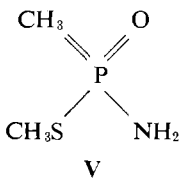
Insecticidal, Anticholinesterase, and Hydrolytic Properties of Phosphoramidothiolates

Gary B. Quistad, T. Roy Fukuto, and Robert L. Metcalf[1]

The insecticidal, anticholinesterase, and hydrolytic properties of a series of phosphoramidothiolates were examined with emphasis on structure–activity relationships. These compounds are surprisingly toxic to houseflies although they are not unusually strong inhibitors of cholinesterase. However, inhibited cholinesterase does not spontaneously recover by regeneration. The mechanism of hydrolysis is explained with a metaphosphate intermediate. Toxicity is directly related to the rate of alkaline hydrolysis and the inhibition of cholinesterase.

Recent reports describing the unusual insecticidal properties of *O*-methyl *S*-methyl phosphoramidothioate and related esters (Chevron Research Corp., 1967; Lorenz *et al.*, 1965) have restimulated interest in phosphoramidates as potential insecticides. Although a number of phosphoramido esters have gained prominence as effective insecticides, including schradan (octamethylpyrophosphoramidate), Zytron (*O*-2,4-dichlorophenyl *O*-methyl *N*-isopropylphoramidothioate) and Ruelene (4-*tert*-butyl-2-chlorophenylmethyl *N*-methylphosphoramidate), basic information concerning the relationship between chemical structure and biological activity for phosphoramidates is, in general, lacking.

O-Methyl *S*-methyl phosphoramidothioate (V, see Table II) is highly toxic to the common housefly, *Musca domestica* L. with a LD_{50} of 1.3 μg. per gram. Because of its simplicity in structure and the



V

difficulty in providing a rationale for its high insecticidal activity on reactivity grounds, a study of a series of analogs of V was initiated. To our knowledge, the mode of action of phosphoramidothiolate esters has not been investigated and this paper represents our initial findings on the toxicological, biochemical, and chemical properties of these biologically active compounds.

MATERIALS AND METHODS

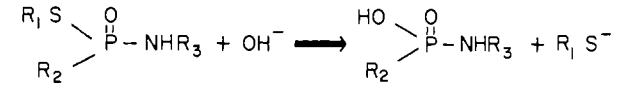
The phosphoramidothionate esters described were prepared according to conventional methods (Mel'nikov and Zen'kevich, 1955) by adding ammonia or the desired amine to the appropriate *O,O*-dialkyl phosphorochloridothioate (Fletcher *et al.*, 1950) in anhydrous ether or 2-butanone. After continued stirring and heating to ensure completion of the reaction, the ammonium chloride or amine hydrochloride was removed by filtration and the product was distilled under reduced pressure. *O,O*-Dialkyl phosphoramidothionates were isomerized to *O*-alkyl *S*-alkyl phosphoramidothiolates by heating the thionate in a several-fold excess of the appropriate alkyl iodide according to Burn and Cadogen (1961).

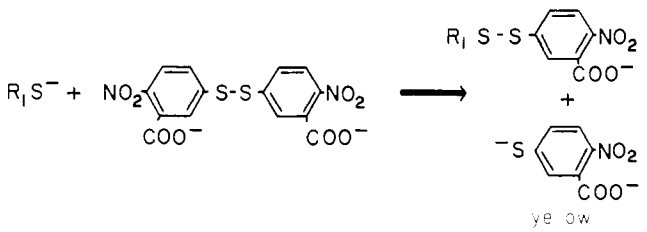
Phosphonamidothiolates also were prepared according to methods described above beginning with the appropriate *O*-alkyl ethylphosphonochloridothionate (Fukuto *et al.*, 1959a). Methyl phosphordiamidothiolate was prepared from methyl phosphorothioic dichloride (Booth *et al.*, 1948) by reaction in the usual manner with ammonia and isomerization with methyl iodide.

Elemental analyses and physical properties are given in Table I. The compounds listed in the table also were characterized by infrared and NMR spectrometry. Microanalyses were by C F. Geiger, Ontario, Calif.

The molar concentration for 50% inhibition (I_{50}) and bimolecular rate constant for inhibition (k_i) were determined for housefly head cholinesterase as described previously (Aldridge and Davison, 1952; Fukuto and Metcalf, 1956). Techniques for determination of insecticidal properties against the female housefly (*Musca domestica* L.) and mosquito larvae (*Culex pipiens quinquefasciatus* Say.) have been described (March *et al.*, 1964; Mulla *et al.*, 1966). Mammalian toxicity was determined according to Hollingworth *et al.* (1967). Three- to 8-month-old female Swiss white mice (26.4 grams per mouse) were treated orally with propylene glycol as the carrying agent.

5,5′-Dithiobis-(2-nitrobenzoic acid), DTNB, was used to follow the hydrolysis of phosphoramidothiolates according to the scheme below.



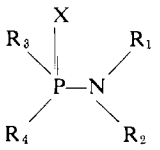


The 5-thio-2-nitrobenzoate anion is yellow and its formation can be followed spectrophotometrically. The reaction with thiol has been shown to be sufficiently rapid so that it is not rate-limiting (Ellman *et al.*, 1961). The hydrolysis buffer (0.0670*M* sodium phosphate, pH 11.5) was prepared with double distilled water which was boiled to remove oxygen and cooled under nitrogen. The substrate (0.05 m*M*) was added in 1 ml. of distilled acetone to the reaction flask. The hydrolysis buffer and reaction flask were preincubated at 30° ± 0.5° C. after flushing with nitrogen. At time zero, 9.0 ml. of hydrolysis buffer was added to the reaction flask,

Department of Entomology, University of California, Riverside, Calif. 92502

[1] Present address, Department of Entomology, University of Illinois, Urbana, Ill. 61801

Table I. Physical Properties of Phosphoramidothionates and Phosphoramidothiolates



	Compound								Analysis	
	R_1	R_2	R_3	R_4	X	M.P., °C	B.P., °C	n_D^{25}	Theory	Found
I.	H	H	CH_3O	CH_3O	S	...	72–73 (1.2 mm)	1.5025	C 17.01	C 17.10
									H 5.68	H 5.01
II.	H	H	C_2H_5O	C_2H_5O	S	...	67–69 (0.15 mm)[a]	1.4845		
III.	H	H	*n*-C_3H_7O	*n*-C_3H_7O	S	...	95–98 (0.15 mm)[b]	1.4782		
IV.	H	H	iso-C_3H_7O	iso-C_3H_7O	S	...	58–59.5 (0.1 mm)	1.4706	C 36.53	C 36.54
									H 8.13	H 8.23
V.	H	H	CH_3S	CH_3O	O	42–45	...	...	C 17.01	C 17.41
									H 5.68	H 5.94
VI.	H	H	C_2H_5S	C_2H_5O	O	50–52	...	...	C 28.38	C 28.68
									H 7.10	H 7.61
VII.	H	H	*n*-C_3H_7S	*n*-C_3H_7O	O	30–31	...	...	C 36.53	C 36.86
									H 8.13	H 8.05
VIII.	H	H	CH_3S	C_2H_5O	O	72.5–73.5[a,c]	...	...		
IX.	H	H	CH_3S	*n*-C_3H_7O	O	47–48.5	...	...	C 28.38	C 28.74
									H 7.10	H 6.80
X.	H	H	CH_3S	iso-C_3H_7O	O	79–80	...	...	C 28.38	C 28.29
									H 7.10	H 7.07
XI.	H	CH_3	CH_3S	CH_3O	O	...	92–93 (0.05 mm)	1.4972	C 23.20	C 23.41
									H 6.45	H 6.51
XII.	CH_3	CH_3	CH_3S	CH_3O	O	...	49–51 (0.2 mm)	1.4819	C 28.38	C 28.82
									H 7.10	H 7.52
XIII.	CH_3	CH_3	CH_3O	CH_3O	S	...	56–58 (3.0 mm)	1.4738	C 28.38	C 28.63
									H 7.10	H 7.54
XIV.	H	CH_3	C_2H_5S	C_2H_5O	O	...	91–93 (0.3 mm)	1.4829	C 32.78	C 32.69
									H 7.70	H 7.68
XV.	CH_3	CH_3	C_2H_5S	C_2H_5O	O	...	86–88 (3.0 mm)	1.4705	C 36.53	C 36.88
									H 8.13	H 8.27
XVI.	C_2H_5	C_2H_5	C_2H_5S	C_2H_5O	O	...	105–107 (4.5 mm)	1.4695	C 42.65	C 43.08
									H 8.89	H 8.98
XVII.	H	H	C_2H_5S	CH_3O	O	...	104 (0.05 mm) falling film still	1.5072	C 23.20	C 22.51
									H 6.45	H 5.78
XVIII.	H	H	C_2H_5O	C_2H_5O	O	49.5–51[d]	...	...		
XIX.	H	H	C_2H_5S	C_2H_5	O	46–48	...	...	C 31.35	C 31.15
									H 7.84	H 7.82
XX.	CH_3	CH_3	C_2H_5S	C_2H_5	O	...	76 (0.3 mm)	1.4959	C 39.76	C 40.07
									H 8.84	H 9.09
XXI.	H	H	CH_3S	C_2H_5	O	65–67	...	...	C 25.88	C 25.48
									H 7.19	H 7.10
XXII.	H	H	CH_3S	NH_2	O	133.5–135.5	...	...	C 9.52	C 9.69
									H 5.56	H 5.44

[a] Burn and Cadogen (1961). [b] Mel'nikov and Zen'kevich (1955). [c] Lorenz *et al.* (1966). [d] Atherton (1947).

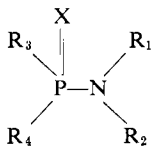
the mixture shaken, and 0.2-ml. aliquots were removed at various time intervals and added to optical test tubes containing 5.6 ml. of sodium phosphate buffer (0.100*M*, pH 6.6) and 0.2 ml. of DTNB (0.01*M*). Absorbance was read after 5 minutes at 412 mμ on a Bausch and Lomb Spectronic 20. The reaction mixture was kept under nitrogen at all times to minimize oxygen-catalyzed disulfide formation. In spite of these precautions, coupling of the alkanethiols could not be avoided but no attempt was made to correct for this side reaction. The pH was constant at 11.5 throughout the assay. The instability of DTNB at high pH precluded its presence in the reaction mixture itself.

The procedure for studying the regeneration of cholinesterase after inhibition by phosphoramidothioate was essentially that of Reed (1968). Housefly heads (100 heads per ml.) were homogenized in 0.1*M* KCl and the homogenate was centrifuged at 13,300 g. for 1 hour at 4° C. in a Sorvall Superspeed RC-2 centrifuge. An acetone solution of inhibitor (0.2 ml., 0.08*M*) was added to 1 ml. of the supernatant and the mixture was allowed to stand 15 minutes at room temperature. The inhibited enzyme mixture was added to a Sephadex G-25 column (1.7 × 9.5 cm.) together with 0.5 ml. of Dextran Blue 2000 indicator (0.1%) and the column was eluted with water. One-hundred microliters of the fraction containing the inhibited cholinesterase was added to a mixture of 2.8 ml. of sodium phosphate buffer (0.1*M*, pH 7.5), 0.2 ml. DTNB (0.01*M*), and 40 μl. of acetylthiocholine (0.075*M*). The effect of 2-pyridinealdoxime methiodide (PAM) on regeneration was determined by adding 30 μl. at $10^{-3}M$ PAM to the above mixture. Controls contained all reagents except enzyme. After equilibration for 5 minutes at 30° ± 0.5° C, absorbance was read at 412 mμ in a Unicam SP 800 recording spectrophotometer. The assay depends on the cholinesterase-catalyzed hydrolysis of acetylthiocholine and the estimation of thiocholine according to Ellman *et al.* (1961).

RESULTS AND DISCUSSION

Cholinesterase Inhibition. The values for anticholinesterase activity, toxicity to the housefly and mosquito larvae, and pseudo-first-order hydrolysis constants in phosphate buffer

Table II. Biological and Hydrolytic Properties of Phosphoramidothionates and Phosphoramidothiolates



	Compound					Fly ChE		*Musca domestica*,	*Culex pipiens quinquefasciatus*,	Pseudo First-Order Constant
	R_1	R_2	R_3	R_4	X	$I_{50}M$	k_eM^{-1} min^{-1}	$LD_{50}\mu g/G$	LC_{50} Ppm	k_o(Min^{-1})
I.	H	H	CH_3O	CH_3O	S	$>1.0 \times 10^{-3}$	...	>500	>10	...
II.	H	H	C_2H_5O	C_2H_5O	S	1.1×10^{-4}	3.2×10^2	>500	>10	...
III.	H	H	n-C_3H_7O	n-C_3H_7O	S	2.1×10^{-4}	2.0×10^2	>500	>10	...
IV.	H	H	iso-C_3H_7O	iso-C_3H_7O	S	$>1.0 \times 10^{-3}$	...	>500	>10	...
V.	H	H	CH_3S	CH_3O	O	3.9×10^{-5}	9.2×10^2	1.3	2.6	3.2×10^{-2}
VI.	H	H	C_2H_5S	C_2H_5O	O	2.3×10^{-5}	1.5×10^3	3.1	3.1	1.1×10^{-2}
VII.	H	H	n-C_3H_7S	n-C_3H_7O	O	1.6×10^{-5}	5.0×10^3	24	3.3	9.7×10^{-3}
VIII.	H	H	CH_3S	C_2H_5O	O	2.4×10^{-5}	2.1×10^3	1.5	1.6	2.6×10^{-2}
IX.	H	H	CH_3S	n-C_3H_7O	O	9.4×10^{-6}	4.9×10^3	4.4	0.70	2.3×10^{-2}
X.	H	H	CH_3S	iso-C_3H_7O	O	1.9×10^{-4}	3.5×10^2	24	>10	8.3×10^{-3}
XI.	H	CH_3	CH_3S	CH_3O	O	$>1.0 \times 10^{-3}$	...	115	>10	1.8×10^{-2}
XII.	CH_3	CH_3	CH_3S	CH_3O	O	$>1.0 \times 10^{-3}$	...	>500	>10	O[a]
XIII.	CH_3	CH_3	CH_3O	CH_3O	S	2.6×10^{-4}	...	>500	>10	...
XIV.	H	CH_3	C_2H_5S	C_2H_5O	O	1.2×10^{-4}	2.1×10^2	49	>10	5.8×10^{-3}
XV.	CH_3	CH_3	C_2H_5S	C_2H_5O	O	1.6×10^{-4}	5.0×10	>500	>10	O[a]
XVI.	C_2H_5	C_2H_5	C_2H_5S	C_2H_5O	O	1.9×10^{-4}	1.5×10^2	195	>10	O[a]
XVII.	H	H	C_2H_5S	CH_3O	O	4.1×10^{-5}	9.9×10^2	1.9	4.5	1.6×10^{-2}
XVIII.	H	H	C_2H_5O	C_2H_5O	O	1.7×10^{-5}	2.5×10^3	>500	>10	...
XIX.	H	H	C_2H_5S	C_2H_5	O	2.3×10^{-6}	2.1×10^4	0.64	4.5	5.2×10^{-2}
XX.	CH_3	CH_3	C_2H_5S	C_2H_5	O	$>1.0 \times 10^{-3}$	...	60	>10	O[a]
XXI.	H	H	CH_3S	C_2H_5	O	2.2×10^{-6}	2.0×10^4	0.85	4.6	1.3×10^{-1}
XXII.	H	H	CH_3S	NH_2	O	6.6×10^{-5}	5.6×10^2	>500	>10	1.3×10^{-1}

[a] No detectable evidence for hydrolysis after 24 hours.

for the series of phosphoramidates are given in Table II. Examination of the data shows that the compounds were, on the whole, not very effective inhibitors of fly head cholinesterase. The strongest anticholinesterases of the series were *S*-methyl ethylphosphonamidothioate (XXI) and the *S*-ethyl analog (XIX) with bimolecular inhibition constants (k_e) of 2.0×10^4 and $2.1 \times 10^4 M^{-1}$ min^{-1}, respectively. Thus, compared to diethyl *p*-nitrophenyl phosphate (paraoxon, k_e $2.7 \times 10^7 M^{-1}$ min^{-1}) and ethyl *p*-nitrophenyl ethylphosphonate (k_e $1.5 \times 10^7 M^{-1}$ min^{-1}) (Fukuto and Metcalf, 1959), XIX and XXI are approximately 1/1000 as effective in inhibiting fly head cholinesterase, although both are significantly more toxic to the housefly.

Somewhat surprising was the effect found for minor modification of structure on anticholinesterase activity, particularly with respect to groups on the nitrogen atom. For example, in comparing compounds V, XI, and XII—*i.e.*, in going from *O*-methyl *S*-methyl phosphoramidothioate (V) to the *N*-methyl (XI) and *N,N*-dimethyl (XII) analogs—we find, at least, a $^1/_{25}$ decrease in anticholinesterase activity. The substantial decrease in activity is difficult to rationalize in terms of the reactivity of the molecule, particularly with respect to V and XI, since the N-CH_3 derivative is only slightly less susceptible to alkaline hydrolysis than the unsubstituted amidate (compare values for k_o). On the other hand, the *N,N*-dimethyl derivative (XII) was completely resistant to alkaline hydrolysis and its poor anticholinesterase activity may be explained on the basis of the stable character of this compound. The absence of anticholinesterase activity in *S*-ethyl *N,N*-dimethyl *P*-ethylphosphonamidothioate (XX), the *N,N*-dimethyl derivative of XIX also may be explained on this basis. Since it is generally accepted that the inhibition of the cholinesterase enzyme by organophosphorus esters takes place *via* a bimolecular reaction between the enzyme and the ester to form a phosphorylated enzyme, phosphoramidothioates of low reactivity—*e.g.*, as estimated by hydrolysis rates—would be expected to be poor inhibitors.

Perhaps even more difficult to explain are the values obtained for cholinesterase inhibition by the series of *O*-ethyl *S*-ethyl phosphoramidothioates VI, XIV, XV, and XVI. Although in this case the *N*-methylphosphoramidothioate (XIV) is approximately one fifth as effective as a cholinesterase inhibitor than the unsubstituted amidothioate (VI), additional substitution to the *N,N*-dimethyl (XV) and *N,N*-diethyl (XVI) analogs resulted in very little change in activity (compare I_{50} values). The weak but significant activity of XV and XVI is unusual in view of the stability of these compounds to alkaline hydrolysis.

As expected, the phosphoramidothionates (I to IV and XIII) were, in general, less effective as anticholinesterases than the thiolates owing to the deactivating effect of the thionate sulfur atom (thiono effect), Bracha and O'Brien, 1968. These compounds were included in the series solely for the purpose of comparing thionates and thiolates. As the table shows, only the thiolates were found to possess insecticidal activity. To our surprise, the single phosphoramidate examined (XVIII) was moderately active as an anticholinesterase. The diamidothioate (XXII) also showed inhibitory activity but neither compound was insecticidal.

Hydrolysis. An important question concerning the reactivity of phosphoramidothiolate esters was the identification of the moiety leaving the phosphorus atom in a displacement reaction—*e.g.*, in reaction with cholinesterase, hydroxide ion or, for that matter, any nucleophile. Based on acidities

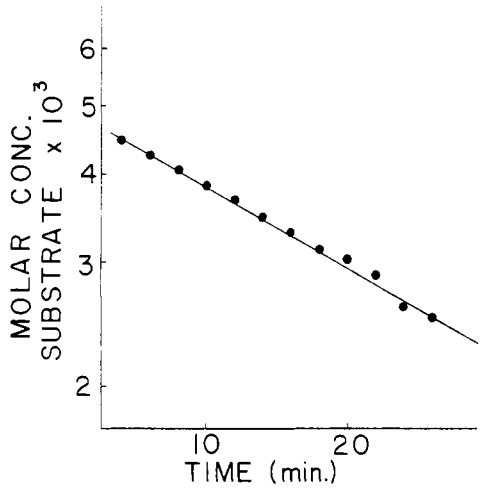


Figure 1. First-order plot showing rate of hydrolysis of *O*-ethyl *S*-methyl phosphoramidothioate in pH 11.5 phosphate buffer

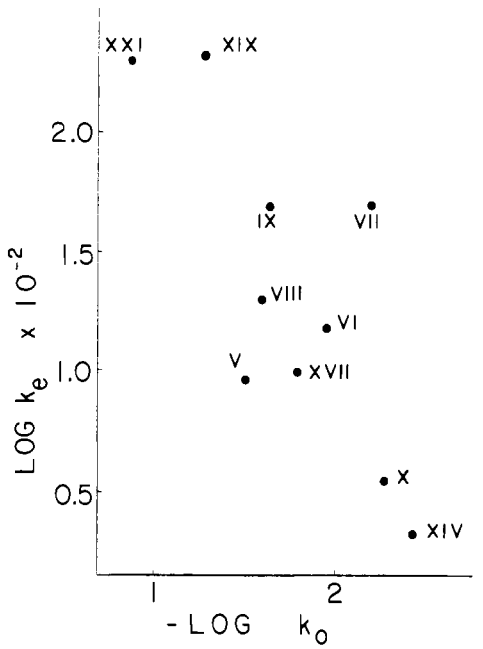


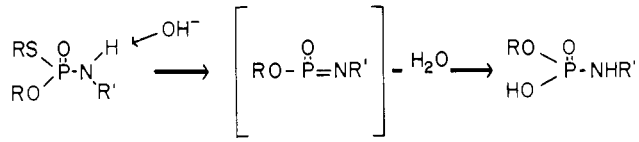
Figure 2. Relationship between bimolecular rate constant for inhibition of fly head cholinesterase (k_e) and pseudo-first-order hydrolysis constant (k_o) in pH 11.5 phosphate buffer

of the conjugate acids of the three types of groups bonded to the central phosphorus atom (pK_a ethanethiol 11, ethyl alcohol 17, $-NH_2$ >20), it was predicted that the alkylthiolate moiety would be most likely to be displaced under alkaline conditions. This expectation was realized by the analytical procedure used (Ellman, 1961) and the satisfactory first-order plots obtained, exemplified by Figure 1, which depicts the rate of hydrolysis of *O*-ethyl *S*-methyl phosphoramidothiolate (VIII) using DTNB as a means of estimating liberated methylthiolate ion.

Several points of interest emerge from the hydrolysis data. The unsubstituted *O*-alkyl *S*-alkyl phosphoramidothiolates and the *N*-methyl derivatives were hydrolyzed, with the liberation of alkylthiolate, faster than anticipated. For example, *O*-methyl *S*-methyl phosphoramidothioate (V) gave a pseudo-first-order hydrolysis constant (k_o) of 3.2×10^{-2} min^{-1} in 0.067 phosphate buffer (pH 11.5). In comparison, k_o for dimethyl *p*-nitrophenyl phosphate (methyl paraoxon), a strong anticholinesterase with I_{50} value of $9.5 \times 10^{-8} M$ (Hollingworth *et al.*, 1967), was 1.6×10^{-2} min^{-1} under the same conditions. Thus, it appears that the phosphoramidothiolates, although approximately of equal reactivity as methyl paraoxon, a strong inhibitor, are substantially less effective as anticholinesterase agents. In contrast to the mono- and unsubstituted amidates, the *N,N*-dialkyl analogs were completely resistant to hydrolysis in the same buffer during a 24-hour period.

The mechanism of phosphoramidate hydrolysis has received considerable attention in recent years (Benkovic and Benkovic, 1967; Garrison and Boozer, 1968; Onley and Caplow, 1967) although phosphoramidothioates appear not to have been studied. Under alkaline conditions phosphoramidates hydrolyze with fission of the P—O bond (Gerrard and Hamer, 1967) and under acidic conditions the P—N bond is broken (Garrison and Boozer, 1968). Two separate mechanisms have been proposed for the alkaline hydrolysis of phosphoramidates (Gerrard and Hamer, 1968; Traylor and Westheimer, 1965). The most obvious mechanism, nucleophilic attack by the base on phosphorus, results in direct displacement of an appropriate leaving group. In this case, the electrophilic character of the phosphorus atom is decreased by $p\pi$-$d\pi$ orbital overlap between phosphorus and nitrogen and the compound should be more resistant to nucleophilic attack compared to an analogous phosphate ester. The other mechanism which has been invoked to explain the high reactivity of unsubstituted or monosubstituted amidates is the formation of the metaphosphate intermediate resulting from attack of base on the amido proton.

The relatively high rates of hydrolysis found for the unsubstituted and *N*-methylphosphoramidothioates and the stability of the *N,N*-dialkyl analogs provide strong support for the metaphosphate mechanism. The high stability of the *N,N*-dialkylphosphoramidothiolates compared to the mono- or unsubstituted amidates is difficult to explain on the basis of polar or steric grounds and, therefore, the following mechanism is proposed.



A similar mechanism has been suggested to explain the rapid rate of hydrolysis of *p*-nitrophenyl *N*-methylcarbamate compared to *p*-nitrophenyl *N,N*-dimethylcarbamate (Bender and Homer, 1965; Dittert and Higuchi, 1963; Fukuto *et al.*, 1967).

The relationship between cholinesterase inhibition (log k_e) and hydrolysis rates ($-\log k_o$) is shown in Figure 2. Although the points are somewhat scattered, the general trend suggests a direct correlation between chemical reactivity and anticholinesterase activity.

Insecticidal Activity. From the toxicity data in Table II, it is obvious that the only compounds showing insecticidal activity are the amidothiolates. The most toxic compound in the series was *S*-ethyl *P*-ethylphosphonamidothioate (XIX) with a LD_{50} to the housefly of 0.64 μg per gram, slightly more toxic than parathion. The *S*-methyl (XXI) analog was slightly less toxic. These two compounds were the strongest anticholinesterases of the series of compounds examined.

Of the phosphoramidothiolates, maximum toxicity to the housefly was associated with the simplest chemical structure. The most toxic compound in this group was *O*-methyl *S*-methyl phosphoramidothioate (V) with a LD_{50} of 1.3 μg per gram. In-

creasing the size of the alkyl group in the alkoxy moiety resulted in a slight but significant decrease in toxicity (compare V, VIII, and IX), although the changes were accompanied by increase in anticholinesterase activity. This disparity between toxicity and anticholinesterase activity within a limited series of compounds has been observed before with other *N*-alkyl phosphoramidates (Fukuto *et al.*, 1963). Branching in the alkoxy moiety resulted in a substantial decrease in toxicity (X). Increasing the size of both alkoxy and alkylthiolate moieties also decreased toxicity and, as expected, the rate of decrease was substantially greater than when changes were made in only one of the groups (compare V, VI, and VII).

Substitution on the amido nitrogen rapidly reduced toxicity (compare V, XI, and XII, also VI, XIV, and XV) and the *N,N*-dimethylphosphoramidothiolates (XII, XV) were ineffective at the highest dosage of 500 μg per gram. The slight but significant toxicity of *O*-ethyl *S*-ethyl *N,N*-diethylphosphoramidothioate (XVI) is unexplained. Disubstitution on the amido nitrogen in a phosphonamidothioate also resulted in lower toxicity but in this case the decrease in activity was not as severe (XX) in spite of complete loss in anticholinesterase activity.

In limited studies *S*-ethyl *P*-ethylphosphonamidothioate (XIX) and *O*-methyl *S*-methyl phosphoramidothioate (V) were found to be somewhat toxic to the white mouse with LD_{50} values of 16 and 27 mg per kilogram, respectively. Obviously these compounds are sufficiently toxic to mammals to warrant caution.

Further examination of the toxicity data shows the existence of a direct relationship between toxicity to the housefly and reactivity of the molecule as expressed by hydrolysis rates. The plot of log LD_{50} against $-\log k_o$ (hydrolysis constant) given in Figure 3 is reasonably (correlation coefficient r = 0.85) linear considering variability in toxicological data and suggests that penetration and detoxication rates are uniform for these compounds in the housefly. A plot of log LD_{50} against log k_e (inhibition constant), although not given here, was not as satisfactory (r = 0.63) but the overall trend indicated a direct correlation between toxicity and anticholinesterase activity.

In spite of these correlations, it is still difficult to rationalize the unusually high toxicity of these compounds in light of their moderate anticholinesterase activity, particularly since none of the more active compounds contain functional groups which are likely to be metabolized *in vivo* to produce more toxic products. A study of the spontaneous and PAM-catalyzed regeneration of fly head cholinesterase inhibited by V and XIX was undertaken to determine the nature of the phosphorylated enzyme and in particular to establish if aging or irreversible phosphorylation was taking place. The inhibited enzyme was isolated by Sephadex chromatography and rates of regeneration were determined in the absence and presence of PAM. The results, summarized graphically in Figure 4, show that spontaneous reactivation of cholinesterase inhibited with either the phosphoramidothiolate V or phosphonamidothioate XIX does not take place, showing that inhibition is essentially irreversible under normal *in vitro* conditions. Evidently, fly head cholinesterase inactivated by V behaves similarly to enzyme inactivated by other phosphate esters such as TEPP, paraoxon, and DDVP with respect to spontaneous regeneration (Mengle and O'Brien, 1960; van Asperen and Dekhuijzen, 1958). The addition of PAM to cholinesterase inhibited by V resulted in a noticeable but only partial recovery of the original enzymatic activity,

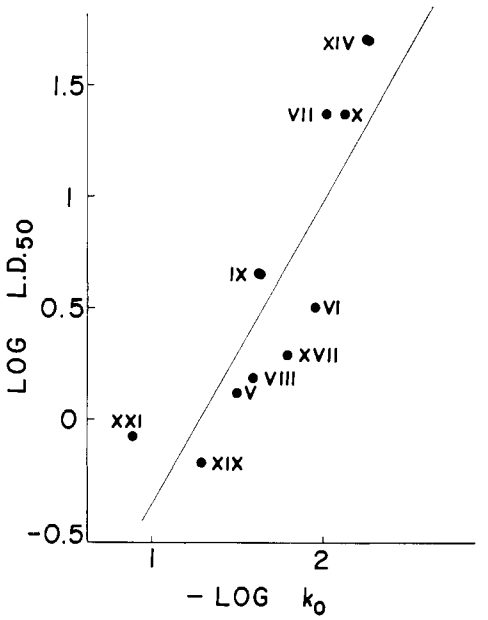


Figure 3. Relationship between housefly toxicity (log LD_{50}) and hydrolysis rate ($-\log k_o$) of phosphoramidothiolates

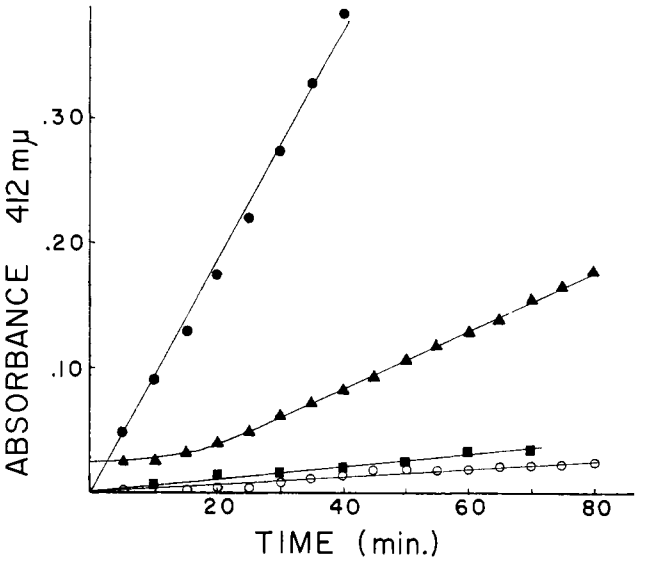


Figure 4. Plots showing the rate of reactivation of fly head cholinesterase inhibited by *O*-methyl *S*-methyl phosphoramidothioate (V) or *S*-ethyl *P*-ethylphosphonamidothioate (XIX) ○, by (XIX) + PAM ■, V + PAM ▲, no inhibitor ●

indicating substantial aging of the inhibited enzyme. Cholinesterase inhibited by XIX could not be reactivated, even after treatment with PAM, indicating that total aging of the enzyme had taken place at a rapid rate. Although this point needs further examination, the possibility remains that aging may be responsible in part for the high toxicity of these compounds. Additional work on the mode of action of these interesting compounds is currently in progress.

LITERATURE CITED

Aldridge, W. N., Davison, A. N., *Biochem. J.* **51,** 62 (1952).
Asperen, K., van, Dekhuijzen, H. M., *Biochem. Biophys. Acta* **28,** 603 (1958).
Atherton, T. J., *J. Chem. Soc.* 674 (1947).
Bender, M. L., Homer, R. B., *J. Org. Chem.* **30,** 3975 (1965).
Benkovic, S. J., Benkovic, P. A., *J. Amer. Chem. Soc.* **89,** 4714 (1967).
Booth, H. S., Martin, D. R., Kendall, F. E., *J. Amer. Chem. Soc.* **70,** 2523 (1948).

Bracha, P., O'Brien, R. D., *Biochem.* **7,** 1545, 1555 (1968).
Burn, A. J., Cadogen, J. I. G., *J. Chem. Soc.* 5532 (1961).
Chevron Research Corp., Neth. Patent Appl. **6,602,588** (Jan. 2, 1967); *C.A.* **67,** 10691*y* (1967).
Dittert, L. W., Higuchi, J., *J. Pharm. Sci.* **52,** 852 (1963).
Ellman, G. L., Courtney, K. D., Andres, V., Featherstone, R. M., *Biochem. Pharmacol.* **7,** 88 (1961).
Fletcher, J. H., Hamilton, J. C., Hechenbleikner, I., Hoegberg, E. I., Sertl, B. J., Cassaday, J. T., *J. Amer. Chem. Soc.* **72,** 2461 (1950).
Fukuto, T. R., Fahmy, M. A. H., Metcalf, R. L., J. AGR. FOOD CHEM. **15,** 273 (1967).
Fukuto, T. R., Metcalf, R. L., J. AGR. FOOD CHEM. **4,** 931 (1956).
Fukuto, T. R., Metcalf, R. L., *J. Amer. Chem. Soc.* **81,** 372 (1959).
Fukuto, T. R., Metcalf, R. L., Winton, Marianne, *J. Econ. Entomol.* **52,** 1121 (1959a).
Fukuto, T. R., Metcalf, R. L., Winton, M. Y., March, R. B., *J. Econ. Entomol.* **56,** 808 (1963).
Garrison, A. W., Boozer, C. E., *J. Amer. Chem. Soc.* **90,** 3486 (1968).
Gerrard, A. F., Hamer, N. K., *J. Chem. Soc., B,* 1122 (1967).
Gerrard, A. F., Hamer, N. K., *J. Chem. Soc., B,* 539 (1968).
Hollingworth, R. M., Fukuto, T. R., Metcalf, R. L., J. AGR. FOOD CHEM. **15,** 235 (1967).
Lorenz, W., Schrader, G., Unterstenkoefer, G., Hammann, I., A.G. Bel. **666,143** (Dec. 30, 1965); *C.A.* **65,** 16864 (1966).
March, R. B., Georghiou, G. P., Metcalf, R. L., Printy, G. E., *Bull. W.H.O.* **30,** 71 (1964).
Mel'nikov, N. N., Zen'kevich, A. G., *J. Gen. Chem. USSR* **25,** 793 (1955).
Mengle, D. C., O'Brien, R. D., *Biochem. J.* **75,** 201 (1960).
Mulla, M. S., Metcalf, R. L., Geib, A. F., *Mosquito News* **26,** 236 (1966).
Onley, I., Caplow, M., *J. Amer. Chem. Soc.* **89,** 6972 (1967).
Reed, W. D., "Kinetics of Acetylcholinesterase Inhibition by Carbamic Acid Esters," Ph.D. dissertation, University of Calif., Riverside, Calif., 1968.
Traylor, P. S., Westheimer, F. H., *J. Amer. Chem. Soc.* **87,** 553 (1965).

Received for review October 17, 1969. Accepted January 8, 1970. Work supported in part by U. S. Public Health Service Research Grant CC-00038 from the Communicable Disease Center, Atlanta, Ga., and from University of California President's Undergraduate Fellowship (awarded to G. B. Quistad).