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COMPARATIVE METABOLISM OF TWO VINYL PHOSPHOROTHIONATE ISOMERS (THIONO PHOSDRIN) BY THE MOUSE AND THE FLY

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Abstract—Fly slices activated *cis*-thiono Phosdrin (mevinphos, dimethyl-l-carbomethoxy-l-propen-2yl phosphorothionate), but this property could not be found in fly homogenates or fly microsomes. Mouse liver slices, liver homogenates or liver microsomes effectively activated *cis*-thiono Phosdrin in an oxygen and reduced pyridine nucleotide dependent system. In contrast, *trans*-thiono Phosdrin was not activated to any measurable extent by fly or mouse liver preparations.

The extent of degradation of Phosdrin isomers (oxygen analogs of, and probable activation products of thiono Phosdrin) by mouse liver slices was found to be severely reduced by the addition to the incubation mixture of the respective thiono Phosdrin isomer.

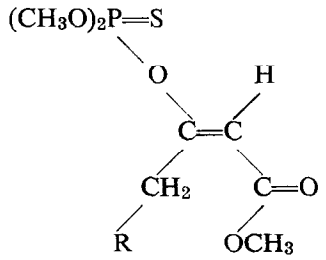
On the basis of present and previous results, an explanation is offered to account for the different toxicity of thiono Phosdrin isomers to the fly and the mouse.

THE TOXIC effects of most organophosphorus compounds are due to the inhibition of cholinesterase and the consequences of this inhibition. Their relative toxicity depends mainly on their effectiveness as cholinesterase inhibitors (usually expressed as I_{50}*) and on their concentration at a given hypothetically vital cholinergic site. This concentration is determined by a number of factors such as degradation, permeability, activation and excretion.

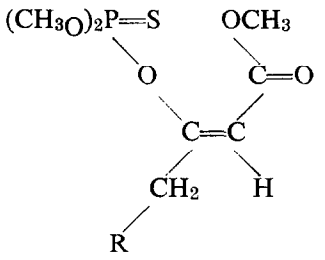
The effectiveness of an organophosphorus inhibitor depends on, among other things, the electrophilic character of its phosphorus atom. Organophosphate compounds that are good electrophilic agents are usually effective cholinesterase inhibitors; the contrary is true for the corresponding organophosphorothionates. In spite of the above, organophosphorothionates are toxic and are able to inhibit cholinesterases *in vivo* because they can be enzymatically oxidized to their corresponding organophosphates; this process is known as activation.[1–3]

A vinyl organophosphorothionate compound, dimethyl l-carbomethoxy-l-propen-2yl phosphorothionate (thiono Phosdrin) occurs as *cis* and *trans* geometrical isomers, the *cis* isomer being more toxic than the *trans*. Both isomers are more toxic to the fly than to the mouse.[4, 5]

* I_{50} = Concentration of organophosphorus compound that produces 50 per cent inhibition of cholinesterase under given experimental conditions.



cis-thiono Phosdrin (R = H)
cis-thiono Bomyl (R = CO — OCH_3)



trans-thiono Phosdrin (R = H)
trans-thiono Bomyl (R = CO — OCH_3)

Recent reports from this laboratory offered an explanation for the toxic effects of Phosdrin and Bomyl (P = O analogs of thiono Phosdrin and thiono Bomyl) to the fly and mouse.[5–7] It was considered important to study the enzymatic oxidation of the thiono Phosdrin isomers by mouse and fly preparations in order to gain a better understanding of their toxic properties.

EXPERIMENTAL

Materials. The mice were female albinos supplied by Rolfsmeyer Farms, Madison, Wisconsin and kept in our laboratory for at least a week (20–25 g, live wt.) before use. Three-day-old susceptible houseflies, *Musca domestica* L., bred in this laboratory were used throughout.

Bovine red cell cholinesterase, $NADPH_2$ and $NADH_2$ were supplied by the Sigma Chemical Co. Chemicals used were of the best quality commercially available.

Organophosphorus compounds. Trans-thiono Phosdrin was synthesized from methylacetoacetate and dimethylphosphorochloridothionate.[8] *Cis*-thiono Phosdrin was prepared by u.v. irradiation of the *trans* isomer and fractionation of the resulting mixture in an alumina column.[4]

Cis and *trans* Phosdrin were prepared as described previously.[6, 9] *Cis*-thiono Bomyl was synthesized from dimethyl acetonedicarboxylate and dimethylphosphorochloridothionate.[5]

Toxicity determination. Toxicity to mice was determined by i.p. injection of the compounds in 0·2 ml corn oil. Toxicity against flies was determined by topical application of 1 μl of an acetone solution to the thorax. Mortality was recorded after 24 hr.

Mouse liver and fly slices. Mice were killed by decapitation and the livers were removed and washed with cold 0·9% KCl. Liver slices were prepared with the aid of a Stadie–Riggs microtome. Flies were chilled, decapitated and longitudinally cut in half with the aid of a sharp blade.

Homogenates. Mouse liver homogenates were prepared in 0·25 M sucrose by using a Potter–Elvehjem unit fitted with a Teflon pestle. Fly homogenates were prepared in 0·25 M sucrose, 0·0125 M phosphate buffer (pH 7·5) by grinding decapitated flies (mixed sexes) in a mortar. The resulting homogenate was filtered through cheesecloth before use.

Microsomes. Mouse liver or fly homogenates prepared as described above were centrifuged at 10,000 *g* for 10 min. The resulting supernatants were spun at 100,000 *g* for 30 min. The pellets (microsomes) were resuspended in 0·25 M sucrose before use.

Incubation procedure. Water solutions of organophosphorus compounds were prepared fresh each day. Thiono Phosdrin isomers and *cis*-thiono Bomyl were suspended in water with the aid of an equal amount of Atlas detergent 8-916P.

Most incubations were carried out in 10-ml Erlenmeyer flasks opened to the air in a water bath shaker. In those experiments where the influence of oxygen on microsomal metabolism was studied, a Warburg water bath was used. The Warburg flasks contained all components of the incubation system in the main vessel except for the microsomal suspension that was put in the side arm. After gassing with nitrogen for 20 min, the microsomal fraction was tipped from the side arm and the incubation was carried on. Gassing was continued through the incubation period. Two kinds of nitrogen were used: (1) commercial nitrogen direct from the cylinder and (2) commercial nitrogen passed through two traps containing Fieser's solution.[10] After incubation, the mixtures were put in a boiling water bath for 3 min. An aliquot was tested against bovine red cell cholinesterase at 25° by using a manometric technique.[6, 11] From standard curves of cholinesterase inhibition (inhibitor concentration vs. per cent inhibition), the amount of organophosphates present in the incubation mixtures was calculated. All results presented in this paper are averages of three or more experiments. Variation between experiments was not more than 5 per cent.

RESULTS

Toxicity of thiono Phosdrin isomers and cis-thiono Bomyl. These results are presented in Table 1. Thiono Phosdrin isomers and *cis*-thiono Bomyl are more toxic to the fly than to the mouse by a factor of 14-42. *Cis*-thiono Phosdrin is more toxic than the *trans* isomer to both species. The LD_{50} values for the thiono Phosdrin isomers are in agreement with those of Spencer.[4]

TABLE 1. TOXICITY OF THIONO PHOSDRIN ISOMERS AND *cis*-THIONO BOMYL

	LD_{50} (mg/kg)*		Toxicity ratio
	Fly	Mouse	(mouse/fly)
Cis-thiono Phosdrin	3·2	43	13
Trans-thiono Phosdrin	22	930	42
Cis-thiono Bomyl	2·8	56	20

* Topical application to the fly and i.p. injection into the mouse (see Methods).

It is interesting to note that *cis*-thiono Bomyl is more toxic to the fly than to the mouse, whereas its oxygen analog, *cis* Bomyl, is equally toxic to both animals.[6]

Metabolism of thiono Phosdrin isomers by mouse liver and fly slices. Since mouse liver slices and homogenates are known to degrade Phosdrin isomers[6] (see Table 4), the present experiments, at least in the case of mouse liver, reflect the balance between activation of thiono Phosdrin to produce the potent cholinesterase inhibitor, Phosdrin, and degradation of the latter.

Table 2 shows that *cis*-thiono Phosdrin is more extensively activated than the *trans* isomer. Mouse liver and fly slices activated *cis*-thiono Phosdrin to approximately the same extent.

TABLE 2. METABOLISM OF THIONO PHOSDRIN ISOMERS BY MOUSE LIVER AND FLY SLICES

Animal	Wt. of tissue added (mg)	Oxygen analog at the end of incubation* (mμmoles/hr/g wet tissue)	
		cis isomer	*trans* isomer
Mouse	100	70	0
	200	65	—2
Fly	400	62	1

* Calculated from cholinesterase inhibition curves. Incubations were carried out for 1 hr at 37° in a total volume of 2 ml containing: phosphate buffer (pH 7·5), $12·5 \times 10^{-3}$ M; thiono Phosdrin, $4·5 \times 10^{-3}$ M; mouse liver or fly slices. After incubation, an aliquot was tested against cholinesterase (see Methods).

Metabolism of thiono Phosdrin isomers by mouse liver and fly homogenates and microsomes. Table 3 shows that *trans*-thiono Phosdrin was not activated by the mouse liver or fly preparations. Even when mouse liver homogenates or microsomes equivalent to 1 g tissue were used, no activation of *trans*-thiono Phosdrin was found. The

TABLE 3. METABOLISM OF THIONO PHOSDRIN ISOMERS BY MOUSE LIVER AND FLY HOMOGENATES AND MICROSOMES

Tissue	Reduced pyridine nucleotide added	Oxygen analog at the end of incubation* (mμmoles/hr/g wet tissue)			
		Homogenates†		Microsomes‡	
		cis isomer	*trans* isomer	*cis* isomer	*trans* isomer
Mouse liver	$NADH_2$	70	—3	105	—2
	$NADPH_2$	120	2	120	0
Fly	$NADH_2$	—1	0	0	0
	$NADPH_2$	2	—2	0	—2

* Calculated from cholinesterase inhibition curves.

† Incubations were carried out for 1 hr at 37° in a total volume of 1·15 ml containing: phosphate buffer (pH 7·5), $12·5 \times 10^{-3}$ M; thiono Phosdrin, $3·9 \times 10^{-3}$ M; $NADH_2$ or $NADPH_2$, 4×10^{-4}M; mouse liver homogenate equivalent to 50 mg wet tissue; fly homogenate equivalent to 200 mg (approximately 10 decapitated flies). After incubation, an aliquot was tested against cholinesterase (see Methods).

‡ Same conditions as for incubation with homogenates† except for: mouse liver microsomes equivalent to 250 mg liver wet tissue; fly microsomes equivalent to 1 g of decapitated flies; $MgCl_2$, 13×10^{-3} M.

cis isomer was activated approximately to the same extent by mouse liver homogenate and microsomes. The latter observation is unexpected, because mouse liver homogenates are known to degrade Phosdrin isomers very actively,[5, 6] while microsomes do not.[5] A likely explanation of these results is offered in the Discussion. $NADPH_2$

was found to be a little better than $NADH_2$ in supporting the enzymatic oxidation of *cis*-thiono Phosdrin.

Fly homogenates and microsomes were unable to activate *cis*-thiono Phosdrin to any significant extent, indicating that the activity present in the slices was lost upon homogenization of the tissue.

The presence of microsomal inhibitors in insect preparations has been postulated.[12] In order to investigate the presence of an inhibitor or of some other unknown cofactor required for the fly microsomal preparations, mixed microsomes (fly and mouse liver) or microsomal fraction prepared from mixed homogenates (fly and mouse liver) were tested for *cis*-thiono Phosdrin activation. The activity of these preparations was found to be equal to that of mouse liver microsomes alone.

Protective effect of thiono Phosdrin isomers on the degradation of Phosdrin isomers by mouse liver slices. Table 4 shows that *trans* Phosdrin was more extensively degraded

TABLE 4. PROTECTIVE EFFECT OF THIONO PHOSDRIN ISOMERS ON THE DEGRADATION OF PHOSDRIN ISOMERS BY MOUSE LIVER SLICES

Incubation*	Phosdrin isomers degraded (mμmoles/per hr/g wet tissue)†
Cis Phosdrin	2100
Cis Phosdrin + *cis*-thiono Phosdrin	360
Trans Phosdrin	3150
Trans Phosdrin + *trans*-thiono Phosdrin	2000

* Incubations were carried out for 1 hr at 37° in a total volume of 2 ml containing: phosphate buffer (pH 7·5), $12{\cdot}5 \times 10^{-3}$ M; Phosdrin, $1{\cdot}8 \times 10^{-4}$ M; thiono Phosdrin (when added), $1{\cdot}5 \times 10^{-3}$ M; mouse liver slices, 100 mg. After incubation, an aliquot was tested against cholinesterase (see Methods).

† Calculated from cholinesterase inhibition curves.

by mouse liver slices than the *cis* isomer. Both isomers were protected from degradation by their respective thiono analog, the *cis* isomer showing the larger effect. Strictly speaking, these experiments represent a balance between degradation of the phosphates and activation of the added phosphorothionates. However, the magnitude of the observed protection in terms of millimicromoles of Phosdrin could not possibly be explained on the basis of the much less efficient activation process. A calculation of the comparative efficiencies of the degradation and activation processes is presented in the Discussion.

Fly slices (200 mg, wet tissue) under the same experimental conditions of Table 4 were unable to degrade Phosdrin isomers to any significant extent.

Factors influencing cis-thiono Phosdrin activation by mouse liver microsomes. Table 5 shows that the activation of *cis*-thiono Phosdrin by mouse liver microsomes is enzymatic and requires molecular oxygen and reduced NADP. The enzymatic system must have a very high affinity for oxygen, as judged from the severe conditions necessary to eliminate oxygen from the medium, and consequently show its requirement. Mg^{2+} and nicotinamide showed no effect on the system. Mg^{2+} is known to be

present in microsomal preparations.[13] When $NADH_2$ was used instead of $NADPH_2$, the system was found to be more sensitive to the lack of oxygen and Mg^{2+}; the per cent activities with respect to the complete system were 6 and 68 respectively.

TABLE 5. FACTORS INFLUENCING *cis*-THIONO PHOSDRIN ACTIVATION BY MOUSE LIVER MICROSOMES

System*	Activation (% complete system)
Complete†	100
Minus microsomes	1
Minus microsomes (boiled microsomes added)	3
Minus oxygen‡	22
Minus Mg^{2+}	100
Minus nicotinamide	105
Minus $NADPH_2$	—4

* Incubations were carried out for 1 hr at 37° in a total volume of 1·15 ml. Complete system contained: phosphate buffer (pH 7·5), $12·5 \times 10^{-3}$ M; thiono Phosdrin, $3·9 \times 10^{-3}$ M; $NADPH_2$, 4×10^{-4} M; $MgCl_2$, 13×10^{-3} M; nicotinamide, 13×10^{-3} M; microsomes from 250 mg liver (wet tissue). After incubation, an aliquot was tested against cholinesterase (see Methods).

† Complete system produced 114 mμmole oxygen analog/hr/g wet tissue.

‡ Before and during incubation, the flasks were gassed extensively with nitrogen that had been bubbled through Fieser's solution (to trap the small amount of oxygen present in commercial nitrogen). When nitrogen was used directly from the cylinder, 100 per cent activation was obtained.

DISCUSSION

These results illustrate some of the factors contributing to the differences in toxicity of thiono Phosdrin isomers to the fly and the mouse. Thiono Phosdrin isomers are very poor cholinesterase inhibitors, approximately 5000 times less effective than their respective oxygen analogs.[6] Probably the small anticholinesterase activity of thiono Phosdrin is due to a small contamination with the water-soluble oxygen analog, Phosdrin. We have found that when thiono Phosdrin isomers were suspended in water with the aid of a detergent and the suspension was broken by high-speed centrifugation, almost all the anticholinesterase activity was found in the supernatant water phase.

Thiono Phosdrin isomers, being poor inhibitors of cholinesterase, probably are not toxic *per se* but rather because they can be oxidized to their respective oxygen analogs (Tables 2 and 3).

A comparison of Tables 2 and 3 shows that fly preparations lose their activating capacity of *cis*-thiono Phosdrin on homogenization. The activation of *cis*-thiono Phosdrin by mixed microsomal preparations (fly and mouse liver) was found to be equal to that of mouse liver microsomes alone. These experiments indicate a marked difference in stability between the fly and mouse microsomal fraction, rather than the presence of an inhibitor in the fly preparations.

Mouse liver homogenates, supernatants and slices degrade *cis* Phosdrin at approximately 2 μmole/hr/g wet tissue, while *trans* Phosdrin is degraded at about 3 μmole/hr/g (refs 5 and 6 and Table 4). The microsomal fraction, containing most of the activation enzyme(s), activates *cis*-thiono Phosdrin at the rate of approximately 0·1 μmole/hr/g (Table 3). Therefore the rate of degradation is much higher than that of activation. Nevertheless, accumulation of the oxygen analog occurs when *cis*-thiono Phosdrin is incubated with mouse liver homogenates or slices (Tables 2 and 3). This apparent contradiction can be explained if thiono Phosdrin protects the newly formed oxygen analog from degradation. Thiono Phosdrin was indeed shown to reduce the degradation of Phosdrin added to the incubation mixture, with the *cis* isomer exhibiting a greater protective effect than the *trans* isomer (Table 4). It is possible that this effect is due to substrate competition.

Trans Phosdrin has been shown to be degraded mainly to dimethyl phosphate.[5, 7] On the other hand, the *cis* isomer is cleaved to its respective desmethyl derivative in a reduced glutathione-dependent reaction.[5, 7] The protective effect shown by *cis*-thiono Phosdrin in *cis* Phosdrin degradation could possibly be due to a competition for the available glutathione.

In the experiments of Table 3, it is evident that the homogenate and the microsomal fraction activated *cis*-thiono Phosdrin at approximately the same rate, in spite of the fact that microsomes as opposed to the whole tissue do not contain any degrading activity.[7] An explanation can be found if the magnitude of the protective effect of thiono Phosdrin is realized. It should be pointed out that in the experiments shown in Table 4, the ratio of thiono Phosdrin/Phosdrin was approximately 10, while in the activation experiments (Tables 2 and 3) this ratio was at least 500.

Cis-thiono Phosdrin is more toxic than the *trans* isomer, possibly because of a higher activation rate (Table 2) and also because *cis* Phosdrin is approximately 100 times better as a cholinesterase inhibitor than the *trans* isomer.[6]

Both thiono Phosdrin isomers are more toxic to the fly than to the mouse probably because of the following: (a) mouse liver homogenates[6] and slices (Table 4) degrade Phosdrin isomers, whereas fly homogenates[6] and slices show no degrading activity (b) fly-head cholinesterase is 4–5 times more susceptible to inhibition than mouse brain cholinesterase,[6] and (c) inhibited mouse brain cholinesterase is reactivated much faster than the fly enzyme.[6]

Trans-thiono Phosdrin is more toxic than its respective oxygen analog, *trans* Phosdrin, to the fly (Table 1 and refs 4 and 5). Since *trans*-thiono Phosdrin is more lipophilic than *trans* Phosdrin, this apparent contradiction could be due to better penetration of *trans*-thiono Phosdrin through the insect cuticle.

Attempts to demonstrate activation of *cis*-thiono Bomyl by mouse liver microsomes were unsuccessful, probably because microsomes degrade *cis* Bomyl very effectively.[5]

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