Biochemical Pharmacology, Vol. 16 pp. 1703–1710. Pergamon Press Ltd. 1967. Printed in Great Britain

BIOCHEMICAL MECHANISMS IN THE TOXICITY OF THE GEOMETRICAL ISOMERS OF TWO VINYL ORGANOPHOSPHATES

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(*Received* 11 *January* 1967; *accepted* 28 *February* 1967)

Abstract—The geometrical isomers of dimethyl-1,3-dicarbomethoxy-1-propen-2-yl phosphate (Bomyl) have been separated and characterized and their toxicities to the housefly and the mouse have been determined.

The inhibition of bovine red cell, mouse brain, and fly head cholinesterase by the geometrical isomers of mevinphos, dimethyl 1-carbomethoxy-1-propen-2yl phosphate (Phosdrin) and Bomyl was studied. The fly head enzyme is more susceptible to inhibition than the mouse brain or bovine red cell enzyme. *Cis* Phosdrin is about 100 times better as an inhibitor than *trans* Phosdrin; *cis* and *trans* Bomyl are equally good inhibitors.

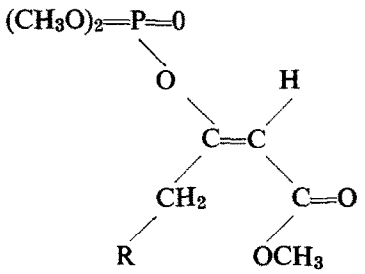
As expected, the rate of reactivation of any of the three enzymes is the same regardless of the dimethyl phosphate used to inhibit it. Inhibited bovine red cell, and mouse brain cholinesterases reactivate faster than the fly head enzyme.

Mouse liver homogenate degrades these compounds faster than fly homogenates. *Trans* Phosdrin is degraded faster than the *cis* isomer, whereas *cis* and *trans* Bomyl are degraded at equal rates. In the light of the present results, an explanation accounting for the differences and similarities in toxicity of Phosdrin and Bomyl isomers is offered.

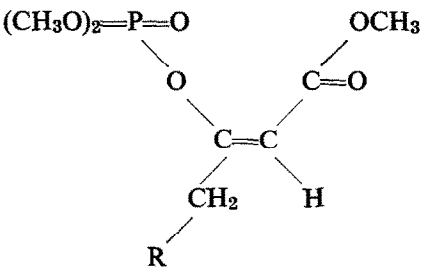
THE TOXIC effects of many organophosphorus compounds are principally due to the inhibition of cholinesterase and the consequences of this inhibition. The first organophosphorus compounds used as insecticides were equally toxic to insects and mammals. The introduction of malathion (*O,O*-dimethyl-s-1,2,-dicarbethoxyethyl phosphorodithioate) initiated a series of insecticides of low mammalian toxicity. This selective toxicity was attributed to a more extensive degradation of these compounds by the mammal, involving the hydrolysis of a carboxyester group.[1] O'Brien[2, 3] showed that selectivity was more or less a general property of carboxyester-containing organophosphorus compounds, the thiono analog being more selective than the corresponding oxygen analog.

A carboxyester vinyl phosphate, mevinphos, dimethyl 1-carbomethoxy-1-propen-2-yl phosphate (Phosdrin), occurring as *cis* and *trans* geometrical isomers, shows slight selectivity.[4] The toxicological data of the Phosdrin isomers also offer an interesting example of how different the biological properties of a compound can be as a consequence of a different spatial arrangement of certain groups. *Cis* Phosdrin is about 50 times more toxic to the fly and 20 times more toxic to the mouse than *trans* Phosdrin.[4]

A new vinyl organophosphorus insecticide, dimethyl 1,3-dicarbomethoxy-1-propen-2-yl-phosphate (Bomyl), is structurally related



cis Phosdrin (R=H)
cis Bomyl (R=CO-O-CH_3)



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

to Phosdrin. Because of this structural similarity it was considered of interest to separate the geometrical isomers of Bomyl and compare their toxicities with those of the Phosdrin isomers. Also, it was considered important to study the inhibition of insect and mammalian cholinesterases by Phosdrin and Bomyl isomers, as well as the degradation of these compounds, in order to gain a better understanding of their toxic properties.

EXPERIMENTAL

Materials

Technical samples of Phosdrin and Bomyl provided by Shell Chemical Co. and Allied Chemical Co., respectively, were the starting materials used to obtain the different geometrical isomers. The mice were female albinos, supplied by Rolfsmeyer Farms, Madison, Wis. and kept in our laboratory for at least a week (20–25 g live weight) before use.

Three-day-old susceptible houseflies, *Musca domestica* L., bred in this laboratory were used throughout. Bovine red cell cholinesterase was supplied by the Sigma Chemical Co. Chemicals used were of the best quality commercially available.

Methods

Separation of the geometrical isomers of Phosdrin and Bomyl. Craig counter-current distribution was used to separate the isomers. A redistilled sample (5 g) of technical Phosdrin or Bomyl was used in the ether–water system (10–ml phases) described by Spencer *et al.*[5] After 60 transfers, the water phase of every fourth tube was sampled and analyzed for phosphorus[6] to determine the location of each isomer.

Cis Phosdrin was obtained from tubes 21–36, which were combined and evaporated under a vacuum, and the trans isomer was obtained from tubes 47–59. The yield of each isomer and the respective boiling points were approximately the same as those reported by Spencer *et al.*[5]

For Bomyl, tubes 11–27 yielded the *cis* isomer (2·7 g; b.p. 130–131°/0·05 Torr.) and tubes 34–54 yielded the *trans* isomer (1·4 g; b.p. 130–131°/0·05 Torr.). The *cis* and *trans* configurations were assigned to Bomyl isomers by comparison of their polarity and infrared spectra with those of Phosdrin. For Phosdrin isomers the

configuration has been established from an examination of magnetic resonance spectra.[7] The rules proposed by Schmerling[8] are used here to designate the specific *cis* and *trans* isomers.

Infrared spectra were determined with a Perkin–Elmer model 21 at 10% (w/v) concentration in CCl_4. The absorption bands found useful to characterize and differentiate the isomers were:

For Phosdrin: *cis* isomer	1655	1245	1140	950	895	cm^{-1}	
trans isomer	1680	1220	1160	980	915	cm^{-1}	
For Bomyl: *cis* isomer	1655	1370	1325	980	945	895	cm^{-1}
trans isomer	1670	1345			950		cm^{-1}

The isomers were stored in a deep freeze and periodic checks were made using infra-red analysis. By contrast with Phosdrin, the *trans* isomer of Bomyl readily isomerizes in light at room temperature.

Toxicity determination. Toxicity of the compounds to the mouse was determined by intraperitoneal injection in 0·2 ml of water; the mortality was recorded after 24 hr, although no change occurred after 1 hr. Groups of 10 mice were used. Toxicity against female flies was determined by topical application of 1 μl of an acetone solution to the thorax. Mortality was recorded after 24 hr. Groups of 20 flies were used.

Inhibition of cholinesterases. A homogenate containing 80 mg of mouse brain or 6 fly heads per ml of phosphate buffer (0·001 M, pH 7·5) freshly prepared was centrifuged at 1700 *g* for 3 min to remove the debris. The supernatant was used as a source of cholinesterase. For the bovine red cell enzyme a water solution of a commercial preparation (see Materials) was used.

The inhibition of the different cholinesterases was manometrically determined at 25°, as described by Vardanis and Crawford.[9] The manometers were read every 10 min for 40 min.

For the reactivation studies, the same manometric technique[9] was used, but the temperature was 35°. The evolution of gas was recorded every 10 min for 70 or 90 min. The per cent inhibition was calculated for each 10-min interval.

Degradation studies. Incubations were carried out in 10-ml Erlenmeyer flasks in a water-bath shaker. Mouse liver homogenates were prepared in 0·25 M sucrose using a Potter–Elvehjem unit fitted with a Teflon pestle. Fly homogenates were prepared in 0·25 M sucrose by grinding decapitated flies (mixed sexes) in a mortar. The resulting homogenates was filtered through cheesecloth before use. After incubation, appropriate dilutions were made, and an aliquot was tested against bovine red cell cholinesterase at 25°. The per cent inhibition of the enzyme is a measure of the organophosphorus compound present.

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 Phosdrin and Bomyl isomers to the fly and mouse

Our first approach to the problem consisted of the determination of the toxicity of Bomyl isomers to the fly and mouse. The results of these experiments are presented in Table 1. The values for the Phosdrin isomers as reported by Spencer[4] are given for comparative purposes.

It is interesting to note that, in contrast with Phosdrin, the Bomyl isomers exhibit very similar toxicity to the two species.

Inhibition and reactivation of cholinesterases

Table 2 shows the results of inhibition of bovine red cell, mouse brain, and fly head cholinesterase with the isomers of Phosdrin and Bomyl. Fly head cholinesterase is

TABLE 1. TOXICITY OF PHOSDRIN AND BOMYL ISOMERS*

	LD_{50} (mg/kg)	
	Fly	Mouse
Cis Phosdrin†	0·27	2·0
Trans Phosdrin†	14·5	45·0
Cis Bomyl	2·3	1·6
Trans Bomyl	2·4	1·4

* Topical application to the fly and i.p. injection into the mouse (see Materials and Methods).
† Data of Spencer.[4]

TABLE 2. INHIBITION OF BOVINE RED CELL, MOUSE BRAIN AND FLY HEAD CHOLINESTERASE BY PHOSDRIN AND BOMYL ISOMERS

Enzyme source†	Phosdrin		Bomyl	
	cis	*trans*	*cis*	*Trans*
	(Results as pI_{50} at 25°, 30′)*			
Bovine red cell	6·75	4·75	7·52	7·50
Mouse brain	7·16	5·25	7·75	7·80
Fly head	7·77	5·77	8·50	8·50

* pI_{50} at 25°, 30′ = minus log of concentration of vinyl organophosphate to produce 50% inhibition of the enzyme at 25° in 30 min.

† Bovine red cell enzyme, 0·240 mg of commercial preparation per flask; mouse brain enzyme, the equivalent of 48 mg of brain per flask; fly head enzyme, the equivalent of 4 fly heads per flask. Under the assay conditions control flasks evolved 110–130 μl. CO_2 in 30 min.

much more susceptible to inhibition than bovine red cell and mouse brain enzyme. *Cis* Phosdrin is about 100 times better as an inhibitor than the *trans* isomer, whereas both Bomyl isomers are equally good inhibitors.

Cholinesterase inhibited by organophosphates is a phosphorylated enzyme, probably a phosphoryl ester of a serine residue at the active site.[10] Reactivation can occur by hydrolysis of this ester in a reaction that follows first-order kinetics.

Fig. 1 shows the reactivation of bovine red cell, mouse brain, and fly head cholinesterases inhbited by the isomers of Phosdrin and Bomyl. The different slopes indicate dissimilar reactivation rates. The figure also shows that regardless of the dimethyl phosphate used to inhibit any one of the enzymes the rate of reactivation is approximately the same. Our results for the three enzymes are in agreement with those reported

by Aldridge and Davison,[11] for the reactivation of erythrocyte cholinesterase inhibited with different dimethyl phosphates.

Degradation of Phosdrin and Bomyl isomers by mouse liver and fly homogenate

Carboxyester-containing organophosphorus cholinesterase inhibitors can be detoxified by hydrolysis of the carboxy or phosphate ester. The resulting negatively charged

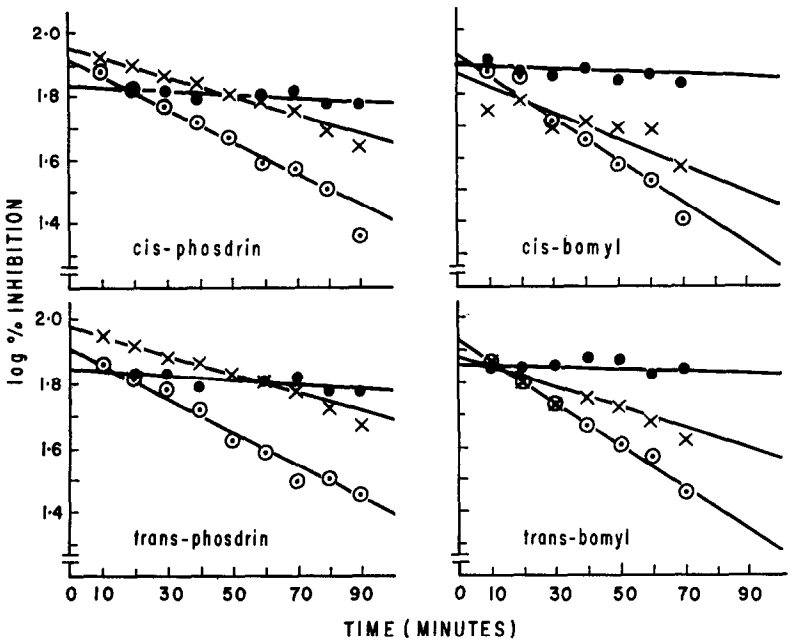


FIG. 1. Reactivation of bovine red cell, mouse brain, and fly head cholinesterase inhibited by Phosdrin and Bomyl isomers. Key: ●—●, fly head enzyme; ×—×, mouse brain enzyme; ⊙—⊙, bovine red cell enzyme. Enzymes were incubated with the inhibitor for 30 min at 35° before the substrate acetylcholine bromide was added. The molar concentrations of the organophosphorus compounds in the incubation mixture with bovine red cell, mouse brain, and fly head enzyme, respectively, were *cis* Phosdrin: 2×10^{-7}, 10^{-7}, and 2×10^{-8}; *trans* Phosdrin: 2×10^{-5}, 10^{-5}, and 2×10^{-6}; *cis* and *trans* Bomyl; $6{\cdot}4 \times 10^{-8}$, 2×10^{-8}, and $6{\cdot}4 \times 10^{-9}$.

molecule is a poor inhibitor of cholinesterase because of the repulsion of the negative charge at the anionic site and a decreased electrophilic character of the phosphorus group.

Table 3 shows the degrading activity of mouse liver and fly homogenates on the isomers of Phosdrin and Bomyl. Fly homogenates showed no measurable degradation of the compounds but mouse liver was very active. *Trans* Phosdrin was degraded faster than the *cis* isomer; *cis* and *trans* Bomyl were degraded at approximately the same rate.

DISCUSSION

These results illustrate some of the factors contributing to the differences and similarities in toxicity of Phosdrin and Bomyl isomers to the fly and mouse.

Cis Phosdrin is more toxic than the *trans* isomer (Table 1), possibly because of its stronger inhibition of cholinesterase (Table 2) and also, in the case of the mouse,

because of the somewhat slower degradation of the *cis* isomer by liver homogenates (Table 3).

Both Phosdrin isomers are more toxic to the fly than to the mouse (Table 1). This selectivity can be understood on the basis of the following considerations: (a) mouse

TABLE 3. DEGRADATION OF PHOSDRIN AND BOMYL ISOMERS BY MOUSE LIVER AND FLY HOMOGENATES

Organo-phosphate	Concentration* (M)	Incubation†	Detoxication‡ (%)
Cis Phosdrin	$2{\cdot}35 \times 10^{-4}$	Mouse liver	48
		Fly	0
		Control	0
Trans Phosdrin	$2{\cdot}35 \times 10^{-4}$	Mouse liver	80
		Fly	0
		Control	0
Cis Bomyl	$3{\cdot}2 \times 10^{-4}$	Mouse liver	77
		Fly	0
		Control	0
Trans Bomyl	$3{\cdot}2 \times 10^{-4}$	Mouse liver	80
		Fly	0
		Control	0

* Concentration of the vinyl organosphosphate in the initial incubation mixture.

† Incubation in 0·0125 M phosphate buffer (pH 7·5) at 35°. Mouse liver homogenate equivalent to 50 mg. Fly homogenate equivalent to 20 flies. For Phosdrin isomers the incubation volume was 1·15 ml and the incubation time 60 min; for Bomyl isomers, 1 and 30 respectively. At the end of incubation appropriate dilutions were made and the mixtures were tested in the cholinesterase assay as explained in Materials and Methods.

‡ Calculated from bovine red cell inhibition curves (log % inhibition versus inhibitor concentration). Controls represent incubations carried out with homogenates that were treated in a boiling water bath for 3 min.

liver homogenates degrade both isomers, whereas fly homogenates show no degrading activity (Table 3); (b) inhibited mouse brain cholinesterase is reactivated much faster than the fly head enzyme (Fig. 1); (c) fly head cholinesterase is four to five times more susceptible to inhibition than mouse brain cholinesterase (Table 2) under conditions in which inhibition follows first-order kinetics (i.e. the inhibitor concentration is in large excess with respect to the enzyme concentrations).

Both Bomyl isomers, by contrast with Phosdrin, are equally toxic (Table 1) because of their similarities in anticholinesterase activity and degradation rate (Tables 2 and 3).

Bomyl isomers have similar toxicity to the fly and the mouse (Table 1), in spite of the findings that both isomers are degraded more rapidly by mouse liver homogenates than by fly homogenates (Table 3) and that fly head cholinesterase is more susceptible to inhibition than mouse brain enzyme (Table 2).

The LD_{50} values of Bomyl isomers to the fly are unexpectedly high, compared with that of *cis* Phosdrin (Table 1), considering that Bomyl isomers appear to be better inhibitors of fly head cholinesterase than *cis* Phosdrin. Possibly these comparatively high LD_{50} values are due to slower absorption through the insect cuticle.

Cholinesterases from several sources show different susceptibility to organophosphorus inhibitors (Table 2). O'Brien[12] proposed that cholinesterases vary with respect to the distance between the anionic and esteratic sites. The effectiveness of an organophosphorus inhibitor depends partly on the electrophilic character of the phosphorus group (that interacts with the esteratic site) and also on the presence of a group that interacts electrostatically or through noncoulombic forces (e.g. Van der Waals, or hydrophobic) with the anionic site. For ideal binding the latter group should be at a distance from the phosphorus equal to the distance between the anionic and esteratic sites of the enzyme. Possibly, for Phosdrin and Bomyl isomers the interaction of a methyl carboxyester group with the anionic site of cholinesterases plays an important role in binding. The distance between the methyl carboxyester group(s) of Bomyl isomers and of *cis* Phosdrin and their respective phosphate groups is approximately the same (Table 4) and is in the range of distance (4·5–5·9 Å) reported to exist between

TABLE 4. DISTANCES BETWEEN THE PHOSPHATE AND THE CARBOXYESTER GROUPS OF PHOSDRIN AND BOMYL*

	Phosdrin		Bomyl	
	cis	*trans*	*cis*	*trans*
Range of distances	4·3–5·2	2·2–4·4	4·4–5·2	4·4–5·2
Average	4·75	3·3	4·8	4·8

* Distances (Å) given are between the centers of the phosphorous atom and the carbonyl carbon. At any possible configuration the molecules have the carbonyl carbon (Phosdrin) or at least one of the carbonyl carbons (Bomyl) in the range of distances shown.

the anionic and the esteratic site of insect cholinesterase.[12] In the case of *trans* Phosdrin, this distance is much smaller and consequently this compound is a poorer inhibitor of insect cholinesterase than *cis* Phosdrin or the Bomyl isomers.

The rate of aqueous reactivation of a given enzyme (bovine red cell, mouse brain or fly head cholinesterase) is approximately the same regardless of the organophosphate used to inhibit it (Fig. 1). This fact further supports the idea that the inhibited enzyme is a phosphorylated one.

Further experiments with these vinyl organophosphorus insecticides are under way in an attempt to gain a better undetstanding of their toxicity and mode of action.

Acknowledgements—The authors wish to thank Mr. J. Pawlik for his help in the determination of toxicity to flies. Thanks are also due to Dr. R. M. Krupka for reviewing this manuscript. One of us, A. Morello, acknowledges financial assistance during part of this work from Allied Chemical Corp.

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