



General mechanism for the action of phosphate esters as antiesterase agents.

The best materials for this purpose would appear to be TEPP, because of its hydrolytic instability, and certain substituted vinyl phosphates (*55, 66, 67*), because of their volatility and biological instability.

The search for insecticides with persistent residual properties has created an apparent paradox. The chemical must be very stable to hydrolysis in order to persist when exposed to weathering on the plant surface or to the varying hydrogen ion concentration of the plant sap. Yet the organophosphate must be a reactive phosphorylating agent to block the cholinesterase in the poisoning process. The insecticide must be stable, yet unstable. Meeting these requirements necessitates the production of a stable insecticide which will undergo a metabolic conversion within the organism to form a more reactive phosphorylating agent.

The following discussion is an attempt to correlate certain of the available data on the metabolism of organophosphate insecticides in relation to antiesterase activity, stability, and residual properties. In order to orient this review, the various reactions involved in the antiesterase activity of organophosphates are considered separately. The general mechanism for the action of phosphate esters as antiesterase agents is as follows (*8, 114*) and the specific case with acetylcholinesterase is illustrated in Figure 1.

Reaction 1. Metabolic Activation of Organophosphorus Esters

The toxicity of organophosphorus esters has been recognized since 1932 (*159*) and their myotic and anticholinesterase properties since 1939 (*106*). Until 1950 it was generally as-

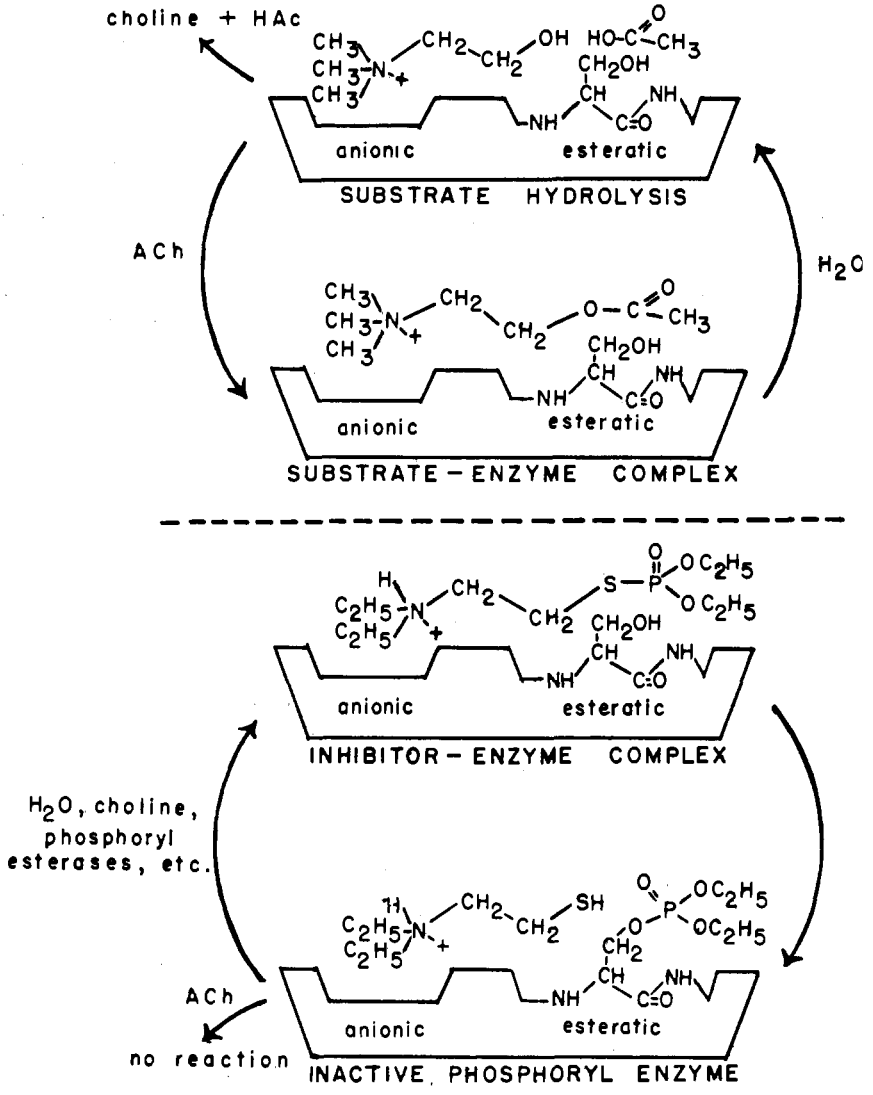


Figure 1. Hydrolysis of acetylcholine and organophosphates by cholinesterase (based on *8, 102,* and *150*)

sumed that the applied organophosphate *per se* was the active antiesterase involved in the poisoning process. In 1950 two research groups (*83*, *98*) and in the following year several other research groups, reported observations that were inconsistent with this assumption (*30*, *58*, *79*, *85*, *110*, *117*, *129*, *138*). The intensive studies of the past half decade on the metabolic conversions of organophosphorus esters to form more or less active antiesterases in vivo have served to establish certain general relationships.

Experimental Approaches. Several experimental approaches have proved useful in investigating the metabolism of organophosphates. To establish the conversion of a phosphate by an organism to a more active antiesterase, several of the approaches should be used concurrently, as each in itself does not constitute proof of the biological significance of any given proposed mechanism.

The first approach is an attempt to correlate the in vitro anticholinesterase activity with the in vivo toxicity of the phosphorus compound. With several small series of organophosphorus compounds it has been found that increased anticholinesterase activity is generally associated with greater toxicity to certain insects (*179*) and mammals (*129*, *147*). Correlations based on the limited data available have been questioned on statistical grounds (*59*). Where neutral organophosphate esters are toxic in vivo and yet inefficient as in vitro anticholinesterase agents, their metabolic conversion to more active antiesterases is suspected (*129*, *149*, *150*). Figure 2 illustrates the correlation and also brings out certain of the discrepancies involved. Toxicity in the figure is expressed as the logarithm of the number of micromoles of phosphate ester per kilogram producing 50% mortality (based on the data for intraperitoneal or subcutaneous administration to rats or mice). Anticholinesterase activity is expressed as pI_{50} (negative logarithm of the molar concentration of organophosphate required to effect 50% inhibition of the acetylcholinesterase of a mammalian serum or plasma). Where adequate data are available, a general relationship between in vivo and in vitro effects is found with the tetralkyl, and dialkyl, dialkylamidopyrophosphates, the alkyl phosphonates, and the dialkyl substituted vinyl and dialkyl substituted phenyl phosphates. The phosphoramides and phosphorothioates usually display an in vitro anticholinesterase activity far too low to account for their in vivo toxicity. Where all the enzymatic and mortality determinations are made by the same investigator under identical conditions, this approach yields useful information (*129*), but where the work of many laboratories is compared (as has been done in Figure 2), the variables are too great to yield a good correlation.

The relationship of in vivo toxicity and in vitro antiesterase activity appears to be affected by the species and sex of the animal tested (*91*, *120*, *150*, *180*, *209*), the route of administration (*9*), impurities present in the organophosphate (*9*, *78*, *80*, *181*), the varying susceptibility of organophosphates to hydrolysis by plasma and sera esterases (*4*, *5*, *18*, *24*), the different rates of combination of organophosphates with cholinesterase in relation to their concurrent breakdown by the blood esterases (*5*, *23*, *25*), and the specificity of the cholinesterases involved (*8*, *45*, *46*, *73*, *180*). Furthermore, certain types of organophosphates may poison by other than anticholinesterase effects (*153*, *221*), or have secondary effects that may be as important in the poisoning process as the anticholinesterase activity (*226*).

A second approach to the problem is a comparison between the degree of tissue cholinesterase inhibition occurring in vivo and that resulting from the addition of the same amount of organophosphate to the tissue in vitro. Experiments of this type have proved satisfactory with the blood of vertebrates (*7*, *9*, *99*, *197*) and insects (*58*). The method is not applicable where the inhibitor readily dissociates from the esterase; the degree of in vivo inhibition cannot be determined under such conditions (*156*). The organophosphate must be free of traces of active antiesterase impurities, the purification often necessitating selective hydrolysis (*11*) and column or paper chromatography. A modification of this method for studying in vivo activation is to determine the antiesterase specificity difference between the applied chemical and its active metabolite (*196*), a situation which will frequently occur because of the great selectivity of the esterases for both substrates and inhibitors (*8*, *23*, *72*).

Another method, probably the most frequently used for determining the mechanism of metabolic activation, is the incubation of a whole organ, tissue slices, or homogenates with the organophosphate and comparing the antiesterase activity of the organophosphate before and after incubation (*52*, *83*, *155*, *178*, *200*, *201*).

A fourth approach for metabolism studies is through studying the change in solubility properties of the organophosphate within the biological system through partitioning of the derivatives recovered from the organism between an organic solvent and water (*56*, *115*, *122*, *123*, *228*), or by chromatographic separation and subsequent assay of the metabolites formed within the tissue (*63*, *95*, *96*, *170*, *171*, *182–184*). Somewhat comparable techniques have been used to demonstrate in vivo activation of systemic insecticides within plants where a decomposition curve is established for the organophosphate by parallel specific chemical or radiochemical, antiesterase, and insect bioassay analysis. Where the three assay methods give the same results, the material merely degrades to less active antiesterases (*47*, *55*), but where the antiesterase or bioassay shows more toxicant than is evident from the chemical analysis, a metabolic activation has occurred (*53*, *184*). The use of radioactively tagged materials has greatly facilitated this type of study.

Oxidation of Dialkylphosphor-

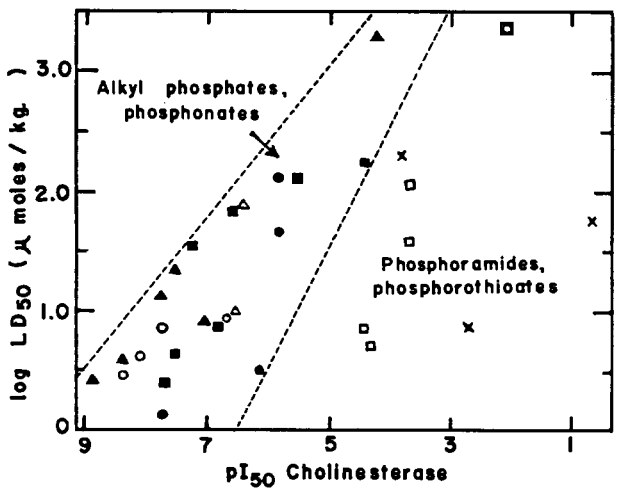


Figure 2. Relation of in vitro anticholinesterase activity and mammalian toxicity in mice and rats

○ Tetraalkyl pyrophosphates (*81*)
△ Dialkyl diamido pyrophosphates (*81*, *129*)
■ Dialkyl phenyl phosphates or phosphorothiolates (*9*, *124*)
● Dialkyl vinyl phosphates (*47*, *48*)
▲ Dialkyl or monoalkyl, monoamide phosphonates (*13*, *129*)
□ Dialkyl phosphorothioates (*9*, *48*, *84*, *124*)
X *N*-substituted phosphoramides (*48*, *129*)

Table I. Reactions Involved in Metabolism of Organophosphate Triesters

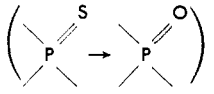
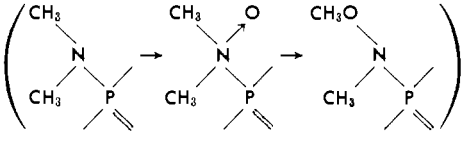
Compound	Biological System	Method[a]	References
Phosphorothioate oxidation 			
$(C_2H_5O)_2P(S)Cl$	Roach nerve	2	(*58*)
$(CH_3O)_2P(S)O\phi—NO_2\text{-}p$	Rabbit	2	(*9*)
	l.s.[b] or homog.	1	(*74, 75, 178*)
	Roach tissues	1, 5	(*180, 178*)
$(C_2H_5O)_2P(S)O\phi—NO_2\text{-}p$	Rabbit	2	(*9, 196*)
	l.s. or homog.	1, 5	(*74, 75, 79, 96*)
	Insects	1	(*164, 224*)
	Insect tissues	1, 6	(*30, 58, 155, 178*)
$(\text{iso-}C_3H_7O)_2P(S)O\phi—NO_2\text{-}p$	Rabbit	2	(*9*)
$(CH_3O)P(S)(O\phi—NO_2\text{-}p)_2$	Rabbit	2	(*196*)
$(C_2H_5O)P(S)(\phi)(O\phi—NO_2\text{-}p)_2$	Roach gut	1	(*178*)
$(C_2H_5O)_2P(S)O\phi—NH_2.HCl\text{-}p$	Roach nerve	2	(*58*)
$(C_2H_5O)_2P(S)$-4-methyl-7-hydroxycoumaryl	Rabbit	2	(*9*)
$(C_2H_5O)_2P(S)OC_2H_4OC_2H_5$	l.s., roach intestine	1	(*48*)
$(C_2H_5O)_2P(S)OC_2H_4SC_2H_5$	Mouse and roach tissues	1, 5	(*171*)
$(C_2H_5O)_2P(S)SCH_2SC_2H_5$	Plants	2, 5	(*41*)
	l.s., roach intestine	1	(*41*)
$(C_2H_5O)_2P(S)SCH_2SC_3H_7\text{-iso}$	l.s.	1, 5	(*48*)
$(C_2H_5O)_2P(S)SCH_2C(O)NH_2$	Roach intestine	1	(*178*)
$(CH_3O)_2P(S)SCH(CH_2C(O)OC_2H_5)C(O)OC_2H_5$	l.s., roach intestine	1	(*48, 178*)
Dimethylphosphoramide oxidation 			
$[(CH_3)_2N]_3P(O)$	Plants	4	(*122*)
$[(CH_3)_2N]_2P(O)F$	Rabbit	2	(*9*)
	l.s.	1	(*52*)
$[(CH_3)_2N]_2P(O)OP(O)[N(CH_3)_2]_2$	Various insect tissues and homog.	1	(*38, 85, 200, 201*)
	Various insects and tissues	2, 5	(*52, 54, 85*)
	Plants	2, 5	(*53, 54, 83, 108, 110, 118*)
	Various mammals	2	(*9, 83, 98, 99, 149*)
	l.s. and homog.	1, 3	(*51, 52, 56, 74, 75, 83, 87, 98, 99, 170*)
$[(CH_3)_2N]_2P(O)O—\phi—NO_2\text{-}p$	l.s.	1	(*52*)
$[(CH_3)_2N]_2P(O)OP(O)(OC_2H_5)[N(CH_3)_2]$	l.s.	1	(*52*)
Thioether oxidation (—P—OR_1SR_2 or —P—SR_1SR_2 → sulfoxide → sulfone)			
$(C_2H_5O)_2P(O)SC_2H_4SC_2H_5$	Mouse tissues	1, 5	(*171*)
	Roach tissues	1, 5	(*171*)
	Plants	1, 2, 5	(*95, 184, 225*)
$(C_2H_5O)_2P(S)OC_2H_4SC_2H_5$	Mouse tissues	1, 5	(*171*)
	Roach tissues	1, 5	(*171*)
	Plants	1, 2, 5	(*95, 183, 184*)
$(C_2H_5O)_2P(S)SCH_2SC_2H_5$	l.s.	1, 5	(*41*)
	Roach intestine	1, 5	(*41*)
	Plants	1, 2, 5	(*41*)
Triphenyl phosphate octivation (possible introduction of —OH and conjugate formation)			
$(\phi—O)_3P(O)$	Rat	2	(*197*)
$(o\text{-}CH_3—\phi—O)_3P(O)$	Rabbit, chicken, rat	2	(*7, 175, 197*)
	l.s. and homog.	1, 4	(*7, 74, 197*)
Carboxylic ester hydrolysis (—P—$OR_1C(O)OR_2$ → —P—$OR_1C(O)OH$ + R_2OH)			
$(CH_3O)_2P(O)OC(CH_3)=CHC(O)OCH_3$ *cis* and *trans*	Plants	2, 5	(*55*)
$(CH_3O)_2P(S)SCH(CH_3C(O)OC_2H_5)C(O)OC_2H_5$	Chicken in vivo	5	(*169*)
	Roach intestine in vitro	5	(*169*)
$(CH_3O)_2P(O)CH[OC(O)CH_3]CCl_3$	Insect tissues	3	(*13*)
Phosphoryl phosphatases ($R_1R_2P(O)X$ → $R_1R_2P(O)OH$ + HX)			
$(CH_3O)_2P(O)F$	Plasma and liver esterases	3, 5	(*174*)
$(CH_3O)(C_2H_5O)P(O)F$	Plasma and liver esterases	3, 5	(*174*)
$(C_2H_5O)_2P(O)F$	Plasma and tissue esterase	3, 5	(*174, 190*)
$(C_2H_5O)[(CH_3)_2N]P(O)F$	Plasma esterases	3	(*24*)
$(\text{iso-}C_3H_7O)_2P(O)F$	Plasma, tissue esterases	3, 5	(*21, 23, 24, 25, 174, 187, 189, 190, 191, 192*)
	Mammals in vivo	5, 6	(*61, 174, 213*)
	Microorganisms	3, 5	(*188*)

Table I. Reactions Involved in Metabolism of Organophosphate Triesters (*cont'd*)

Compound	Biological System	Method[a]	References
Phosphoryl phosphatases ($R_1R_2P(O)X \rightarrow R_1R_2P(O)OH + HX$)			
$(n\text{-}C_4H_9O)_2P(O)F$	Kidney esterases	3, 5	(*190*)
$[(CH_3)_2N]_2P(O)F$	Mammals in vivo	5	(*202*)
$[(CH_3)_2N](C_2H_5O)P(O)Cl$	Plasma esterases	3	(*24*)
$[(CH_3)_2N](CH_3O)P(O)CN$	Plasma esterases	3	(*18, 24*)
$[(CH_3)_2N](C_2H_5O)P(O)CN$	Plasma and tissue esterases	3, 5	(*18, 21–26*)
$[(CH_3)_2N](i\text{-}C_3H_7O)P(O)CN$	Plasma esterases	3	(*24*)
$(CH_3O)_2P(O)C(OH)CCl_3$	Plasma esterases	4, 5	(*13*)
	Plants, insects, mammals in vivo	2, 4, 5	(*13, 82*)
$(CH_3O)_2P(O)C[OC(O)CH_3]CCl_3$	Plasma esterases	4	(*13*)
	Plants, insects, mammals in vivo	4	(*13*)
$(CH_3O)_2P(O)OCH{=}CCl_2$	Plasma esterases	4	(*13*)
	Plants, insects, mammals in vivo	4	(*13*)
$(CH_3O)_2P(O)OC(CH_3){=}CHC(O)OCH_3$	Plasma esterases	4	(*47, 55*)
cis and *trans*	Plants, insects, mammals in vivo	2, 4, 5	(*47, 48, 55*)
$(C_2H_5O)_2P(O)O\phi\text{—}NO_2\text{-}p$	Plasma esterases	3, 5	(*5, 24, 193*)
$(C_2H_5O)_2P(S)O\phi\text{—}NO_2\text{-}p$	Mammals in vivo	5	(*97, 101, 145, 198, 203*)
$(C_2H_5O)_2P(O)SC_2H_4SC_2H_5$	Plants in vivo	4, 5	(*183, 184*)
$(C_2H_5O)_2P(S)OC_2H_4SC_2H_5$	Plants in vivo	4, 5	(*183, 184*)
$(C_2H_5O)_2P(S)SCH_2SC_2H_5$	Plants in vivo	4, 5	(*41*)
$[(CH_3)_2N]_2P(O)OP(O)[N(CH_3)_2]_2$	Plants in vivo	4	(*122*)
$[(CH_3)_2N]_2P(O)OP(O)[N(CH_3)_2]OP(O)[N(CH_3)_2]_2$	Plants in vivo	4	(*122*)
$(C_2H_5O)[(CH_3)_2N]P(O)OP(O)[N(CH_3)_2](OC_2H_5)$	l.s.	1	(*52*)
$(C_2H_5O)_2P(O)OP(O)[N(CH_3)_2]_2$	l.s.	1	(*52*)
$(C_2H_5O)_2P(O)OP(O)(OC_2H_5)[N(CH_3)_2]$	l.s.	1	(*52*)
$(C_2H_5O)_2P(O)OP(O)(OC_2H_5)_2$	l.s.	1	(*52*)
	Plasma esterases	3	(*24, 186*)
	Mammals in vivo	6	(*213*)

[a] Nature of evidence.
1. In vitro change in anticholinesterase activity.
2. In vivo change in anticholinesterase activity or specificity.
3. Acid liberation measured manometrically in bicarbonate buffer.
4. Solubility changes.
5. Chemical differentiation of products.
6. Miscellaneous physiological observations.

Liver slice.

amides. The phosphoramides may act *per se* as anticholinesterase agents, or a preliminary in vivo oxidation may be required for their toxic action. No monoalkyl phosphoramide has been extensively studied in this respect. The dialkylamido alkoxy phosphoryl cyanides appear to inhibit cholinesterase and poison without preliminary activation (*129*); whereas certain dialkyl phosphoramides shown in Table I require an activation step. The chemical information on this metabolism is almost exclusively from studies with schradan. That an oxidative metabolism was involved in the activation of schradan was originally proposed by DuBois, Doull, and Coon (*83*) and Hartley (*117*), and demonstrated by Casida, Allen, and Stahmann (*50, 51, 56*). The initial active product from this metabolism in a wide variety of biological tissues appears to be the monophosphoramide oxide of schradan (*50–52, 54, 56, 227, 228*). The responsible enzyme system, which is different than trimethylamine oxidase (*51, 201*) and has been termed a phosphoramide oxidase system (*52*) is present in many insect tissues (*54, 200, 201*) but is restricted to the liver of rats (*60, 92*) and possibly other mammals (*51*). Although the ability of tissues to oxidize schradan is largely lost when the tissue is homogenized (*51, 83, 201*), homogenates of both rat liver (*74, 75*) and insect gastric caeca (*38*) have been reported to activate schradan when fortified with nicotinamide, magnesium, and either diphospho- or triphosphopyridine nucleotides. The oxidizing system of mammalian liver is in the microsome plus supernatant fraction and requires diphosphopyridine nucleotide and magnesium cofactors in vitro (*74*).

An antagonist of the in vitro oxidizing system, β-diethylaminoethyl diphenylpropylacetate hydrochloride, prolongs the survival time of schradan-poisoned rats (*48, 74*). The *N*-oxide of schradan rapidly isomerizes under biological conditions to the less active anticholinesterase *N*-methoxide (*227, 228*). The loss of anticholinesterase activity of this *N*-oxide may therefore be due to both hydrolysis and isomerization. The *N*-methoxide serves insecticidally as a "weaker schradan" (*110*) and might possibly undergo another similar activation series (*228*). Although the substrate specificity of this enzyme system has not been studied in detail, the phosphoramides shown in Table I give an indication that a large number of similar compounds may be activated. No evidence has been reported for the metabolic activation of any monoalkyl phosphoramide. This may explain the similar quantitative toxicology and species specificity to insects of the isosteres, *O,O*-diisopropyl phosphorofluoridate (DFP) and monoisopropyl phosphorodiamidic fluoride (Isopestox) (*158*). However, the very marked species specificity for poisoning with schradan (*158, 200, 201, 207*) cannot be explained on the basis of the ability of the different forms to metabolize schradan to the toxic *N*-oxide (*54, 201*). Whether in vivo oxidation of the nitrogen also occurs with the analogous anticholinesterase *N*-alkyl carbamic and sulfamic anhydrides (*121, 219*) has not as yet been established. *N*-oxides and methylated derivatives might also be considered as possible metabolites with the wide variety of phosphate triesters containing nitrogen in an aliphatic or heterocyclic side chain.

Oxidation of Phosphorothioates. In a similar manner to the dialkyl phosphoramides, the phosphorothioates may act *per se* as antiesterase agents or may require a preliminary metabolic activation. The phosphorothioates react with cholinesterase in the same way as the corresponding phosphates and this reactivity is proportional to the hydrolytic lability of the phosphoric anhydride bond (*8, 10*). As the phosphorothioates are more stable to hydrolytic attack than the corresponding phosphates, they are generally less active antiesterase agents. This relation is discussed in more detail later. Diggle and Gage in 1951 (*78*) noted the high in vivo and low in vitro anticholinesterase activity of the phosphorothioate, parathion, and demon-

strated that incubation with liver slices produced a potent anticholinesterase agent (*79*). The enzyme system responsible for this activation is present in liver slices (*79*, *178*) and in a variety of insect tissues (*178*). When suitably fortified with magnesium and diphosphopyridine nucleotide, liver homogenates also increase the anticholinesterase activity of parathion (*74*, *75*). It has not been ascertained whether the schradan and parathion activating enzyme systems are the same (*74*).

The metabolic product from both insect (*178*) and mammalian tissues (*96*) has been characterized as an oxidized derivative, the corresponding *O,O*-dialkyl *O*-*p*-nitrophenyl phosphate. The phosphorothioates and phosphorodithioates which have been reported to undergo a biological activation, presumably by oxidation, are shown in Table I. Again the substrate specificity of this enzyme system has not been extensively studied. It would appear that *O,O*-diisopropyl *O*-*p*-nitrophenyl phosphorothioate is less readily activated in vivo than the dimethyl or diethyl analogs (*9*). It would further appear that honeybees might be less efficient in oxidatively metabolizing diisopropyl *p*-nitrophenyl phosphorothioate and diisopropyl phosphorothioic anhydride than houseflies, based on the in vivo toxicity of the phosphates and phosphorothioates compared with the in vitro anticholinesterase activity for the corresponding phosphates (*179*). *O,O*-diethyl *O*-*p*-nitrophenyl phosphate (paraoxon) and *O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate are similar in quantitative toxicology and species specificity with a variety of insect forms (*158*), indicating that such a species specificity in oxidizing phosphorothioates is less important with dimethyl and diethyl analogs than with the diisopropyl compounds.

Yet, not all phosphorothioates require a preliminary metabolic activation. *O,O*-diethyl phosphorofluorothioate and *O,O*-di-*n*-propyl phosphorothioic anhydride directly phosphorylated and inhibited the esteratic activity of chymotrypsin (*138*), although the activity of the latter compound might have been due to impurities. *O,O,O*-triethyl phosphorothioate is a more active antiesterase and more potent insecticide than the corresponding phosphate (*58*). *O,O*-diethyl *O*-ethyl-2-mercaptoethyl phosphorothioate is apparently metabolized in insects and mammals to the corresponding active antiesterase sulfoxide and sulfone which account for the toxicity of the insecticide rather than being metabolized to the various phosphate oxidation products (*171*). The combination rate of phosphorothioates with cholinesterase is essentially the same as that for phosphates of similar hydrolytic stability and stereochemical configuration (*8*, *10*). Phosphorothioate oxidation therefore appears to serve as a mechanism of activation only in those cases where the phosphorothioate *per se* or other phosphorothioate metabolites are not sufficiently active (or reactive) anticholinesterase agents to effect the poisoning.

Thioether Oxidation. The systemic insecticide, demeton, has been extensively investigated as to the intermediate anticholinesterase agents formed on degradation in plants and animals. This insecticide is a mixture of about equal parts of *O,O*-diethyl *O*-ethyl-2-mercaptoethyl phosphorothionate (Systox or the thionate isomer) and *O,O*-diethyl *S*-ethyl-2-mercaptoethyl phosphorothiolate (Isosystox or the thiolate isomer) (*93*, *100*, *125*, *126*, *181*, *241*). On treatment of plants with the phosphorothiolate or -thionate compounds essentially none of the unchanged isomers can be found in the plants after a few days, but each isomer has been converted to at least three different nonionic metabolic products (*123*). The recent comprehensive studies of Metcalf and coworkers (*95*, *171*, *183*, *184*) have helped to elucidate the nature and toxicological significance of Systox and Isosystox metabolites.

In a wide variety of biological tissues the phosphorothionate was oxidized, first to the sulfoxide, and then to a further oxidation product, probably the phosphorothionate sulfone. Although the sulfoxide and sulfone of *O,O*-diethyl *O*-ethyl-2-mercaptoethyl phosphate were not definitely eliminated as metabolites, their presence was considered unlikely on the basis of toxicity and anticholinesterase determinations. Similarly the phosphorothiolate metabolism involved a thioether oxidation to the sulfoxide and sulfone. The liver was the principal organ for oxidative metabolism of the Systox isomers in the mouse, while the insect intestine, muscle, and nerve were active in this biological oxidation. Thus, the enzymes responsible for the thioether oxidation of the Systox isomers appear to be more generally distributed among the organ systems of insects than of mammals.

A similar case occurs with the phosphoramide and phosphorothioate oxidizing enzymes. Conversion of the phosphorothiolate and -thionate to the corresponding sulfoxides and sulfones increased the anticholinesterase activity of these materials, whereas that of the phosphate was reduced on formation of the sulfoxide and sulfone (*171*). The possibility exists however, that impurities may have affected certain of these anticholinesterase results. The sulfoxide group in each case served to stabilize the material to in vitro alkaline hydrolysis.

Further evidence for the stability of these oxidative metabolites is the fact that they accumulate in plants after formation from the Systox isomers, such that the limiting factor in the residual action of the isomers within plants appears to be the biological stability of these oxidized derivatives. The phosphorothiolate isomer, or its metabolites, persists about twice as long in plant tissues before hydrolysis as the phosphorothionate isomer or its metabolites (*183*, *184*); yet the phosphorothiolate isomer is more susceptible to hydrolytic attack in vitro than the phosphorothionate isomer (*95*). This can be explained by the observation that the phosphorothiolate sulfoxide is much more stable to hydrolysis than the phosphorothionate sulfoxide, while the sulfones of the two isomers are similar in stability (*95*).

As the limiting step in the degradation within plants would appear to be the stability of the oxidized derivatives, and as the sulfone of the thiolphosphate is the most active antiesterase of the materials currently considered to be formed within the plant, the use of the anticholinesterase method for determination of the residual hazard of demeton treated plants would essentially be a determination of the amount of phosphorothiolate and -thionate sulfoxides and sulfones present (*184*). In addition to the oxides, the methosulfate of Isosystox is a potent insecticidal anticholinesterase agent (*94*). The substitution of selenium or oxygen for sulfur in the thioether side chain of the Systox type molecule also yields active insecticidal materials (*219*).

Triphenyl Phosphate Activation. Tri-*o*-cresyl phosphate (TOCP) is metabolized by rabbits, rats, and chickens in vivo to an active anticholinesterase (*7*, *175*, *197*). The antiesterase metabolites are produced primarily in the liver as demonstrated in vivo (*7*, *197*) and in vitro (*7*). The same liver suspensions effecting in vitro activation of parathion and schradan will also activate TOCP (*74*). Two or more metabolites are formed, one of which is at least 6000 times more active than the original compound as an inhibitor of pseudocholinesterase (*197*). Triphenyl and tri-*o*-chlorophenyl phosphates also appear to be activated and on analogy with the metabolism of other aromatics it has been proposed without experimental evidence that the activation of these substituted phenyl phosphates involves introduction of a hydroxyl group in the para position, followed by a sulfate or glucuronide formation (*197*).

Carboxylic Ester Hydrolysis. Not all metabolic changes within the organism prior to the hydrolysis of the phosphoric anhydride bond effect an increase in the antiesterase activity. The initial site of in vivo attack in plants on the insecticidal cis and trans isomers of *O,O*-dimethyl 1-carbomethoxy-1-propen-2-yl phosphate appears to be an enzymatic hydrolysis of the carboxylic ester, followed by a cleavage of the vinyl phosphate bond (*55*). Accordingly, the limiting factor in the residual persistence

of these phosphate esters in plants would appear to be the susceptibility of this carboxylic ester group to enzymatic hydrolysis. Detoxification of malathion [*O,O*-dimethyl-*S*-(1,2-dicarbethoxyethyl) phosphorodithioate] also involves an enzymatic hydrolysis of the carboxylic ester groups, the mechanism of decomposition being more complex in mammals than in insects (*169*).

O,O-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (Bayer $L_{13/59}$ or Dipterex) is readily dehydrochlorinated by alkali and rearranges to form the more active antiesterase *O,O*-dimethyl 2,2-dichlorovinyl phosphate (*37*, *165*, *173*). Many insects can enzymatically dehydrochlorinate such trichloroethanes as DDT to the corresponding substituted vinyl derivatives (*177*), and it has been speculated that $L_{13/59}$ might also be converted to its vinyl derivative in vivo by insect dehydrochlorinase enzymes (*29*). However, studies with insects, plants, and mammals have shown that the metabolic breakdown involves direct cleavage of the phosphonate bond rather than a dehydrochlorination and rearrangement to form the vinyl derivative (*13*). The toxicity of *O,O*-dimethyl 2,2,2-trichloro-1-acetoxyethyl phosphonate to rats and several insect species appears to be due to enzymatic hydrolysis of the acetyl group to form the more potent anticholinesterase Bayer $L_{13/59}$ with its α-hydroxyl available for hydrogen bonding with the esterase (*13*). Thus carboxylic ester hydrolysis may serve either to increase or decrease the anticholinesterase activity of the phosphate insecticides.

Influence of in vitro Chemical Changes on Stability and Activity. The susceptibility of neutral organophosphate esters to isomerization and in vitro oxidation means that chemical changes other than those occurring within the organism may affect their toxicity. Among these are impurities present from the manufacturing process and isomerization or decomposition products formed prior to the actual exposure of the organism to the insecticide. Phosphorothioates isomerize to more active antiesterase agents in the presence of heat or ultraviolet light. This isomerization with dialkyl *p*-nitrophenyl phosphorothioates yields *S*-alkyl derivatives (*172*, *181*, *204*, *219*, *242*). However, with *O,O*-diethyl and *O,O*-dimethyl *O*-ethyl-2-mercaptoethyl phosphorothionates and *O,O*-diethyl *O*-β-diethylaminoethyl phosphorothionate, isomerization occurs to form the corresponding dialkyl *S*-ethyl-2-mercaptoethyl and *S*-β-diethylaminoethyl phosphorothiolates (*100*, *102*, *115*, *126*, *220*, *241*). Isomerization of phosphorodithioates would presumably form only the *S*-alkyl derivatives. Since the isomerides present in even a 99.5% pure phosphorothioate may greatly alter the anticholinesterase activity of the material (*9*, *11*, *78*) and may have a different in vivo antiesterase specificity than the phosphorothioates or their metabolites (*196*) it has been very difficult to interpret some of the earlier data on in vitro anticholinesterase activity of phosphorothioates.

Impurities, such as tetraethyl pyrophosphate, may interfere with the determination of the in vitro anticholinesterase activity of an organophosphate when present in concentrations as low as 0.025% of the organophosphate (*10*, *63*, *65*). Other isomerization reactions may reduce the anticholinesterase activity such as occurs with the schradan-*N*-oxide conversion to the less active schradan-*N*-methoxide in the presence of dilute acid or alkali or on heating under anhydrous conditions (*227*, *228*). Certain cis-trans isomeric vinyl phosphates also isomerize with concurrent change in their anticholinesterase activity (*47*, *55*). Schradan is slowly oxidized on contact with air to an anticholinesterase agent (*228*). The Systox isomers oxidize in the presence of light (*64*) to yield the corresponding sulfoxides and sulfones (*95*).

Chemical duplication of these activation mechanisms might appear to be an ideal way to enhance the toxicity of these insecticides, since the chemical would no longer be dependent on the variable metabolic efficiency of the organism to effect its poisoning. The lag in the poisoning process necessitated by formation of sufficient metabolite to block the important esterases might also be eliminated. It has been noted that systemic insecticides generally penetrate into the plant with greater ease in their more water-soluble forms (*49*, *115*, *116*, *183*). Since the oxidative metabolites would in most cases be more water-soluble than their precursors, application of the oxidation products might allow the toxicants to be more effectively absorbed and translocated by the plant. However, such chemical duplication has not generally increased the efficiency of the toxicant, because of the instability of the products formed.

Isomerization of the phosphorothioates usually increases their anticholinesterase activity but decreases their toxicity to insects and mammals (*9*, *124*, *172*, *181*, *241*). The instability of the *S*-alkyl derivatives appears to explain the inefficient in vivo compared with in vitro anticholinesterase activity (*177*, *181*). Isomerization of the phosphorothionate isomer of Systox to the phosphorothiolate markedly increases the anticholinesterase activity and susceptibility to alkaline hydrolysis (*95*); however, this also increases the toxicity to insects (*131*, *183*) and effects a longer residual action in plants (*183*, *184*). The explanation for the efficiency of the phosphorothiolate may lie in the greater stability to alkaline hydrolysis of the sulfoxide of the phosphorothiolate than that of the phosphorothionate, and the similar stability for the sulfones of the two isomers (*95*). Thus, with the Systox isomers, the chemical duplication of the oxidative processes occurring within the organism (*95*) may actually yield certain advantages in the practical utilization of this type of insecticide.

Chlorination of schradan yields very unstable derivatives which are low in insecticidal toxicity despite a high anticholinesterase activity because of this instability (*222*). Oxidation of schradan yields a very active antiesterase, but may either decrease or increase its toxicity depending on the method of administration to the organism (*56*, *119*). The *N*-oxide of schradan is almost a million times more active as an antiesterase than schradan, but its halflife as an anticholinesterase agent at pH 7.8 is only 18 minutes (*228*); considerable loss may therefore occur before it arrives at the final site of action. The oxidation derivatives of schradan also vary in their antiesterase specificity (*56*, *228*). It would thus appear that these metabolic activations within the organism are usually essential to assure that a fairly stable organophosphate may arrive in proximity to the final site of action so that the unstable antiesterase formed may react before being destroyed.

Reaction 2. Combination of Antiesterase with Enzyme The mechanism for the antiesterase action of organophosphates has been primarily investigated with cholinesterase, chymotrypsin, and trypsin. Chymotrypsin appears in α, β, and γ forms. Each of these forms and a periodate oxidation product of chymotrypsin appear to react with the organophosphate in the same manner (*136*, *137*). However, the cholinesterases from both mammalian (*16*) and insect tissues (*45*, *46*) represent a "family" of enzymes, each with its own pattern of specificity for inhibitors and substrates. Two general types of cholinesterases have been differentiated; the true or acetylcholinesterase hydrolyzes acetyl choline selectively and possesses both an anionic and esteratic site (*194*, *238*), while the pseudocholinesterase attacks a variety of choline esters and the anionic type site is of lesser significance in its action (*194*). Most of the experimental evidence implicates the true or acetyl type of cholinesterase as the important esterase in organophosphate poisoning. Yet many other acetyl esterases are also very susceptible to inhibition by phosphate esters (*4*, *45*, *130*, *162–164*, *176*, *195*, *234*). Since the physiological function of most of these acetyl esterases is not clearly understood, the contribution of their inhibition in the poisoning process is at present only speculative.

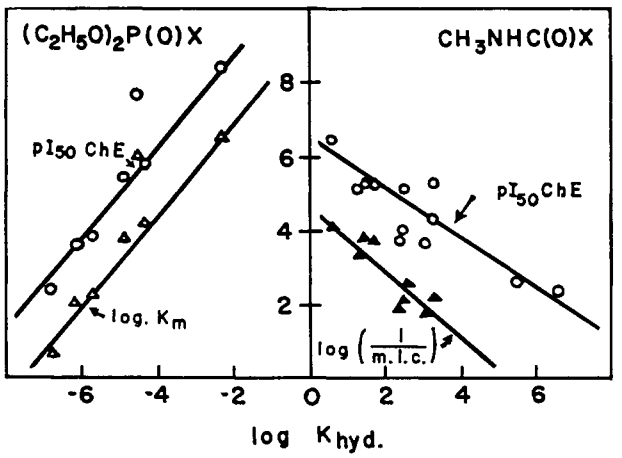
Enzymes are generally considered to

enter into combination with their substrates to form an unstable complex, which then dissociates to yield the reaction products. Such an intermediate reversible stage in the combination of the enzyme with the substrate (or inhibitor) is often demonstrable through a change in the reaction kinetics. Studies with DFP, parathion, and paraoxon have shown that the rates of progressive cholinesterase inhibition follow the characteristics of bimolecular reactions and yield inhibited enzymes which do not show significant reversibility (*2*). On the other hand, *O,O*-diethyl 8-quinolyl phosphorothioate (which reacts much more slowly with cholinesterase) displayed an initial progressive reversible phase of inhibition showing monomolecular reaction characteristics, followed by a very slow bimolecular reaction to yield the irreversibly inhibited enzyme (*2*). Purification of this phosphorothioate showed that the material(s) responsible for the reversible inhibition were present only in the impure sample; chemical nature of these impurities has not been determined (*11*).

A formation and dissociation of a reversible cholinesterase-antiesterase complex has been suggested to explain the very brief period of in vivo serum cholinesterase inhibition occurring in the poisoning of mammals with *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (*82*). However, the cause of in vivo reversal of this cholinesterase inhibition is more likely the rapid cleavage of the phosphonate bond by certain serum esterases, which serves quickly to reduce the level of effective antiesterase in the blood. The cholinesterase which has been inhibited can then be reactivated without a further source of inhibiting organophosphates (*13*).

The quaternary nitrogen-containing and cationic tertiary amide-containing organophosphates (*28, 94, 102, 221*) might also be expected to have a more pronounced reversible stage of inhibition, because of possible orientation on both the anionic and esteratic sites of acetylcholinesterase. Of the tertiary amide phosphates reported n the literature, the one referred to by Ghosh and Newman (*102*) is unique in the variety of salts that it will form. Yet the final stage of inhibition with the dialkyl phosphates containing quaternary amide groups is the same as that with neutral organophosphates with the same dialkyl phosphoryl group (*28*), as the ease of reactivation of the inhibited esterases has been shown to be similar.

The mono- and dialkyl carbamates inhibit cholinesterase by competing with acetylcholine for the active sites of the enzyme. The unhydrolyzed carbamate effects the inhibition rather than the alkylcarbamoxy group binding at the esteratic site after hydrolysis. Evidence of this is found in the inverse relationship of the anticholinesterase activity and the susceptibility to alkaline hydrolysis of certain methylcarbamate derivatives of substituted phenols (*156*, and Figure 3). This inverse correlation of stability and antiesterase activity would not be strictly expected to hold throughout the wide variety of alkylcarbamates that have been studied as anticholinesterase agents (*1, 28, 86, 208*), as the steric relationship of the groupings will also determine the efficiency of combination at the anionic site and the degree of enzyme-inhibitor interaction.



$(C_2H_5O)_2P(O)X$. pI_{50} with sheep erythrocyte cholinesterase; log K_m (bimolecular rate constant for reaction of phosphate ester with cholinesterase) $CH_3NHC(O)X$. pI_{50} with fly brain cholinesterase; log $\left(\frac{1}{m.l.c.}\right)$ based on median lethal concentration in % to thrips

Figure 3. Relation between log hydrolysis constant and anticholinesterase activity and toxicity (based on *8, 10,* and *156*)

The very low antiesterase activity of acetates and other esters compared with carbamates appears to be due, at least in part, to the relatively great resistance of the carbamates compared with the acetates to hydrolysis by the enzyme (*28*). High inhibitory activity of methyl carbamates is associated with stabilization of the carbamoxy group (*156*), so that the inhibitor when bound on the active site of the esterase will be more stable to hydrolysis than the normal substrates. However, the steric relationships can be very critical, as certain monomethyl carbamates which are hydrolytically unstable are very active anticholinesterase agents (*28*). No information has appeared in the literature on the mode of antiesterase activity of the toxic alkyl sulfamates (*121, 219*).

The ease of reaction of the organophosphate with cholinesterase will depend on the steric hindrance for the approach of the antiesterase to the active esteratic site, on the nature of the binding force to the enzyme surface, and on the phosphorylation efficiency of the "activated organophosphate substrate" on the enzyme surface. These factors vary considerably with each different esterase. For example, it has been shown with both mammalian (*16*) and insect (*45*) tissues that the cholinesterases might be considered as a family of enzymes with widely divergent substrate specificity and susceptibility to inhibitors. Both the anionic and esteratic sites would appear to be structurally hindered to a much greater extent in true than in pseudocholinesterase (*8, 28, 105*). If the phosphorus of the inhibitor is considered to replace the acyl carbon of the choline ester, relative substrate specificity and inhibitor studies with true and pseudocholinesterase indicate that a similar process may be involved in both hydrolysis of the choline ester and inhibition by the organophosphorus compound (*3, 6, 8*). The two processes appear to differ only in that the rate-limiting step with acetylcholine is the formation of the acylated enzyme, whereas with organophosphate inhibition the rate-limiting step is the hydrolysis of the phosphoryl enzyme (*6, 17, 39, 113, 152*).

The nature of the esteratic site of enzymes is poorly understood. Organophosphate antiesterases have proved an invaluable tool in studying these sites. Neither trypsinogen nor chymotrypsinogen reacts with DFP, nor are their potential esteratic activities affected by DFP (*142, 143*). The active esteratic center of the precursor appears to be covered (*112*) in such a way that the organophosphate cannot react until after the cyclic polypeptide chains are opened, as is done by carboxypeptidases (*104, 199*). Chymotrypsinogen has no *N*-terminal residues; chymotrypsin has several *N*-terminal residues, which on reaction with DFP are reduced in number to only isoleucine and valine (*211*). Trypsinogen, with valine as the *N*-terminal residue, also frees additional residues on conversion to trypsin, but the *N*-terminal residues of DFP-inhibited trypsin were found to consist only of isoleucine (*211*). DFP-inhibited trypsin combines in a weaker combination with proteinaceous trypsin inhibitors from soybeans and other sources than with trypsin *per se* (*140*). α-Chymotrypsin reactivation from organophosphate inhibition progresses as if the antiesterase were combined in two forms of different stability (*68, 69*). This could

be attributed to two different types of sites being attacked or to two forms of α-chymotrypsin. Chymotrypsin which has reacted with paraoxon until it displays no proteolytic, amino acid esterase, or amidase activity will still hydrolyze *p*-nitrophenyl acetate and *p*-nitrophenyl ethyl carbonate (*113*). This hydrolysis of *p*-nitrophenyl esters will also occur with certain tyrosine derivatives, protamine sulfate, and insulin, indicating that the initial reaction of these nitrophenyl phosphates, carbonates, and acetates may be through an amino or hydroxyl group of a tyrosine derivative (*113*).

Studies on the competition of urea in the phosphorylation of trypsin by paraoxon have led to the hypothesis that paraoxon reacts with trypsin at a site other than that involved in the combination of trypsin with the protein substrate. Normally the two sites are in close proximity so that diethyl phosphorylation interferes with the accessibility of the second site to the substrate. Urea may alter the configuration of the trypsin through unfolding or fragmentation so that the two sites are separated and free to exert their characteristic activities (*229*). It has been recently shown (*32*) that the reaction of nitrophenyl acetates with chymotrypsin is a mole for mole acetylation reaction yielding an esteratically inactive enzyme which can be readily reactivated under mild hydrolytic conditions. There is a rapid combination of enzyme and substrate in the reaction of acetyl-L-phenylalanine ethyl ester with chymotrypsin such that a measurement of the dissociation constant of the over-all reaction shows this combination reaction to be independent of the subsequent hydrolysis step (*111*). Acetylation of most of the amino groups in trypsin (*143*) altered the reaction of this enzyme with DFP. Histidine has been suggested as the initial site of attachment of phosphate esters to chymotrypsin based on competition experiments (*107, 230, 231*) and the role of histidine in the enzymatic activity of chymotrypsin (*235*). Apparently several amino acid residues on the enzyme surface of trypsin and chymotrypsin may be involved in combination with the organophosphate, but more extensive data may show one among them to be of primary importance.

Cholinesterase inhibition by organophosphates is dependent on a free esteratic site on the enzyme for combination with the inhibitor. The protective action of acetylcholine and other reversible cholinesterase inhibitors (*44, 62*) against enzyme inactivation by organophosphorus compounds illustrates that the esteratic site must be free; it further suggests that these phosphate esters have a very low affinity for the enzyme, but once the compound is attached to the enzyme, a second process occurs by which the enzyme is irreversibly inactivated by phosphorylation (*15, 20*). The efficiency of acetylcholine in reducing the cholinesterase inhibition varies with different organophosphates (*208*). The protection of cholinesterase by procaine, pantocaine, and pyrocatechol has been suggested to be due to the preferential phosphorylation of these phenols rather than the esteratic site of cholinesterase, at which site phenolic groups may also be involved (*15, 151*). Support for this hypothesis is found in the observations that phenols directly catalyze the hydrolysis of organophosphates (*14, 15, 20, 135*); but amines (*233*), imidazoles (*230, 231*), and metal chelates (*232*) are also active in this respect. Basic groups have also been proposed to function in the initial attachment of the phosphate at the esteratic site (*151, 235*). Many different initial and ultimate sites of attachment may be involved, as evidenced by the wide diversity of properties among the cholinesterases.

The nature of the combination site of true and pseudocholinesterase with organophosphates appears to differ (*73*), and even among the true cholinesterases two types of combination have been demonstrated (*73*). The attachment of the organophosphate to psuedocholinesterases may be through an amino group, based on the susceptibility of this attachment to acid and tryptic hydrolysis (*73*). Reactivation of true cholinesterase displays neither acid nor basic catalysis; further, there are two types of combination sites on this esterase, the more stable of the inhibited sites occurring on that portion of the true cholinesterase which is most stable to peptic hydrolysis. The two types of combination of the organophosphate with cholinesterase are evident from reactivation studies both in vitro and in vivo (*72, 73*). The organophosphates have thus provided an excellent experimental tool to investigate the complex nature of the active surface of cholinesterases.

The final enzyme-phosphate combination is through a covalent bond rather than a surface adsorption or binding, based on the following evidence.

Among a series of related diethyl phosphates the compounds more unstable to hydrolysis were the most efficient cholinesterase inhibitors (*10, 176*, and Figure 3). A somewhat similar relationship has been demonstrated with certain dimethylphosphoramides and chymotrypsin (*50, 227*).

The reaction involves one mole of organophosphate per mole of esterase. This has been shown with chymotrypsin and DFP, TEPP, diisopropyl phosphoric anhydride, dipropyl phosphorothioic anhydride, diethyl phosphorofluorothioate, diphenyl chlorophosphate, paraoxon, *O,S*-diethyl *p*-nitrophenyl phosphate, and the mono-*N*-oxide of schradan (*50, 56, 88, 112–114, 138, 142–144, 151, 152*); trypsin and DFP, paraoxon, and *O,S*-diethyl *p*-nitrophenyl phosphate (*70, 136, 152*); and horse liver esterase with DFP (*40*). Parathion (*45, 138*) and certain dimethyl phosphoramides (*52, 138*) display low antiesterase activity with chymotrypsin. This poor inhibition by schradan has made it possible to use chymotrypsin as a trapping agent for active antiesterases formed from this material (*50, 228*). Such a technique may have broader application where the identity of the most active antiesterase in a mixture is to be determined. The molar ratio for combination of organophosphates with cholinesterase is uncertain, as the pure enzyme has never been isolated. A mole for mole ratio was indicated with hexaethyl tetraphosphate and purified human plasma cholinesterase where acetone precipitation was used to purify the inhibited enzyme (*42*). However, with DFP, if a one to one ratio occurred, the equivalent weight of the reacting cholinesterase would be 63,000 for an electric eel preparation (*185*) and 26,000 for a human plasma preparation (*139*). These equivalent weights may be low because of incompleteness of precipitation with the trichloroacetic acid method utilized (*139*).

Enzymatic and chemical degradation of phosphorylated chymotrypsin has yielded diisopropyl phosphorylserine in 30% yield (*216*) combined in a phosphoserylglycine unit (*214*). Phosphorylserine has also been isolated in 40% yield from DFP-inhibited cholinesterase (*215, 217*).

The use of structurally specific nucleophilic reagents for in vitro reactivation of the inhibited esterases is also indicative of the cleavage of a covalent bond during dephosphorylation. Of particular interest is the efficiency of nicotinohydroxamic acid methiodide (*239, 240*), choline, and hydroxamic acid in cholinesterase reactivation with the possible formation of such derivatives as dialkyl phosphorylcholine in the course of dephosphorylation of the esterase (*237*).

The energy of activation for reactions involving inhibition or reactivation of the esterases falls in a range characteristic of a covalent type bond between the organophosphate and the esterase. For example, an activation energy of 10 to 11 kcal. per mole has been found for the inhibition of the true cholinesterase of erythrocytes by a variety of diethyl phosphates and phosphorothioates, and a value of about 14 kcal. per mole for their reactivation (*8*). Inhibition of pseudocholinesterase by octaisopropyl pyrophosphoramide displayed an activation energy of 14.5 kcal. per mole (*73*). The energy of activation for the reaction of paraoxon with trypsin

was 15.5 kcal. per mole and with chymotrypsin was 21.6, while *O,S*-diethyl *p*-nitrophenyl phosphate gave an activation energy of 11.3 kcal. per mole (*152*). The reactivation of diethyl phosphoryl chymotrypsin has an activation energy of 11.4 kcal. per mole (*68*).

A wide variation in the efficiency of antiesterases might be anticipated from the specificity of the cholinesterases and the variety of steric relationships found in organophosphates. Since all the antiesterase organophosphates appear to inhibit by phosphorylating the esteratically active site in a mole for mole reaction, the varying efficiency of inhibitors will lie in the relative ease of approaching and orienting on this site, in the ease of reaction with this site, and in the stability of the reversible and irreversible complexes formed. Rate combination studies with a single enzyme source have yielded much valuable information. For example the rate constant for paraoxon with sheep erythrocyte cholinesterase was 1.1×10^6 moles^{-1} min.$^{-1}$ while that for the phosphorothioate analog was 1.2×10^2 (*11*). The constant for *O*-ethyl *O*-*p*-nitrophenyl benzene phosphonate was 6.1×10^6 with purified eel acetylcholinesterase, while that for its phosphorothioate analog was 1.3×10^3 moles^{-1} min.$^{-1}$ (*134*). A more extensive study with related neutral diethyl phosphates showed a direct relationship between the logarithm of the bimolecular rate constants compared to the negative logarithm of the hydrolysis constants, such that the more labile the organophosphate to hydrolysis the more efficient as an in vitro anticholinesterase agent (*8, 10–12*). Yet such a relationship would not hold where the stability was altered by changing the alkyl group, because the orientation on the enzyme surface as well as the phosphorylating reactivity would then be changed. Furthermore, the portion of the phosphate freed upon hydrolysis also determines the orientation. This is evident from the observation that paraoxon is more efficient as an anticholinesterase than anticipated on the basis of its stability compared with other diethyl substituted phenyl phosphates (*8, 10*). Similar specificity probably occurs with certain diethyl phosphoryl compounds containing quaternary nitrogen groups (*28, 102*), the schradan-*N*-oxide (*51*) and the methosulfates of the Systox-type compounds (*94*). Thus, broad scale relationships of stability and antiesterase activity fail to be of significance because of the specificity encountered in forming the initial complex of the organophosphate with the esterase.

An attempt to correlate certain reactions involved in phosphate and phosphorothioate antiesterase activity is shown in Table II. Several of the reactions indicated are purely speculative. Phosphites may be toxic to insects (*166, 167*) and mammals (*127, 153*). An investigation of a small series of phosphites $(RO)_3P$, phosphinates $R_2(RO)P(O)$, and phosphonates $R(RO)_2P(O)$ indicated that their toxic effect on mammals was not characteristic of an anticholinesterase action, but rather of a stimulation and depression, or depression alone, of the central nervous system (*153*). There is no evidence indicating an in vivo oxidation of trisubstituted phosphites to trisubstituted phosphates. It is generally considered that arsenates must first be reduced to arsenites before arsenical poisoning results (*43*). The phosphates and phosphorothioates (*8, 129, 153, 179*) and certain phosphonates (*13, 45, 82, 120*) appear to effect their toxic action primarily through an anticholinesterase effect. Although certain phosphorothioates can be oxidized in vivo to the corresponding phosphate, this does not mean that such an oxidation is essential for their antiesterase activity and toxicity. Careful chromatographic purification of phosphorothioates to remove active phosphate impurities leaves a definite residual anticholinesterase (*6, 8, 48*), apparently due to the phosphorothioate *per se*, since the inhibitory activity is related to the stability of the material in the same way as with the more reactive analogous phosphates (*8, 11*). Certain phosphorothioates react with chymotrypsin in a mole-for-mole phosphorylation reaction (*138*). A comparison of the toxicity to the cockroach of triethyl phosphate and triethyl phosphorothioate with the in vivo and in vitro cholinesterase inhibition by the two compounds (*58*) indicates that the phosphorothioate was probably the effective poison in this case.

Table II. Generalized Reactions of Thiophosphates with Esterases

	Active Antiesterase	Reaction Products
A. Phosphates $R_1OR_2P(O)OX$	$R_1OR_2P(O)OX$	$R_1OR_2P(O)OEst + HOX$
B. Phosphorothiolates $R_1OR_2P(O)SX$	$R_1OR_2P(O)SX$	$R_1OR_2P(O)OEst + HSX$ or $R_1OR_2P(O)SEst + HOX$
C. Phosphorothioates $R_1OR_2P(S)OX$	$R_1OR_2P(S)OX$	$R_1OR_2P(S)OEst + HOX$
	$\xrightarrow{[O]} R_1OR_2P(O)OX$	$R_1OR_2O(O)OEst + HOX$
	$\xrightarrow{\Delta} R_1OR_2P(O)SX$	$R_1OR_2P(O)OEst + HSX$ or $R_1OR_2P(O)SEst + HOX$
	$\xrightarrow{\Delta} R_1SR_2P(O)OX$	$R_1SR_2P(O)OEst + HOX$
D. Phosphorodithioates $R_1OR_2P(S)SX$	$R_1OR_2P(S)SX$	$R_1OR_2P(S)OEst + HSX$ or $R_1OR_2P(S)SEst + HOX$
	$\xrightarrow{[O]} R_1OR_2P(O)SX$	$R_1OR_2P(O)OEst + HSX$ or $R_1OR_2P(O)SEst + HOX$
	$\xrightarrow{\Delta} R_1SR_2P(O)SX$	$R_1SR_2P(O)OEst + HSX$ or $R_1SR_2P(O)SEst + HOX$

In addition to the potential in vivo oxidation of phosphorothioates, they may be isomerized in vitro to the types of derivatives shown in Table II (*181*). Based on enzyme reactivation studies (*8*), the PSR′ bond of a *p*-nitrophenyl phosphorothiolate combined with cholinesterase to yield the same stability of enzyme-phosphorus bond as when inhibited by the corresponding phosphate compound. This means that the same groups were ultimately attached to the enzyme with the phosphorothiolate and the phosphate; the PSR′ bond was therefore cleaved by the enzyme in the same way as by alkaline hydrolysis. However, certain phosphorothioates, [including $(CH_3O)_2P(S)SP(S)(OCH_3)_2$, $(CH_3O)_2P(S)SSP(S)(OCH_3)_2$, malathion, and one or both of its half esters] are cleaved by strong alkali to yield the dithio acid salt. This reaction serves as the basis for determination of these compounds through formation of the colored copper-dimethyl-dithiophosphate complex from the hydrolyzed phosphate (*27, 148*). The enzymatic cleavage of these phosphorodithioates has not been thoroughly investigated but should be of particular interest because of the possibility of enzymatic cleavage to form a *P—S*-cholinesterase bond.

Reaction 3. Reactivation of Phosphoryl Esterase Enzyme

The phosphorylated esterase is subject to both enzymatic and nonenzymatic hydrolysis. This is diagrammatically represented in Figure 1 and Table II. Where the esterase inhibited is of little physiological significance, or

where the phosphoryl enzyme is so unstable that the phosphate group is readily released, the esterase serves as a detoxifying enzyme. The general mechanism appears to be the same for the detoxifying enzymes and for those inhibited by the organophosphate and then reactivated as with cholinesterase (*8*). The difference lies in the specificity patterns and the rates of the reactions involved. The phosphoric anhydride bond of paraoxon is split by the A-esterase of mammalian sera and other tissues (*5*) and by plasma aromatic esterases (*193*), these possibly being the same enzymes. The very small difference in an esterase which is inhibited and one which detoxifies is seen with the A- and B-esterases of human sera, the former of which readily loses the phosphate group and thus hydrolyzes paraoxon while the latter forms a more stable phosphoryl enzyme and is inhibited (*4*, *5*). A single plasma esterase will hydrolyze DFP, TEPP, paraoxon and *p*-nitrophenyl acetate (*186*). However, Augustinsson and coworkers (*18*, *21*, *22*, *24*) have demonstrated at least three esterases in purified human plasma which can hydrolyze trisubstituted organophosphates, one attacking TEPP, another paraoxon, and a third of broader specificity.

Human plasma esterases will slowly hydrolyze such vinyl phosphates as *O,O*-dimethyl 2,2-dichlorovinyl phosphate and the cis and trans isomers of *O,O*-dimethyl 1-carbomethoxy-1-propen-2-yl phosphate; a rapid enzymatic hydrolysis occurs with *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate and its acetyl derivative (*13*, *48*). The phosphoryl fluorides such as DFP are cleaved by esterases present in a variety of animal tissues and microorganisms (*61*, *174*, *188*, *190–192*). Several of these enzymes have been purified and shown to be activated to different degrees by magnesium, manganous, and calcium ions (*187*, *189*). An enzyme system from human serum hydrolyzing a phosphoryl cyanide utilized strontium and barium ions, while the system from kidney utilized manganous and calcium ions (*26*).

No isolated enzyme system hydrolyzing trisubstituted phosphorothioates has as yet been demonstrated. In vivo studies with parathion indicate that this phosphorothioate is rapidly decomposed in mammals to yield *p*-nitrophenol (*97*, *101*, *198*). A portion of the parathion is probably oxidized to paraoxon, but a sulfur-containing metabolite is formed, which is of low volatility compared to parathion, is absorbed on an anion exchange resin, and is precipitable by ammonium molybdate, as might be expected of a thiophosphoric acid derivative (*145*). Since phosphorothioates will react directly with esterases (*8*, *138*), it would appear that these materials, when in a form with a suitable stability, might also be hydrolyzed by certain of these detoxifying enzymes. Enzyme preparations hydrolyzing mono- and diesters but not triesters of phosphoric acid are known, as are ones hydrolyzing mono- and di- but not triesters of phosphorothioic acid (*90*).

The ease of reactivation of the inhibited esterase is determined by the specificity of the esterase and by the various groupings introduced into the enzyme on inhibition. The reactivation differences with esterases of varying substrate specificities is as anticipated (*8*). In general, the reactivation of pseudocholinesterases occurs more rapidly than true cholinesterases (*73*). But even with cholinesterases judged to be similar by their substrate specificity, the rate of reactivation after inhibition by the same organophosphate varies considerably (*8*, *73*). The active site in true cholinesterase is generally considered to be sterically hindered to a much greater extent than with pseudocholinesterase (*8*, *105*), and yet either type of esterase may be the more readily reactivated depending on the tissue source (*8*, *73*).

The ease of reactivation is not totally dependent on the steric hindrance at the active site, and possibly different amino acids or peptides are involved in the bonding of the phosphate in the different esterases. Determination of the stability to varying hydrogen ion concentration and tryptic and peptic hydrolysis of the phosphoryl enzyme bond with pseudo- and true cholinesterase has indicated that different amino acid groups may be involved in the bonding with these different esterases (*73*). The reactivation of the dimethyl phosphoryl esterase is more rapid than of the diethyl phosphoryl esterase, which in turn is more rapid than that with the diisopropyl derivative as shown with both true cholinesterase and chymotrypsin (*8*, *68*, *119*). Inhibition of pseudo and true cholinesterase by diisopropyl and diisopropylamino phosphates is essentially irreversible (*73*). With pseudocholinesterase, the inhibition by diethyl phosphates is more rapidly reversed than that by di-*n*-propyl phosphates, but that by dimethyl, diisopropyl, and diisopropylamino phosphates is essentially irreversible (*73*). No explanation of the unusual stability of the dimethyl phosphoryl pseudocholinesterase is available.

Where the residue finally introduced into the enzyme is the same, the ease of reactivation of the esterase is independent of the source of the organophosphate group (*8*, *28*). The order of increasing hydrolytic stability with dialkyl *p*-nitrophenyl phosphates is *S*-alkyl phosphate, *S*-phenyl phosphorothiolate, phosphate, and the most stable is the phosphorothioate (*8*, *181*, *182*). The order of stability of the thio derivatives varies since with the Systox-type molecule the phosphorothiolate is intermediate in stability between the phosphate and phosphorothioate (*95*). It might therefore be expected that the *S*-alkyl reaction product with the esterase would be the most readily reactivated, the phosphorothioate least, and the other two materials identical and intermediate if they both hydrolyzed to combine with the enzyme in the same way.

The relatively low in vivo toxicity of *S*-alkyl phosphates compared to a high in vitro anticholinesterase activity (*181*) may be due in part to their rapid enzymatic hydrolysis. The *S*-ethyl isomer of parathion reacts with trypsin at a rate commensurate with its stability (*152*), but is a less active anticholinesterase than its stability might indicate and does not give the usual first-order kinetics on reaction with the esterase (*11*). Thiophosphates vary considerably in their antiesterase activity, and in the type of residue that ultimately serves to phosphorylate the esterase. Accordingly the groupings present in the individual thiophosphate may be a very important factor in governing the cholinesterase reactivation and recovery of the organism from poisoning after the initial dose has been largely excreted or decomposed.

Isomerization reactions which occurred on the surface of the inhibited enzyme might allow an esterase to be inhibited very slowly but reactivated rapidly, or the reverse situation of a rapid reaction and a slow reversal. An example of the first situation would be on phosphorylation by a dialkyl phosphorothioate which on isomerization after combination with the enzyme might yield the less hydrolytically stable *S*-alkyl phosphoryl enzyme. However, no evidence is available to indicate that phosphorothioates can be isomerized in vivo to their *S*-alkyl phosphate derivatives. The second case could occur where the esterase was inhibited by a compound such as schradan-*N*-oxide, which would introduce an *N*-oxide group into the enzyme; this *N*-oxide could then isomerize to form a more hydrolytically stable *N*-methoxide, and thus effect prolonged esterase inhibition (*228*). Also transphosphorylation within the active site of the esterase to different amino acid residues might yield a rapid inhibition and slow reversal, or *vice versa*.

The rate of reactivation is also influenced by the anionic site on cholinesterase. With hydroxamic acids it has been shown that the anionic site serves to bind and orient the reactivation catalyst (*212*, *236*).

The enzymatic reactivation of a phosphoryl esterase was first noted in vitro with TEPP-inhibited citrus acetylesterase, which could be reactivated

by citrus pectinesterase (*141*). Trypsin catalyzes the reactivation of phosphoryl pseudocholinesterase (*73*). Reactivating serum esterases have been studied in some detail (*12, 19, 73*).

Enzymatic detoxification may partially explain several observations on organophosphate poisoning. The low relative toxicity of *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate to mammals may be due to the ease of hydrolysis of the phosphonate by mammalian serum esterases (*13*). Acquired resistance of insects to organophosphates (*57, 76, 77, 161, 168*) might be a result of adaptive detoxifying enzymes. Certainly a large proportion of the organophosphate is lost by detoxification when applied to mammals (*213*). The interaction of chlorinated hydrocarbons and phosphate insecticides in the poisoning of mammals (*31*) and the ability of such materials as piperonyl butoxide to synergize the organophosphates (*128*) may depend on an interference with esterases attacking the organophosphates.

Reaction 4. Fate of Acid Residue Liberated on Phosphorylation of Esterase

As already considered, the phosphate portion which remains attached to the esterase is probably the more physiologically important part of the inhibiting molecule. Enzymatic cleavage of TEPP yields diethyl phosphoric acid (*88, 186*) which is essentially nontoxic to mammals (*160*). The acids formed on enzymatic hydrolysis of dialkyl phosphoryl fluorides and cyanides (*21, 61, 142, 174, 190*) might contribute to the deleterious effects from these compounds. Enzymatic cleavage of *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate yields the hypnotic, trichloroethanol; but this alcohol is conjugated with glucuronic acid and excreted as urochloralic acid (*13*). Hydrolysis of *O,O*-dimethyl 2-carbomethoxy-1-propen-2-yl phosphate in plants apparently yields methanol, acetoacetic acid, and dimethyl phosphoric acid (*55*).

The Systox isomers and *O,O*-diethyl *S*-ethylmercaptomethyl phosphorodithioate are metabolized and hydrolyzed within plants to diethyl phosphoric and thiophosphoric acids and the sulfoxides and sulfones of the liberated alcohols (*41, 184*). Paraoxon and *S*-ethyl parathion yield *p*-nitrophenol on enzymatic hydrolysis (*151*). In vivo hydrolysis of parathion may yield diethyl phosphorothioic acid and *p*-nitrophenol (*97, 145*). The *p*-nitrophenol liberated from parathion in vivo is reduced to *p*-aminophenol and excreted as the sulfate or glucuronide (*203*). The phosphate triesters are usually far more toxic than the component parts of the residues released on enzymatic hydrolysis, such that enzymatic cleavage can be considered as an almost total detoxification.

On the cleavage of schradan-*N*-oxide by chymotrypsin, the phosphate moiety apparently combines with the enzyme at random (*50*). Although a random combination occurs with the "one-site esterase," chymotrypsin, this in itself is no indication that the combination with cholinesterase would also be at random, for the *N*-oxide has been shown to be more selective in its ability to inhibit cholinesterase than chymotrypsin (*228*). If this were due to an orientation of the *N*-oxide on the cholinesterase surface at the anionic site, then only a given half of the molecule would be liberated. Further studies on the inhibition by and hydrolysis of mixed anhydrides are needed.

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