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INSECTICIDE METABOLISM AND MODE OF ACTION

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Abstract - The metabolism, both in vitro and in vivo, of the chiral isomers of the phosphonodithioate insecticide fonofos was examined. The absolute configurations of the fonofos isomers and relevant oxidation products were established by x-ray diffraction analysis and chemical correlation. Oxidation of fonofos to the corresponding oxon in the presence of mouse liver mixed-function oxidase occurred predominantly with retention of configuration. The difference in toxicities of the fonofos enantiomers to insects and mammals is rationalized in terms of stereoselectivity in target site inhibition by the oxon, stereoselectivity in fonofos to fonofos oxon conversion and detoxication by other enzymes.

INTRODUCTION

Intoxication of insects and mammals exposed to insecticides involves a complex sequence of events, but the principal steps in the poisoning process doubtlessly are (1) penetration and translocation to the site of action, (2) metabolic activation or detoxication, and (3) target site inactivation or perturbation. The intrinsic toxicity of a toxicant is determined by its rate of arrival at the active site and magnitude of interaction between the toxicant and active site. Metabolic oxidation reactions are of considerable significance in the in vivo modification of insecticides in plants and animals. The various oxidation reactions which take place, e.g. olefin epoxidation, thioether oxidation to sulfoxides and sulfones, N- or O-dealkylation, aliphatic and aromatic hydroxylation, and phosphorothionate desulfuration (P=S to P=O conversion), result in both activated and detoxified products and are mediated by the mixed-function oxidases (MFO) (Ref. 1, 2).

In the pure state, phosphorothionate esters such as parathion are poor inhibitors of the cholinesterase enzymes owing to the deactivating influence of the thionate sulfur atom on the reactivity of the phosphorus center (Ref. 2). By contrast, the corresponding phosphate ester, paraoxon, is a very potent anticholinesterase, and the toxic action of parathion and related insecticides is attributable to the phosphate ester formed in vivo by oxidative desulfuration. Because of its obvious importance, this metabolic reaction has received considerable attention (Ref. 3 and references therein).

In order to elucidate the mechanism of the desulfuration reaction, we have examined the stereochemistry of P=S to P=O conversion effected by chemical model oxidation systems and mouse liver MFO, using the resolved isomers of fonofos (O-ethyl S-phenyl ethylphosphonodithioate). This report is concerned with the stereochemical aspects of the chemistry, metabolism, and mode of action of the chiral isomers of fonofos.

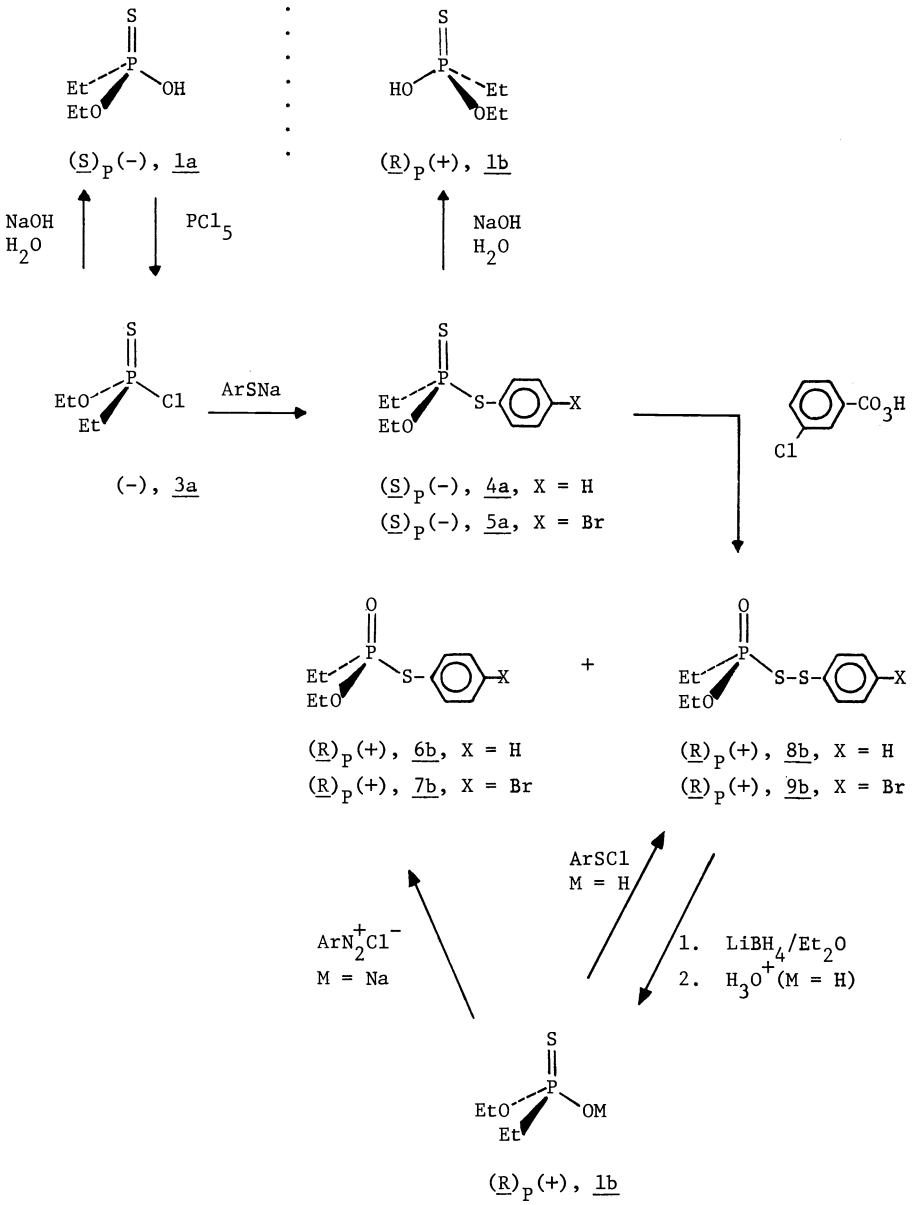
CHEMISTRY

Synthesis and determination of absolute configuration

The various reactions used in the synthesis of the chiral isomers of fonofos relevant to this study are indicated in Scheme 1 for one of the enantiomers. Values of observed (neat) and specific rotations of all enantiomeric materials are presented in Table 1. The absolute configuration of the resolved starting material, i.e., enantiomeric O-ethyl ethylphosphonothioic acid (1a or 1b), was established by x-ray diffraction analysis of the (-)-α-phenethylammonium salt of the acid (2a), obtained after repeated crystallizations. The absolute configuration of the acid 1a was established as $(S)_P$ by relating it to the known configuration of the (S)-(-)-α-phenethylammonium ion (Ref. 4, 5, 6). Since the phosphonothioic acid 1a is $(S)_P$, its enantiomer 1b is $(R)_P$.

Treatment of 1a or 1b with phosphorus pentachloride in carbon tetrachloride afforded (-)- and (+)-O-ethyl ethylphosphonochloridothioate, respectively. The reaction probably proceeded with a small amount of racemization since treatment of the chloridothioate 3a with sodium hydroxide in aqueous dioxane resulted in 1a with 7-15% reduction in optical rotation.

Scheme 1



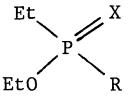
Reaction between the enantiomeric O-ethyl ethylphosphonochloridothioate (3a and 3b) and the sodium salt of benzenethiol or p-bromobenzenethiol resulted in the corresponding enantiomers of fonofos (4a and 4b) and the p-bromo analogs (5a and 5b). The absolute configuration of the (-)-rotating isomer 5a [α_D^{24} -257.90° (neat after melting)] was determined by x-ray analysis and established as $(S)_P$. It should be pointed out that the unit cell contained two molecules of fonofos which were related to each other by a pseudo center of symmetry. Fortunately, the nature of the molecular arrangement in the crystal was such that the heavy atoms in the two molecules in the unit cell deviated slightly from being centrosymmetrical, and this allowed the determination of the absolute configuration of 5a. Since 5a is $(S)_P$, 5b is $(R)_P$.

The configurations of the fonofos enantiomers (4a and 4b) were assigned on the basis of their chemical similarity to the p-bromo analogs (5a and 5b). For example, both 4a and 5a were prepared from (-)-O-ethyl ethylphosphonochloridothioate, which in turn was synthesized from $(S)_P$(-)-O-ethyl ethylphosphonothioic acid (1a). Further, alkaline hydrolysis of 4a and 5a both returned $(R)_P$(+)-O-ethyl ethylphosphonothioic acid (1b), the expected isomer from three inversion reactions. Rotations (neat, degrees arc) for the various products in the three-reaction cycle involving the preparation and alkaline hydrolysis of fonofos (4a) and the p-bromo analog (5a) are given as follows:

α_D^{24}	1a	3a	4a	1b
	-14.50°	-77.71°	-149.20°	+11.43°
α_D^{24}	1a	3a	5a	1b
	-14.94°	-82.50°	-257.99°	+14.65°

Based on the rotations of the starting acid 1a and the recovered acid 1b, the three-reaction cycle involving fonofos proceeded with about 21% racemization. The reaction cycle was significantly cleaner with the p-bromo analog where only 2% racemization was observed.

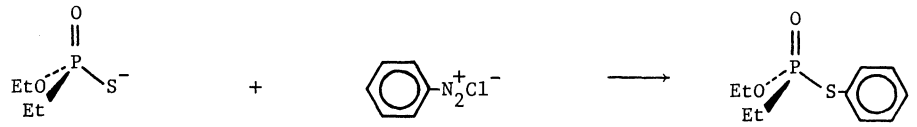
TABLE 1. Physical constants and values of observed (neat) and specific rotations in degrees arc for the compounds of general structure



Structure	No.	Absolute configuration	Observed α_D^{24} (neat)	Specific[a] $[\alpha]_D^{24}$
X = S R = OH	1 1a 1b	Racemic $(S)_P$ $(R)_P$	- -15.45 +14.82	- - -
X = S R=O$^-$H$_3$N$^+$CHC$_6$H$_5$ (CH$_3$)	2a 2b	$(S)_P$ $(R)_P$	- -	-12.78[b] +12.40[b]
X = S R = Cl	3 3a 3b	Racemic - -	- -82.50 +78.65	- - -
X = S R = SC$_6$H$_5$	4 4a 4b	Racemic $(S)_P$ $(R)_P$	- -181.50 +155.98	- +138.40[c] -114.56
X = S R=SC$_6$H$_4$-p-Br	5 5a 5b	Racemic $(S)_P$ $(R)_P$	- -257.90[d] +258.13	- +130.67[c] -129.53
X = O R = SC$_6$H$_5$	6 6a 6b	Racemic $(S)_P$ $(R)_P$	- - -	- -121.70 +112.6
X = O R=SC$_6$H$_4$-p-Br	7 7a	Racemic $(S)_P$	- -	- -107.87
X = O R = SSC$_6$H$_5$	8 8a	Racemic $(S)_P$	- -32.30	- -68.27
X = O R=SSC$_6$H$_4$-p-Br	9 9a	Racemic $(S)_P$	- -65.50	- -138.38[e]

Note a. In cyclohexane solvent unless specified.
Note b. In methanol solvent.
Note c. A change in sign of rotation from the neat measurements also was observed in other solvents.
Note d. Rotation of melted material was measured.
Note e. In carbon tetrachloride solvent.

The enantiomeric oxons of fonofos (6a and 6b) and corresponding p-bromo analog (7a) were prepared by the reaction between the sodium salt of 1a or 1b and the respective aryl diazonium chloride. This reaction undoubtedly proceeds by reaction between the aryl carbonium ion and the anion of 1a or 1b and bonds connected to the phosphorus atom are not involved. Therefore, the configurations of the oxon should be identical to that of the acid. Since the configuration of the acid 1a by x-ray analysis was determined to be $(S)_P$, 6a and 7a are $(S)_P$ and 7b is $(R)_P$.



1a $(S)_P$ 6a $(S)_P$

The configurations of (-)-phenyl ethyl(ethoxy)phosphinyl disulfide (8a) and the corresponding p-bromo analog (9a) were assigned by two independent methods. The first was based on their synthesis from the appropriate arylsulfenyl chloride and the acid 1a (see Scheme 1). As in the case of the synthesis of the oxons, the chiral phosphorus atom is not involved in this reaction and 8a and 9a should have the same configuration as 1a. Second, lithium borohydride reduction of 8a or 9a, resulting in cleavage of the S-S bond, returned 1a with greater than 99% optical retention in both cases. Observed rotations (neat, degrees arc) of products involved in this reaction cycle are given as follows:

α_D^{24}	1a -14.50°	8a -32.30°	1a -14.40°
α_D^{24}	1a -14.50°	9a -65.50°	1a -14.45°

These reactions show that the configurations of 8a and 9a are identical to 1a and, therefore, they are $(S)_P$.

Oxidation with m-chloroperoxybenzoic acid

Oxidation of the enantiomers of fonofos (4a and 4b) with m-chloroperoxybenzoic acid in dichloromethane gave fonofos oxon and phenyl ethyl(ethoxy)phosphinyl disulfide as the principal products (Ref. 7). Other materials isolated were unreacted fonofos, diphenyl disulfide, and diphenyl disulfide oxide. Data for the specific rotations (cyclohexane) of the oxon and phosphinyl disulfide isolated from oxidation of 4a and 4b are presented in Table 2. From

TABLE 2. Specific rotations in cyclohexane (degrees arc) of the chiral products obtained from peracid oxidation of fonofos enantiomers

Fonofos enantiomer[a]	$[\alpha]_D^{24}$, Peracid Products	
	oxon	disulfide
4a, S_P	+66.17 (c 0.141)	+55.40 (c 2.22)
4a, $(S)_P$	+66.32 (c 0.117)	+54.32 (c 1.81)
4b, $(R)_P$	-57.87 (c 1.30)	-51.86 (c 1.84)

Note a. $[\alpha]_D^{24}$ of synthesized 4a and 4b were +138.20 (c 0.302) and -114.56 (c 0.309), respectively.

the sign and magnitude of the specific rotations of the oxon and disulfide isolated, it is apparent that $(S)_P$-fonofos (4a) was converted predominantly to $(R)_P$-fonofos oxon (6b) and $(R)_P$-phenyl ethyl(ethoxy)phosphinyl disulfide (8b). Conversely, $(R)_P$-fonofos (4b) was converted primarily to 6a and 8a.

An approximation of the stereospecificity of the peroxy acid oxidation of fonofos was made from the specific rotations of the products and an estimate of the optical purity of the enantiomeric fonofos, synthesized oxon and phosphinyl disulfide. Thus, the oxidation of 4a to 6b was estimated to occur with about 77% retention of configuration and oxidation of 4a to 8b with about 90% inversion.

TOXICOLOGICAL STUDIES

Toxicity

Toxicity data for the racemic and optically active enantiomers of fonofos and fonofos oxon

against susceptible house flies (NAIDM strain), Culex mosquito larvae, and white mice are presented in Table 3. Included also are values for the relative toxicity ratio, defined as the LD_{50} or LC_{50} of the racemate or less toxic enantiomer divided by the corresponding values for the more toxic isomer, i.e., the more toxic isomer was given the value of unity. In the case of fonofos, the $(R)_P$ isomer was more toxic against all test animals than the $(S)_P$

TABLE 3. Toxicity of the enantiomers and racemates of fonofos and fonofos oxon against house flies, mosquito larvae and white mice

Compound	House fly LD_{50} (µg/g)	Ratio	Mosquito larvae LC_{50} (ppb)	Ratio	Mouse (oral) LD_{50} (mg/kg)	Ratio	Mouse (i.p.) LD_{50} (mg/kg)	Ratio
Fonofos racemic	12.0	1.9	31.0	1.2	14.0	1.3	4.8	1.3
$(S)_P$	25.0	4.0	45.0	1.8	32.0	3.4	7.5	2.0
$(R)_P$	6.3	1.0	25.0	1.0	9.5	1.0	3.8	1.0
Fonofos oxon racemic	8.0	2.0	92.0	1.8	21.0	3.5	0.94	1.3
$(S)_P$	4.0	1.0	50.0	1.0	6.0	1.0	0.72	1.0
$(R)_P$	48.0	12.0	610.0	12.2	38.0	6.3	1.86	2.6

enantiomer and the racemates were intermediate in toxicity. The difference in toxicities of the two isomers evidently varied with the test animal and largest difference was observed with the house fly (toxicity ratio of 4). In comparison, the toxicity ratio for mosquito larvae was only 1.8. While the toxicity ratios are not large, there is little doubt that chirality of the phosphorus center has a significant effect on the toxicity of the fonofos isomers.

Much larger differences in toxicity were observed between the fonofos oxon isomers and higher toxicity was found with the $(S)_P$ enantiomer. For example, $(S)_P$-oxon was approximately 12-fold more toxic to house flies relative to the less toxic $(R)_P$ enantiomer. The same toxicity ratio was observed with mosquito larvae. By oral administration $(S)_P$-fonofos oxon was 6-fold more toxic to mice than the $(R)_P$-oxon but only a 2.6-fold difference was observed after intraperitoneal administration.

Cholinesterase inhibition

The bimolecular rate constants (k_i) for the inhibition of bovine erythrocyte (BAChE), electric eel (EEAChE), house fly-head (HFAChE) acetylcholinesterases, horse serum (HSChE) and cholinesterases from different mouse tissues are presented in Table 4. Relative values for

TABLE 4. Values for the bimolecular rate constant for the inhibition of different cholinesterase enzymes by racemic and enantiomeric fonofos oxon

Enzyme	$k_i \times 10^{-5}$ ($M^{-1}min^{-1}$) racemic	$(S)_P$	$(R)_P$
1. Electric eel AChE	28 (3.3)[a]	78 (9.3)	8.4 (1)
2. Bovine erythrocyte AChE	16 (4.6)	32 (9.0)	3.5 (1)
3. Fly-head AChE	130 (14.8)	550 (62.5)	8.8 (1)
4. Bee-head AChE	-	12 (10.9)	1.1 (1)
5. Horse serum ChE (ASCh)[b]	7.5 (2.5)	13 (4.3)	3.0 (1)
(BuSCh)[c]	6.2 (1.9)	13 (4.0)	3.2 (1)
6. Mouse brain ChE	26.0 (26)	49 (49.0)	1.0 (1)
7. Mouse whole-blood ChE	31.0 (22)	85 (56.7)	1.4 (1)
8. Mouse serum ChE (ASCh)	1.4 (4.7)	3.7 (13.2)	0.3 (1)
(BuSCh)	1.7 (3.4)	4.1 (5.3)	0.8 (1)
9. Mouse liver ChE	-	4.4 (10.5)	0.4 (1)

Note a. Values in parentheses represent relative rate constants.
Note b. Acetylthiocholine was used as substrate.
Note c. Butyrylthiocholine was used as substrate.

k_i, i.e., where the anticholinesterase activity of the less active $(R)_P$-fonofos oxon isomer is given the value of unity, are presented parenthetically. These values or k_i ratios pro-

vide a measure of the relative inhibitory potency of the oxon isomers. For example, the k_i ratio given for the inhibition of HFAChE by $(S)_P$-oxon indicates that this isomer is 62.5-fold more potent as an anticholinesterase than the $(R)_P$ isomer.

The data reveal that variable but significant rate differences exist in the inhibition of ChE by the enantiomers of fonofos oxon against the different ChE enzymes. In every case, $(S)_P$-fonofos oxon was a more potent inhibitor than the $(R)_P$ enantiomer. In general, the $(S)_P$-oxon was approximately 2-fold more effective as an inhibitor than the racemic mixture. This observation is reasonable since most of the anticholinesterase activity in the racemic mixture can be attributed to the more potent $(S)_P$ enantiomer. Since half of the racemic oxon consists of the more active $(S)_P$ form, the inhibitory activity of the racemic mixture should be slightly greater than half of that of the $(S)_P$ enantiomer. The largest difference in anticholinesterase activity between the two enantiomers was found in the inhibition of HFAChE (62.5 fold), followed by a mouse whole-blood (56.7 fold) and brain (49 fold) ChE. Further, with a k_i value of 5.5×10^7 $M^{-1}min^{-1}$ for the $(S)_P$ isomer, HFAChE evidently was most sensitive to inhibition among the various enzymes examined. Much smaller differences in k_i values were observed between the enantiomers for the inhibition of serum ChE (no. 5 and 8 in Table 4). Compared to HFAChE, the serum cholinesterases were substantially less sensitive to inhibition by the $(S)_P$-oxon and this was the principal cause for the smaller k_i differences between the $(S)_P$ and $(R)_P$ isomers. Differences in k_i values between the isomers for the inhibition of BAChE and EEAChE were intermediate to those for HFAChE and serum ChE. Overall, the order of differences in k_i between the isomers were HFAChE > BAChE > HSChE.

The rate differences observed in enzyme inhibition were qualitatively consistent with the differences observed in toxicity between the isomers, i.e., the stronger ChE inhibitors were more potent toxicants. Comparison of house fly toxicity in Table 3 with HFAChE inhibition data in Table 4 shows a 12-fold difference in toxicity between $(S)_P$- and $(R)_P$-fonofos oxon but a 62.5-fold difference in anticholinesterase activity. Similar relationships also were found for mouse toxicity and mouse brain or blood ChE inhibition, e.g., a 6-fold difference in mouse oral toxicity (2.6-fold intraperitoneally) was observed between the $(S)_P$- and $(R)_P$-fonofos oxons compared to 50- and 60-fold differences in the inhibition of the mouse brain and mouse blood cholinesterases. The smaller difference in toxicity between the enantiomers compared to inhibition rates suggests that other processes involved in intoxication and detoxication, particularly the latter, also are affected by chirality.

In vivo inhibition of cholinesterases

The in vivo inhibition of ChE in white mice and house flies after treatment with the enantiomers and racemates of fonofos and fonofos oxon was examined. Figures 1 and 2 provide plots showing enzymatic activity after different time intervals of brain and whole-blood ChE obtained from mice treated with 3.0 and 0.6 mg/kg of the enantiomers and racemates of fonofos and fonofos oxon, respectively. The results reveal a significant difference in the inhibition of brain and whole-blood ChE from mice treated with the two fonofos enantiomers. During the initial 2-4 hr after treatment, both brain and blood ChE were inhibited in mice treated with $(R)_P$-fonofos at a faster rate and to a greater extent than the $(S)_P$ isomer. As expected, the rate of inhibition by racemic fonofos was in between that found for the enantiomers. At the common time interval of 2 hr after treatment, more than 80% of the brain and blood ChE

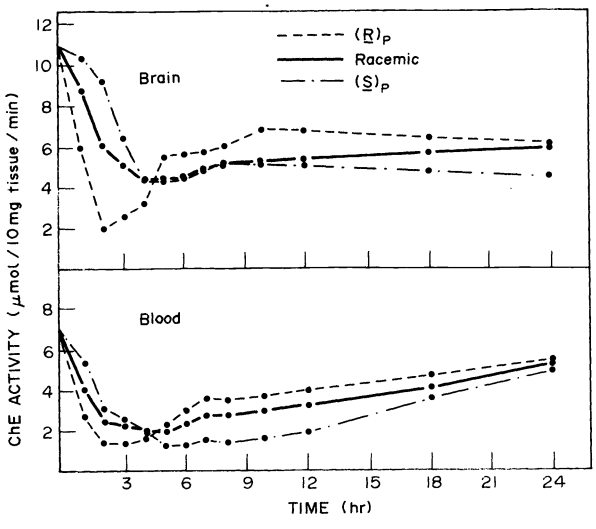


Fig. 1. Level of cholinesterase activity in mouse brain and blood at various time intervals after intraperitoneal administration of 3 mg/kg racemic and enantiomeric fonofos. Each point represents the mean of 3 replications.

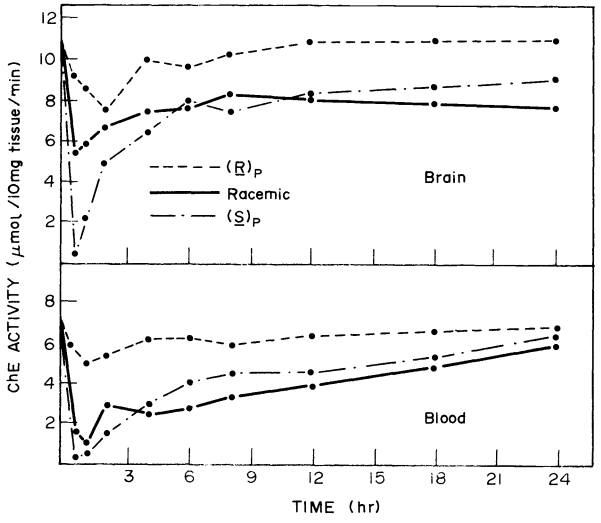


Fig. 2. Level of cholinesterase activity in mouse brain and blood at various time intervals after intraperitoneal administration of 0.6 mg/kg racemic and enantiomeric fonofos oxon. Each point represents the mean of 3 replications.

obtained from mice treated with $(R)_P$-fonofos was inhibited, compared to 15 and 60% inhibition of the respective enzymes obtained from mice treated with the $(S)_P$ isomer. The initial relative rates of inhibition, therefore, are in good agreement with the differences in toxicity between the enantiomers of fonofos (see Table 3).

Stereoselective inhibition of brain and whole-blood ChE obtained from mice treated with 0.6 mg/kg of the enantiomers of fonofos oxon also was observed. The ChE depression curves in Fig. 2 show that the cholinesterases from mice treated with $(S)_P$-oxon were inhibited at a faster rate and to a greater extent than mice treated with racemic or $(R)_P$-oxon. At the same interval of 30 min after treatment, $(S)_P$-oxon inhibited approximately 95% of the brain and blood ChE, while approximately 18 and 30% inhibition of the respective enzymes was observed with the enantiomeric $(R)_P$-oxon. Maximum inhibition of both blood and brain ChE by $(S)_P$-oxon occurred 30 min after treatment. This also appeared to be the situation with the inhibition of blood ChE by the $(R)_P$-oxon, but inhibition of brain ChE was slower and 2 hr were required before maximum inhibition was attained. Beginning at the point of maximum inhibition, a gradual increase of ChE activity occurred and in general a major portion of the original cholinesterase activity was recovered after 24 hr. As in the case of fonofos, good agreement between toxicity of the enantiomers or racemate and *in vivo* ChE inhibition was evident.

Stereoselectivity in *in vivo* inhibition of HFAChE also was observed. Figure 3 presents

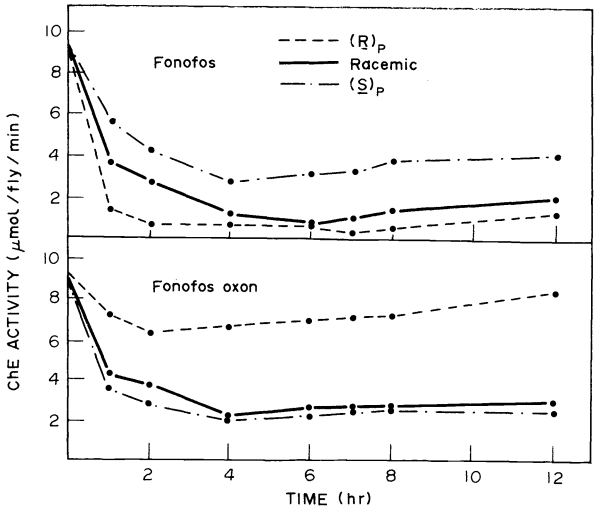


Fig. 3. Level of cholinesterase activity in house fly-head at various time intervals after topical administration of 10 μg/g racemic and enantiomeric fonofos, and 5 μg/g racemic and enantiomeric fonofos oxon. Each point represents the mean of 3 replications.

results for the in vivo inhibition of HFAChE after topical application of house flies with 10 and 5 µg/g of the enantiomers and racemates of fonofos and fonofos oxon, respectively. HFAChE was inhibited at a faster rate and extent by the more toxic $(R)_P$-fonofos or $(S)_P$-fonofos oxon than their enantiomers. In contrast to the mouse enzymes, very small amounts of recovery or regeneration of inhibited HFAChE was observed. The results obtained from the in vivo ChE inhibition studies were qualitatively consistent with the results obtained from toxicity and in vitro ChE inhibition studies. The more toxic enantiomers of fonofos and fonofos oxon inhibited HFAChE both in vivo and in vitro to a greater extent than the less toxic enantiomer.

METABOLISM

In vitro metabolism of the chiral isomers of fonofos in the presence of mouse liver microsomal mixed-function oxidase

Prior to the study of the chiral isomers of fonofos, the racemate was first examined to determine the conditions for maximum conversion of fonofos to fonofos oxon. Using racemic ^{14}C-phenyl fonofos, maximum enzymatic activity was found between pH 7.4 and 7.8 and 0.1 M Tris buffer was found superior to 0.1 M phosphate. A time course study showed that the amount of fonofos oxon formed increased linearly for approximately 30 min and then slowly leveled off to a plateau after 2 hr. As expected, NADPH was essential to metabolic activity. Based on the amount of fonofos being metabolized, the concentration of fonofos for maximum MFO activity was approximately 96 µM or 120 µg per 5 ml incubation mixture. However, maximum efficiency for fonofos oxon formation was observed at a fonofos concentration of about 59 µg per 5 ml (48 µM). Therefore, this concentration was selected for subsequent experiments on the stereochemical aspects of the oxidative desulfuration reaction. Besides fonofos oxon, the other metabolic products observed from the use of ^{14}C-phenyl fonofos were diphenyl disulfide (DPDS) and minor amounts of diphenyl disulfide oxide (also see Table 5).

Data for the amounts of fonofos oxon and DPDS isolated per unit weight of mouse liver microsomes (µg fonofos equivalents per mg protein) in the absence and presence of esterase inhibitors and other additives are presented in Table 5. The essentiality of NADPH in the

TABLE 5. Effect of cofactors and inhibitors on the in vitro microsomal oxidation of racemic ^{14}C-phenyl fonofos

	Amounts of metabolic products (µg fonofos equivalent/mg protein)			
	Oxon	DPDS	DPDS-oxide	Total
Control[a]	0.76[b]	0.85	0.07	1.68
-NADPH	-	-	-	-
CO-Air (1:1)	0.07	0.02	0.02	0.11
+ p-Nitrophenyl phosphate (1 mM)	0.66	0.62	0.02	1.30
+ Bis-p-nitrophenyl phosphate (1 mM)	0.80	0.65	0.02	1.47
+ Tri-p-nitrophenyl phosphate (1 mM)	0.71	0.85	0.04	1.60
+ Paraoxon	0.81	0.64	0.03	1.48
+ Tributyl phosphorotrithioate (1 mM)	0.44	0.40	0.04	0.88

Note a. The standard incubation mixture contained 0.1 M Tris buffer pH 7.8, G-6-P (1 mM), NADPH (0.5 mM), G-6-P dH (1 EU), mouse liver microsomal suspension (17.5 mg) and fonofos enantiomer (59 µg). Incubation was carried out at 37.5° for 2 hr.

Note b. Each value represents the average of two replications.

metabolic reaction is clearly indicated and this, along with the drastic reduction in products observed in a carbon monoxide atmosphere, provided evidence that the metabolic reactions were catalyzed by an NADPH-dependent microsomal MFO enzyme system. In the control reaction, significantly larger amounts of DPDS were obtained relative to fonofos oxon. Owing to the possibility that the formation of DPDS was catalyzed by an esterase present in the microsomal fraction (Ref. 8), i.e., via esterase-catalyzed hydrolysis of fonofos or fonofos oxon to benzenethiolate and subsequent oxidative coupling of the benzenethiolate to DPDS, the effect of a variety of esterase inhibitors on the relative yields of products was examined. Although the addition of some of these inhibitors, particularly paraoxon and bis-(p-nitrophenyl) phosphate, resulted in reversing the relative amounts of products compared to the control reaction, the magnitude of the reversal was not large enough to warrant use of inhibitors in subsequent studies with the chiral isomers. The reversal of products, however, appeared to be caused by decreased formation of DPDS, indicating that microsomal esterases contributed to a small extent to DPDS formation. Since DPDS was not formed in the absence of NADPH, esterases probably are involved in the hydrolysis of fonofos oxon but not fonofos.

Comparable control experiments using ^{14}C-ethoxy-fonofos largely supported the results obtained with the ^{14}C-phenyl labeled fonofos. In this case ethyl ethylphosphonothioic acid (ETP) and ethyl ethylphosphonic acid (EOP), besides fonofos oxon, were obtained as metabolic products. In the absence of NADPH, no ETP or EOP was detected, again providing support for an oxidative pathway for the formation of degradation products as well as oxon.

After establishment of optimum conditions for conversion of fonofos to fonofos oxon, the stereochemistry of the oxidative desulfuration of $(\underline{S})_P$- and $(\underline{R})_P$-fonofos was investigated. Two separate sets of 300 individual incubations (59 µg enantiomer per incubation) with $(\underline{S})_P$-fonofos and a single set of the same number of incubations with $(\underline{R})_P$-fonofos were carried out. From a total of 17.5 mg $(\underline{S})_P$-fonofos (300 X 59 µg) approximately 3 mg of fonofos oxon was isolated from each set. A larger yield of 5.3 mg oxon was obtained from the $(\underline{R})_P$-fonofos. The oxons obtained from each fonofos enantiomer were determined to be >99% pure (disregarding isomeric content) as estimated by autoradiography of a tlc plate and by glc analysis. Optical rotation measurements in cyclohexane solvent using a 1 dm cell gave the following values for each oxon: from $(\underline{S})_P$-fonofos, +0.148° (2.98 mg) and +0.190° (3.07 mg); from $(\underline{R})_P$-fonofos, -0.369° (5.33 mg). Calculated specific rotations ($[\alpha]_D^{25}$) are presented in Table 6, along with those of the starting fonofos enantiomers. The degree of stereospeci-

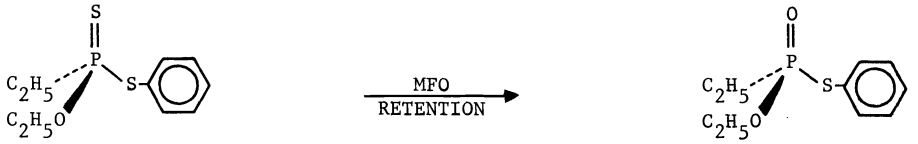
TABLE 6. Specific rotation of starting $(\underline{R})_P$- and $(\underline{S})_P$-fonofos and respective oxons isolated after incubation of each fonofos isomer with mouse liver microsomal mixed-function oxidase

Fonofos	$[\alpha]_D^{25}$ [a]	Fonofos Oxon	$[\alpha]_D^{25}$	Stereospecificity
$(\underline{S})_P$	+ 138.40°(c 0.301)	$(\underline{R})_P$	+ 49.78°(c 0.298)	72% retention
$(\underline{S})_P$	+ 138.40°	$(\underline{R})_P$	+ 61.83°(c 0.307)	77% retention
$(\underline{R})_P$	- 114.56°(c 0.309)	$(\underline{S})_P$	- 69.29°(c 0.533)	79% retention

Note a. Rotations were determined in a Rudolph high precision polarimeter at 25° using a 1 dm cell, cyclohexane solvent, and sodium D line.

ficity of the desulfuration reaction was calculated by comparing the specific rotations of the isolated oxons with those of the oxons [$(\underline{R})_P$ +112.64° (c 1.02), $(\underline{S})_P$ -121.70° (c 0.636)] which were synthesized from the respective enantiomers of O-ethyl ethylphosphonothioic acid. Owing to differences in the specific rotations of the synthesized isomers of fonofos and fonofos oxon, these values must be regarded as estimates.

The results provided conclusive evidence that mouse liver MFO catalyzed conversion of fonofos to fonofos oxon proceeded predominantly with retention of configuration, as depicted below with $(\underline{S})_P$-fonofos.



$(\underline{S})_P$-fonofos $(\underline{R})_P$-fonofos oxon

Stereoselective rate differences in metabolism

Because of the general phenomenon of stereoselectivity in enzyme interactions with chiral substrates and inhibitors, the effect of chirality on the relative susceptibilities of the fonofos enantiomers to oxidative metabolism was investigated. The relative amounts of fonofos isomers metabolized under optimal and identical conditions of mouse liver MFO activity after different time intervals are presented graphically in Fig. 4. The plots clearly reveal a faster rate of metabolism for $(\underline{R})_P$- than for $(\underline{S})_P$-fonofos.

Similar plots showing the relation between time and amounts of DPDS and oxon obtained from each fonofos enantiomer and racemate are presented in Figs. 5 and 6, respectively. Consistent with the values for the disappearance of fonofos, larger amounts of both DPDS and fonofos oxon were obtained at each interval from $(\underline{R})_P$- than from $(\underline{S})_P$-fonofos. Based on the plots in Figs. 5 and 6, it appears that the substantial difference in the rate of metabolism of the fonofos isomers is attributable more to the difference in the reaction leading to DPDS than to fonofos oxon. Thus, while $(\underline{R})_P$-fonofos was metabolized at a faster rate than its enantiomer, a significantly larger fraction of the product was in the form of degraded material.

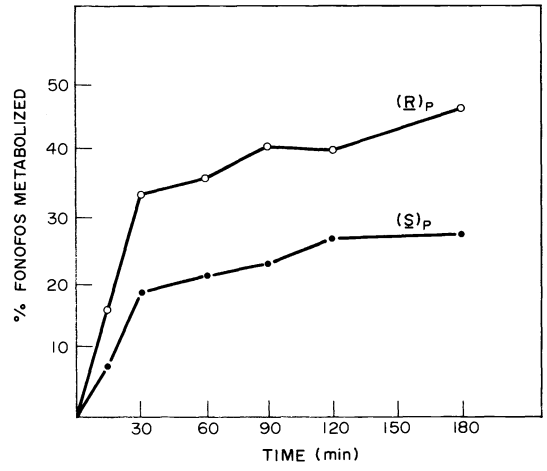


Fig. 4. Time course of the in vitro metabolism of $(S)_P$- and $(R)_P$-fonofos by mouse liver microsomal mixed-function oxidase system. Each value represents the average of two replications.

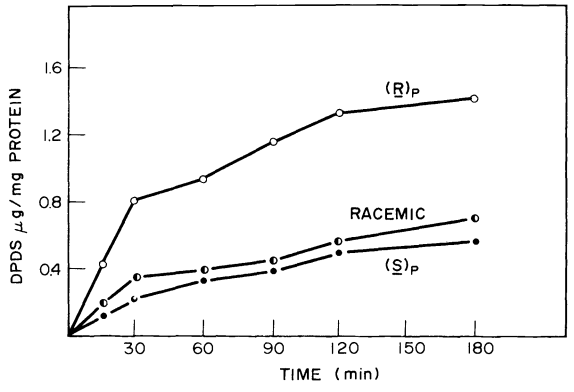


Fig. 5. Time course of the formation of diphenyl disulfide (DPDS) in μg fonofos equivalent for the in vitro metabolism of racemic $(S)_P$- and $(R)_P$-fonofos by mouse liver microsomal mixed-function oxidase. Each value represents the average of two replications.

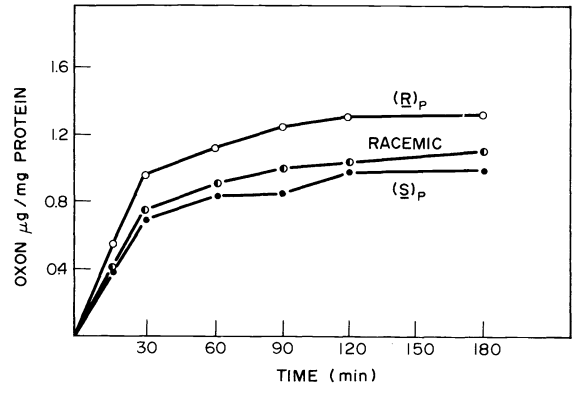


Fig. 6. Time course of the formation of fonofos oxon in μg fonofos equivalent for the in vitro metabolism of racemic (◐), $(S)_P$- (●), and $(R)_P$-fonofos (○) by mouse liver microsomal mixed-function oxidase. Each value represents the average of two replications.

Further evidence confirming the greater susceptibility to metabolism of $(R)_P$-fonofos compared to its enantiomer was found in studies with racemic ^{14}C-fonofos. When the racemate was incu-

bated with liver MFO, the unmetabolized fonofos which was recovered gave a specific rotation of $[\alpha]_D^{25}$ +7.63° (c 1.704 in cyclohexane). By assuming a specific rotation of +138.4° for $(\underline{S})_P$-fonofos, the calculated ratio of $(\underline{S})_P$- to $(\underline{R})_P$-fonofos in the recovered material was approximately 53/47.

Effect of esterases on fonofos chiral isomers

In seeking a satisfactory explanation for the toxicity differences between the chiral isomers of fonofos and fonofos oxon, the possible impact of differences in hydrolytic degradation also was considered. Data for the relative amounts of DPDS produced after incubation for 1 hr of the various ^{35}S-enantiomers with mouse liver homogenate and microsome, rat and mouse blood serum are presented in Table 7. Enzyme incubations were carried out in 0.1 M Tris

TABLE 7. Effect of enzyme and cofactor on the degradation of phenyl-^{35}S-fonofos and fonofos oxon to diphenyl disulfide

	Diphenyl disulfide in % of applied radioactivity[a]			
	$(\underline{S})_P$-Fonofos	$(\underline{R})_P$-Fonofos	$(\underline{S})_P$-Oxon	$(\underline{R})_P$-Oxon
Mouse liver homogenate[b]				
+ NADPH[c]	12.42	20.65	0.58	0.39
- NADPH	N.D.[d]	N.D.	0.15	0.13
Mouse liver microsome				
+ NADPH	14.87	26.74	0.49	0.44
- NADPH	N.D.	N.D.	<0.1	<0.1
Mouse serum	N.D.	N.D.	0.95	0.58
Rat serum	N.D.	N.D.	2.76	1.19

Note a. The data are the average of two separate experiments; each experiment consisted of two replications.
Note b. Amounts of liver and serum protein per incubation are as follows: mouse liver homogenate - 25 mg; mouse liver microsome - 17 mg; rat and mouse serum - 8 mg.
Note c. Final concentration of NADPH was 1 mM.
Note d. Not detected.

buffer at pH 8.0. In the absence of NADPH, neither mouse liver homogenate nor microsome had any effect on fonofos degradation, in agreement with preceding studies on the oxidative activation and degradation of fonofos. Further, fonofos also was stable to the action of mouse and rat serum both in the presence and absence of possible cofactors.

In contrast to fonofos, appreciable degradation of fonofos oxon to DPDS took place in the presence of mouse and rat serum. Significant amounts of DPDS also were formed after incubation of the oxon with liver homogenate and microsomal fraction, particularly when NADPH was present. In all cases ^{35}S-DPDS was the major product observed although two other metabolites of minor significance occasionally were detected. The identity of these metabolites is not known. Although degradation of fonofos oxon to DPDS by mouse and rat serum was slow compared to the oxidative reactions mediated by MFO, the results revealed that $(\underline{S})_P$-fonofos oxon was hydrolyzed at a faster rate than its $(\underline{R})_P$ enantiomer. Plots showing differences in the rate of formation of DPDS obtained from each isomer after incubation with rat serum are given in Fig. 7. From the slopes $(\underline{S})_P$-fonofos oxon evidently was hydrolyzed at a rate more than 2-fold faster than its enantiomer. At the 60-min incubation period 2.76% of the applied radioactivity was converted to DPDS from $(\underline{S})_P$-fonofos oxon, compared to 1.19% from the $(\underline{R})_P$ enantiomer.

Metabolism in the house fly

House flies were treated at 6.5 µg/g of each of the fonofos enantiomers, a dosage which is approximately the LD_{50} for the more toxic $(\underline{R})_P$ isomer and sub-lethal for the $(\underline{S})_P$ isomer. At this dosage, about 20% of the house flies treated with $(\underline{R})_P$-fonofos were down 6 hr after treatment and the remaining flies showed symptoms of cholinergic poisoning. No intoxication symptoms were obvious in flies treated with the $(\underline{S})_P$ isomer.

The rate of fonofos penetration was estimated from the amount of radioactivity recovered from the external surface of treated flies and also from the amounts present internally in the fly. The fonofos enantiomers were rapidly absorbed by the flies but no significant difference in penetration rates between the two enantiomers was observed.

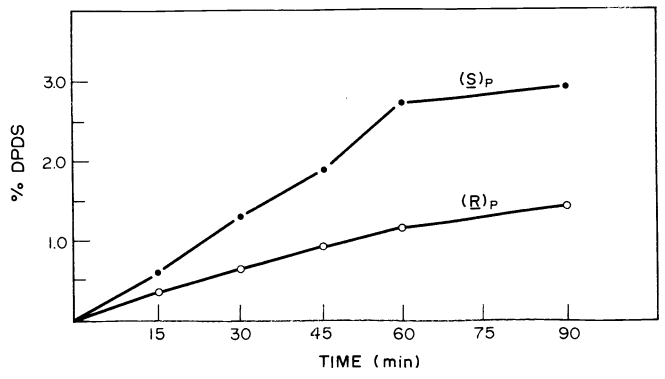


Fig. 7. Time course of the formation of diphenyl disulfide (DPDS) in percent of applied radioactivity for the in vitro metabolism of $(S)_P$- and $(R)_P$-fonofos oxon by rat serum esterase. Each value represents the average of two replications.

House flies were examined for fonofos metabolism 4 hr after topical treatment with 6.5 µg/g ^{35}S-phenyl labeled enantiomer. The external wash, organo-soluble and water-soluble fractions of the fly homogenate were analyzed separately. Data summarizing the nature and amounts of metabolites obtained from the flies are presented in Table 8. Unchanged fonofos was the

TABLE 8. Nature and amounts of metabolites in various fractions from house flies treated with 6.5 µg/g phenyl-^{35}S-fonofos enantiomers

	% of Applied Radioactivity	
	$(S)_P$-fonofos	$(R)_P$-fonofos
External wash		
Fonofos	19.3	21.6
Fonofos oxon	0.7	0.6
Methyl phenyl sulfone	0.5	0.2
Methyl phenyl sulfoxide	5.0	1.7
Origin	9.6	10.9
TOTAL	35.1	35.0
Fly homogenate - Organo-soluble		
Fonofos	28.5	36.1
Methyl phenyl sulfone	1.9	1.4
Origin	1.8	2.1
TOTAL	32.2	39.6
- Water-soluble		
Diphenyl disulfide	0.4	1.2
Fonofos	0.4	3.1
Methyl phenyl sulfone	1.3	1.0
Methyl phenyl sulfoxide	1.7	1.3
3-OH-Phenyl methyl sulfone	0.8	0.7
4-OH-Phenyl methyl sulfone	12.8	6.5
Origin	4.0	1.6
TOTAL	21.4	15.4
Unextractable Radioactivity	7.6	6.4
TOTAL RECOVERY	96.3	96.4

principal constituent in the external wash, along with lesser amounts of fonofos oxon, methyl phenyl sulfoxide and sulfone, and a substantial amount of polar material which remained at the origin of the tlc plate. The polar material was not identified but it probably was a

conjugated metabolite coming from the fly excreta. Fonofos also was the major constituent present internally in the flies, most of it being recovered in the organo-soluble fraction along with methyl phenyl sulfone. Water soluble metabolites were isolated and identified after subjecting the aqueous phase to acid hydrolysis. In addition to the same metabolites found in the other fractions, 3-hydroxyphenyl and 4-hydroxyphenyl methyl sulfones also were detected, the latter being present in greatest abundance. Total recovery of radioactivity was about 96% with both enantiomers.

On the whole, the metabolism of the fonofos enantiomers was qualitatively and quantitatively similar. However, based on the relative amounts of fonofos and 4-hydroxyphenyl methyl sulfone isolated, the less toxic $(\underline{S})_p$-fonofos appeared to be metabolized to a greater extent during the 4-hr holding period than the $(\underline{R})_p$ isomer. The difference is probably attributable to the physical state of the flies, i.e., those treated with $(\underline{R})_p$-fonofos were visibly intoxicated while those treated with the $(\underline{S})_p$ isomer appeared to be normal.

Metabolism of fonofos in the white mouse

Mice were treated orally at two dosages, 4 and 8 mg/kg. At 8 mg/kg $(\underline{R})_p$-fonofos, mice showed severe poisoning symptoms 4 hr after treatment and remained in an intoxicated state during the entire 96 hr metabolism holding period. A total of 5 mice were treated with $(\underline{R})_p$-fonofos and of these only 2 survived the 96 hr holding period. Mice treated with 8 mg/kg $(\underline{S})_p$-fonofos and 4 mg/kg $(\underline{R})_p$-fonofos showed mild symptoms of cholinergic poisoning but recovered after 12 hr. Mice treated with 4 mg/kg $(\underline{S})_p$-fonofos were essentially unaffected. The oral LD_{50} values for $(\underline{R})_p$- and $(\underline{S})_p$-fonofos are 9.5 and 32 mg/kg, respectively.

The rate of excretion of administered radioactivity was monitored in both urine and feces. The results are presented in Fig. 8 for the 2 dosages of 8 and 4 mg/kg. The figures show

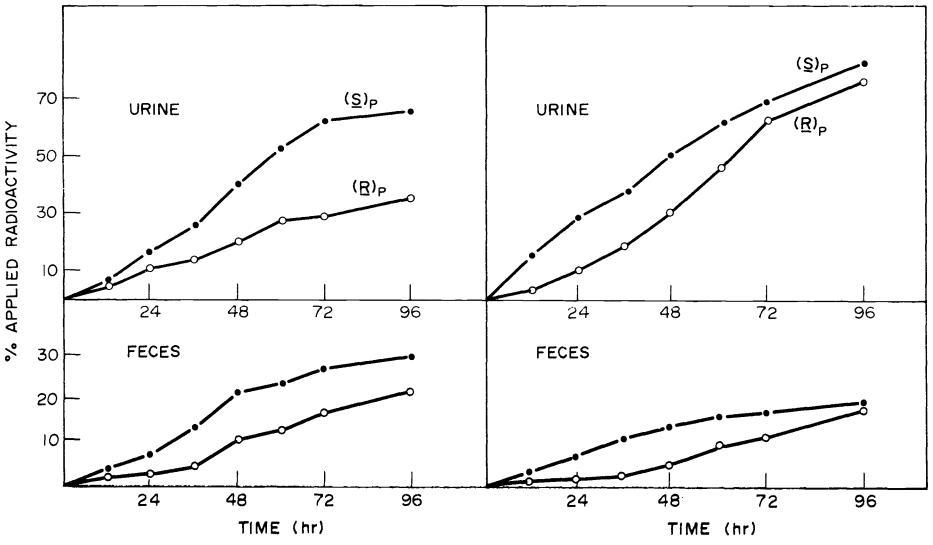


Fig. 8. Plots showing the percentage of applied radioactivity present in urine and feces obtained from female white mice at different time intervals following oral administration of 8 mg/kg (left) and 4 mg/kg (right) $(\underline{S})_p$-fonofos and $(\underline{R})_p$-fonofos.

that at both dosages radioactivity associated with the less toxic $(\underline{S})_p$ isomer was eliminated faster from the mouse than its enantiomer. At the higher dosage of 8 mg/kg, about 95% of administered $(\underline{S})_p$ isomer was eliminated within the 96-hr holding period compared to about 50% for the more toxic $(\underline{R})_p$ isomer. The major route of elimination was via the urine. At the lower dosage of 4 mg/kg, the initial rate of elimination was substantially smaller for the $(\underline{R})_p$ isomer, but the rate gradually increased during the holding period and the total recovery of administered radioactivity for the two isomers was about the same after 96 hr. About 3-fold more radioactivity was eliminated from the mouse treated with the $(\underline{S})_p$ isomer than its enantiomer during the first 24 hr after treatment.

Table 9 contains data on the nature and total amounts of ^{35}S-labeled metabolites present in the urine and feces of mice 96 hr after treatment with the fonofos isomers at the two dosages. The expected metabolites were found, including unchanged fonofos, fonofos oxon, methyl phenyl sulfoxide in the organo-soluble fraction, and 3-hydroxyphenyl and 4-hydroxyphenyl methyl sulfone in the aqueous fraction. Small amounts of diphenyl disulfide and diphenyl disulfide oxide also were detected in the organo-soluble phase. These metabolites

TABLE 9. Total fonofos and metabolites isolated from the urine and feces of mice 96 hr after treatment with 4 mg/kg and 8 mg/kg enantiomers of phenyl-^{35}S-fonofos

	% of Applied Radioactivity			
	4 mg/kg		8 mg/kg	
	$(S)_P$	$(R)_P$	$(S)_P$	$(R)_P$
Diphenyl disulfide	-	-	0.28	-
Fonofos	0.53	0.76	2.67	3.06
Fonofos oxon	0.04	0.65	0.11	0.18
Diphenyl disulfide oxide	0.50	0.75	0.22	-
Methyl phenyl sulfoxide	4.65	3.89	11.08	4.14
Methyl phenyl sulfone	17.72	18.25	16.43	8.58
4-OH-Phenyl methyl sulfone	15.30	14.15	13.85	8.70
3-OH-Phenyl methyl sulfone	13.16	13.40	15.69	7.74
Origin	26.30	28.75	17.60	12.20
Unextractable (urine)	16.10	14.70	14.67	8.42
TOTAL	94.3	95.3	92.6	53.0

were not reported in previous studies with rats and racemic fonofos (Ref. 9, 10). A relatively large amount of radioactivity remained as a polar unknown at the origin of the tlc plate or as unextractable water soluble materials even after acid hydrolysis. These probably were degraded and conjugated products of fonofos.

The water-soluble fraction contained large amounts of 3- and 4-hydroxyphenyl methyl sulfone. These were excreted as conjugated products and were liberated by acid hydrolysis. These metabolites were reported previously by McBain et al. (Ref. 9, 11).

At the lower dosage of 4 mg/kg, very little difference in the metabolism of $(R)_P$- and $(S)_P$-fonofos was observed. For all practical purposes, the amounts of each metabolite obtained from the fonofos isomers after treatment at 4 mg/kg are the same. However, notably lesser amounts of metabolites were formed from $(R)_P$-fonofos than from its enantiomer at 8 mg/kg. This is doubtlessly attributable to the severely intoxicated state of the mouse treated with the $(R)_P$ isomer. Evidently, the normal excretory and metabolic functions of the animal were inhibited.

Metabolism of fonofos oxon in mice

Mice were treated orally with 4 mg/kg ^{35}S-phenyl labeled $(S)_P$- and $(R)_P$-fonofos oxon. This dosage is sublethal for the $(R)_P$-oxon and is approximately the LD_{20} value for the more toxic $(S)_P$ isomer. The LD_{50} values of $(S)_P$- and $(R)_P$-fonofos oxon are 6 and 38 mg/kg, respectively. Animals treated with the $(S)_P$ isomer showed cholinergic poisoning symptoms but recovered after 12 hr. Animals treated with $(R)_P$-oxon were normal.

As in the case of fonofos, differences in the excretion rates of radioactivity from mice treated with the two oxon isomers also were observed (Fig. 9). The major route of excretion was via the urine. Excretion was slower in the mouse treated with the more toxic $(S)_P$ isomer

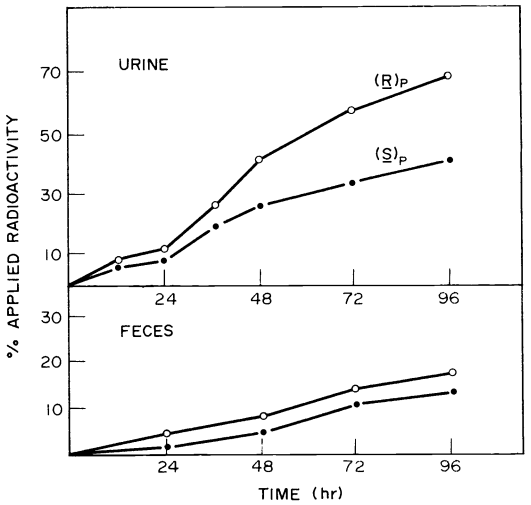


Fig. 9. Plots showing the percentage of applied radioactivity present in urine and feces obtained from female white mice at different time intervals following oral administration of 4 mg/kg ^{35}S-$(S)_P$ (•) and $(R)_P$ (o) isomers of fonofos oxon.

and the total recovery of administered radioactivity at the end of 96 hr was 56%. In comparison, 89% of the administered $(\underline{R})_P$ isomer was recovered. Again, the difference in elimination rates is probably a result of the different toxicological conditions of the mice.

Table 10 presents data on the nature and amounts of ^{35}S-labeled metabolites in the urine and

TABLE 10. Nature and amounts of metabolites recovered from urine and feces of white mice treated with 4 mg/kg of each enantiomer of phenyl-^{35}S-fonofos oxon

	% of Applied Radioactivity			
	Urine		Feces	
	$(\underline{S})_P$	$(\underline{R})_P$	$(\underline{S})_P$	$(\underline{R})_P$
Organo-soluble				
Fonofos oxon	0.54	0.38	0.14	-
Methyl phenyl sulfoxide	1.75	3.62	0.60	0.09
Methyl phenyl sulfone	3.90	9.34	0.86	2.16
Origin	0.24	2.48	2.05	1.93
TOTAL	6.43	15.82	3.65	4.18
Water-soluble				
4-OH-Phenyl methyl sulfone	11.15	19.75	2.84	3.76
3-OH-Phenyl methyl sulfone	9.49	14.91	2.43	3.14
Origin	6.46	8.56	3.58	4.30
TOTAL	27.10	43.22	8.85	11.20
Total Unextractable	6.82	9.72	3.75	4.77
Total Recovery	40.35	68.76	16.25	20.15

Total recovery of applied radioactivity

$(\underline{S})_P$-fonofos oxon 56.6%

$(\underline{R})_P$-fonofos oxon 88.9%

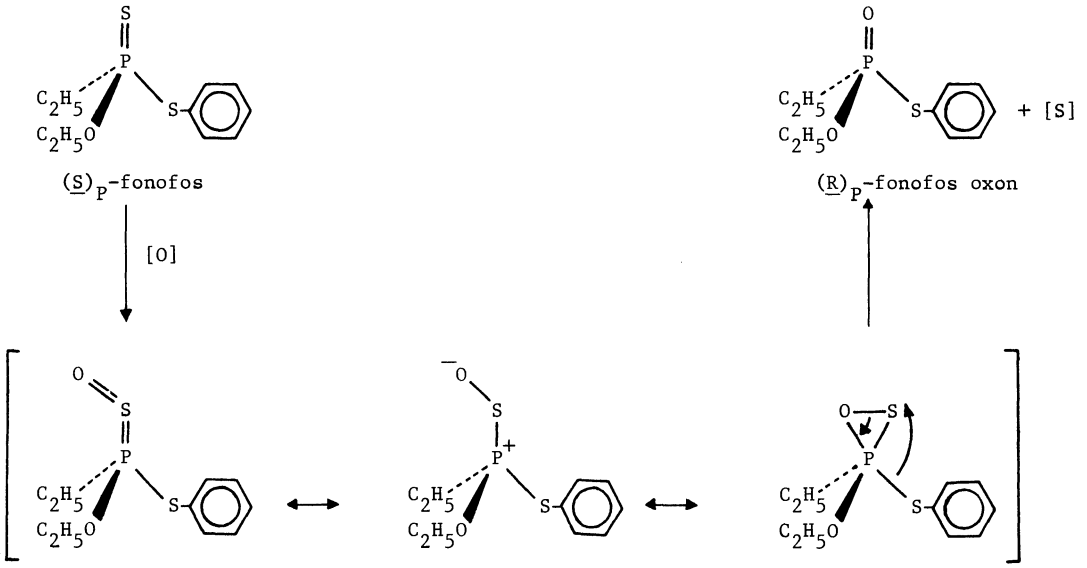
feces of mice treated with the oxon enantiomers. The metabolites identified were methyl phenyl sulfoxide and sulfone, and 3- or 4-hydroxyphenyl methyl sulfone, along with a small amount of unchanged oxon. Diphenyl disulfide and diphenyl disulfide oxide which were found in the fonofos metabolism studies were not detected. Because of the larger amount of radioactivity excreted from mice treated with $(\underline{R})_P$-oxon, proportionately larger amounts of each metabolite were recovered from $(\underline{R})_P$- than from $(\underline{S})_P$-oxon treated mice. The relative amounts of metabolites, i.e., relative to the total amount of radioactivity excreted, remained fairly constant and there appeared to be little difference in the pattern of metabolism between the two enantiomers.

DISCUSSION

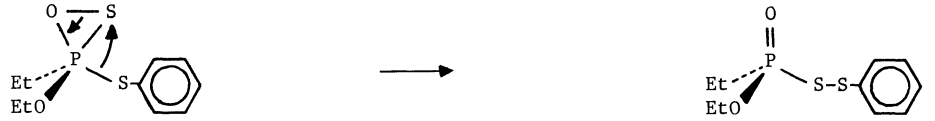
The elucidation of the stereochemical course of the MFO-catalyzed conversion of fonofos to fonofos oxon was not only essential to the explanation of the order of toxicity of the fonofos isomers, but was also of theoretical significance. Based on the stereochemical results, a mechanism consistent with the preferential retention of configuration observed for the desulfuration reaction may be suggested. The mechanism is similar to that proposed by Ptashne and Neal (12) for the MFO-catalyzed conversion of parathion to paraoxon in which initial attack by oxygen on the thiono-sulfur to give the S-oxide was postulated. Closure of the S-oxide to the three-membered ring with subsequent loss of sulfur gives the oxon with retention of configuration.

The stereospecific oxidative desulfuration of O-ethyl O-2-nitro-5-methylphenyl N-isopropylphosphoramidothioate (S-2571) and cyanofenphos to the corresponding oxon by the action of rabbit and rat liver microsomes and NADPH recently was reported by Ohkawa *et al.* (13, 14). Incubation of levorotatory S-2571 resulted in levorotatory oxon.

The conversion of $(\underline{S})_P$-fonofos (4a) to $(\underline{R})_P$-fonofos oxon (6b) by the action of m-chloroperoxybenzoic acid occurred predominantly with retention of configuration of the phosphorus atom. In contrast, oxidation to the rearrangement product, $(\underline{R})_P$-phenyl ethyl(ethoxy)-phosphinyl disulfide (8b) took place with predominant inversion of configuration. In the



case of the reaction leading to the oxon, the stereochemical course was identical to that observed with mouse liver MFO. While it is possible that fonofos oxon and phenyl ethyl-(ethoxy)phosphinyl disulfide are formed by two separate reactions between fonofos and m-chloroperoxybenzoic acid, the same 3-membered ring intermediate also may account for the stereochemical result as indicated below.



8b

Thus, the stereochemistry of both oxidation products may readily be accounted for by the three-membered ring intermediate. This intermediate is possible by considering hybridization of an sp^3 orbital with a d_{xy} orbital, giving two spd bonds at 71° and three sp^3 bonds (Ref. 15). Hybridization of this type is compatible with the angular strain associated with a three-membered ring. The same intermediate was suggested by Herriott (16) to explain retention observed in the peroxy acid oxidation of l-menthyl methylphenylphosphinothioate to the corresponding phosphinate.

It is well known that chirality at the phosphorus atom has a significant effect on the biological activity of phosphonothioate esters, i.e., esters which contain the P=O linkage (Ref. 17). However, fonofos is different in that it contains the P=S moiety and metabolic activation by oxidative desulfuration to the anticholinesterase fonofos oxon is required for intoxication to take place. The toxicological data in Table 3, indicating 2- to 4-fold higher toxicity of $(R)_P$-fonofos to mice and house flies relative to $(S)_P$-fonofos, was of considerable significance since it implied that the oxon was generated in the animal with some degree of stereospecificity. Compared to fonofos, the difference in toxicity between the fonofos oxon enantiomers was greater and $(S)_P$-oxon was 2.6- (i.p.) and 12-fold more toxic to white mice and house flies, respectively, than the $(R)_P$-oxon.

The relative toxicities of $(S)_P$- and $(R)_P$-fonofos oxon to house flies and white mice were qualitatively in agreement with their relative *in vitro* anticholinesterase activities toward fly-head, mouse brain, and blood ChE, i.e., the isomer of higher toxicity was more effective in inhibiting the three enzymes. On a quantitative basis, however, the differences in anticholinesterase activities between the isomers were substantially greater (49- to 60-fold) than the differences in toxicities to house flies and white mice. Since the two isomers differ only in chirality at the phosphorus atom, perhaps a closer relationship between toxicity and anticholinesterase activity might be expected. Although it is difficult to arrive at a quantitative relationship, it appears that *in vivo* ChE inhibition is more closely correlated with toxicity (see Figs. 2 and 3). At the same intraperitoneal dosage of oxon isomers (0.6 mg/kg), the level of maximum inhibition of both brain and blood ChE obtained from mice treated with $(S)_P$-oxon was approximately 3-fold greater than enzymes obtained from mice treated with the $(R)_P$ enantiomer. Maximum inhibition, however, occurred at different time intervals after treatment and the relationship was further complicated by the differ-

ences in recovery rates of the inhibited enzymes. Nevertheless, agreement between *in vivo* inhibition of mouse ChE and intraperitoneal toxicity was good.

The relationship between *in vivo* inhibition of HFAChE and toxicity of house flies treated with the two isomers was less satisfactory. Maximum reduction of HFAChE in flies treated with 5 μg/g $(R)_P$-oxon was about 35% compared to about 80% for flies treated with the same amount of $(S)_P$-oxon, a slightly greater than 2-fold difference (see Fig. 3). This difference is substantially smaller than the 12-fold difference observed for house fly toxicity. Examination of the plots in Fig. 3 shows that HFAChE inhibited by $(R)_P$-oxon recovered to a significantly greater extent (compare activities at 12 hr) and it is possible that ChE depression over longer time periods should be considered in relating *in vivo* inhibition with toxicity.

The smaller differences in fly and mouse toxicities between $(R)_P$- and $(S)_P$-fonofos may be justified with less difficulty. It is well known that thionate esters in the pure state are poor anticholinesterases and metabolic activation to the corresponding P=O ester is required for intoxication. Studies on the *in vitro* metabolism of the two isomers in the presence of mouse liver microsomal mixed-function oxidase showed that $(R)_P$-fonofos and $(S)_P$-fonofos were converted to the respective $(S)_P$-oxon and $(R)_P$-oxon with 70-80% stereospecificity. Since $(S)_P$-oxon is a stronger anticholinesterase and is more toxic than $(R)_P$-oxon, the order of toxicity of the fonofos isomers is in the right direction. Although metabolic conversion of fonofos to the oxon occurred with predominantly retention of configuration, apparently a significant amount of inversion also took place, leading to partial racemization of the oxon. This undoubtedly contributed to the smaller toxicity ratios between the fonofos isomers.

Further insight into the explanation for the relative toxicities of the fonofos and fonofos oxon isomers was obtained from studies on the relative rates of metabolism. While the results revealed that $(R)_P$-fonofos was metabolized at a rate approximately 2-fold greater than $(S)_P$-fonofos, analysis of the products showed that $(R)_P$-fonofos was converted in greater proportion to the detoxication product DPDS than its enantiomer. Thus, even though $(R)_P$-fonofos was metabolized by mouse liver MFO at a faster rate, the difference in amounts of fonofos oxon formed from the fonofos isomers was not large. Differences in rates of metabolism of chiral organophosphorus esters recently was reported for the *in vivo* metabolism of cyclophosphamide in man (Ref. 18) and rabbit liver MFO catalyzed metabolism of S-2571 (Ref. 13).

Rat and mouse serum were effective in degrading fonofos oxon but not fonofos. Hydrolysis of the enzyme probably was effected by an esterase, e.g. the paraoxon hydrolyzing A-esterase whose presence has been demonstrated in mammalian serum (Ref. 19, 20). The *in vitro* degradation of fonofos oxon by rat or mouse serum was relatively slow compared to the oxidative reactions, but the results clearly showed that $(S)_P$-oxon was degraded at a faster rate than the $(R)_P$-oxon (approximately 2-fold). Although *in vitro* results do not necessarily represent events which take place *in vivo*, the greater susceptibility of $(S)_P$-oxon to enzymatic degradation may account for the smaller difference in toxicities between the fonofos-oxon enantiomers relative to their differences in anticholinesterase activities (50- to 60-fold). Further, since $(R)_P$-fonofos is preferentially metabolized to $(S)_P$-oxon and $(S)_P$-fonofos to $(R)_P$-oxon, the faster rate of degradation of $(S)_P$-oxon may contribute to the even smaller differences in toxicities of the fonofos enantiomers. Thus, the smaller difference in toxicities between the fonofos enantiomers compared to the relative toxicities of the oxons and their anticholinesterase activities may be accounted for by partial racemization in the oxidative desulfuration reaction and faster rate of degradation of $(S)_P$-oxon.

The *in vivo* metabolism of the chiral isomers of fonofos in house flies and white mice was straightforward and, with the exception of diphenyl disulfide and diphenyl disulfide oxide, the same metabolites previously reported by McBain *et al.* (9) and Hoffman *et al.* (10) for rats were also found in this study.

Based on the data available, it appears that the speed and extent to which metabolism occurred in either house flies or mice treated with the various enantiomers of fonofos or the oxon was related to the physiological state of the animal, i.e., metabolism occurred more slowly in distressed animals. In general, metabolism and excretion occurred more rapidly with the less toxic isomers of both fonofos and fonofos oxon. The relative amounts of each metabolite formed from either isomer, however, were similar in all cases. It is not possible to determine from the data whether the less toxic isomer is metabolized at a faster rate than its enantiomer because of stereoselectivity or because of the state of the animal. Unfortunately, owing to the nature of the radioactive label it was not possible to obtain an estimate of the amount of fonofos oxon which was formed in the animals since the various ^{35}S-labeled detoxication products are possible from either fonofos or the oxon. Additional studies using resolved isomers containing a radiolabel in the ethyl ethylphosphonyl moiety are required for the total elucidation of the effect of chirality on fonofos metabolism and toxicity.

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