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Selective Toxicity of Phoxim (Phenylglyoxylonitrile Oxime O,O-Diethyl Phosphorothioate)

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Phoxim (phenylglyoxylonitrile oxime *O,O*-diethyl phosphorothioate) and a series of analogs have been examined for toxicity to insects and the white mouse. These compounds were generally quite toxic to insects and, with the exception of the phosphonate derivative, were virtually nontoxic to mice. The PO esters were all potent inhibitors of both bovine erythrocyte and housefly-head cholinesterase but they were somewhat more effective in inhibiting the latter. Although differences in cholinesterase sensitivity to inhibition may contribute to the selective toxicity of phoxim, the evidence indicates that metabolism may play an even more important role. In mice, phoxim is rapidly degraded to nontoxic hydrolysis products. The most important pathway appears to involve desulfuration of phoxim to PO phoxim which in turn is hydrolyzed with extreme rapidity to diethyl phosphoric acid and critical levels of PO phoxim in the mouse are not reached. In addition, pathways involving cleavage of phoxim to *O,O*-diethyl phosphorothioic acid and hydrolysis of the cyano group to the carboxylic acid also were found to be important. The latter appeared to increase in importance as the administered dose was increased. Relatively large amounts of phoxim and PO phoxim were found internally in susceptible flies but phoxim was rapidly metabolized in organophosphate-resistant flies to nontoxic products. As in the mouse, hydrolysis of the PO phoxim to diethyl phsophoric acid evidently was the predominant pathway for degradation in resistant flies.

INTRODUCTION

Phoxim (phenylglyoxylonitrile oxime *O,O*-diethyl phosphorothioate) is a promising new material which is presently being developed as a wide spectrum insecticide. Based on preliminary performance data (1), phoxim has shown excellent activity for the control of a variety of insects of hygienic and agricultural importance but is remarkably safe to warm-blooded animals with a reported oral LD_{50} value to male rats of 8500 mg/kg. Thus, among the more effective organophosphorus insecticides currently in use or under development, phoxim undoubtedly is one of the compounds least toxic to mammals. In contrast to most other organophosphorus insecticides possessing favorable properties of selectivity, i.e., chemicals which are toxic to insects but nontoxic to mammals, phoxim is unusual in that it is a diethyl ester rather than a dimethyl ester as is found in malathion (diethyl mercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorodithioate), fenitrothion [*O,O*-dimethyl *O*-(4-nitro-*m*-tolyl) phosphorothioate], and others.

Because of its outstanding properties as a selective insecticide, and as part of our con-

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tinuing endeavor to provide rationale for the design of chemicals which are selectively toxic to insects, an investigation on the mode of action of phoxim was initiated. Specifically, this paper is concerned with the selective toxicity of phoxim and analogs in relation to the effect of these compounds on the target enzyme cholinesterase and the metabolism of phoxim in mice and house flies.

MATERIALS AND METHODS

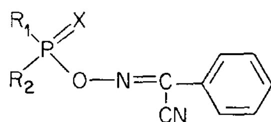
Synthesis. The various intermediates needed for the preparation of phoxim and its analogs were prepared as described below. *O,O*-Dimethyl phosphorochloridothioate [bp 49–52° (1.0 mm), n_D^{25} 1.5282], *O,O*-diethyl phosphorochloridothioate [bp 58–59° (2.5 mm), n_D^{25} 1.4685], and *O,O*-diisopropyl phosphorochloridothioate [bp 69–73° (2.0 mm), n_D^{25} 1.4874] were prepared according to Fletcher et al. (2). *O*-Ethyl ethylphosphonochloridothioate [bp 60–63° (5.0 mm), n_D^{25} 1.4901] was prepared according to Fukuto and Metcalf (3) and diethylphosphinothioic chloride [bp 80–81° (4.0–4.5 mm), n_D^{25} 1.5281] according to Christen et al. (4). Dimethyl phosphorochloridate [bp 50–52° (2.0 mm), n_D^{25} 1.4103], diethyl phosphorochloridate [bp 95–96° (21 mm), n_D^{25} 1.4152], and diisopropyl phosphorochloridate [bp 49–52° (0.7 mm), n_D^{25} 1.4142] were prepared according to McCombie et al. (5). Ethyl ethylphosphonochloridate [bp 61–62° (5.0 mm), n_D^{25} 1.4348] was prepared according to Razumov et al. (6), and diethylphosphinic chloride [bp 41–44° (0.10–0.15 mm), n_D^{25} 1.4646] according to Pollart and Harwood (7). Phenylglyoxylonitrile oxime, mp 124–125°, was prepared according to Perrot (8).

Phoxim and the various analogs listed in Table 1 were prepared by reacting the sodium salt of phenylglyoxylonitrile oxime with the appropriate chloridate in 2-butanone or toluene. The following procedure for the preparation of phoxim (I) is typical. To 5.1 g (0.031 mole) of the dried sodium salt of phenylglyoxylonitrile oxime in 100 ml 2-butanone was added dropwise over a period of 30 min 5.65 g (0.03 mole) *O,O*-diethyl phosphorochloridothioate in 40 ml 2-butanone. The flask was cooled in an ice bath during the addition, and the reaction was carried out under dry nitrogen. The mixture was warmed gently for 2 hr, cooled, washed once with 5% aqueous sodium hydroxide, twice with water, and dried over anhydrous sodium sulfate. Removal of the solvent under vacuum (1.0 mm) gave 6.8 g of a light yellow oil in 76% yield, n_D^{25} 1.5410. The product was not distilled owing to its thermal instability. Thin-layer chromatography using silica gel and a mixture of chloroform: ethyl acetate (9:1) as the developing solvent showed only a trace of impurities. Nmr and infrared spectra also confirmed the structure. Elemental analysis is given in Table 1. The PO analogs were purified by distillation in a falling-film molecular still.

^{32}P-Labeled phoxim was prepared from labeled *O,O*-diethyl phosphorochloridothioate (Radiochemical Centre, Amersham, England) according to the procedure above but on a smaller scale. Two labeled samples were prepared with specific activities of 4,371 and 9,730 cpm/μg as estimated by gas-flow counting. Thin-layer chromatographic analysis showed that each preparation was 99.0% pure. The potassium salt of *O,O*-diethyl phosphorothioic acid was prepared according to Mastin et al. (9) and diethyl phosphoric acid was prepared by hydrolysis of tetraethyl pyrophosphate according to Toy (10). ^{32}P-*O,O*-Diethyl phosphorothioic acid was obtained by alkaline hydrolysis of ^{32}P-phoxim as follows. Approximately 100 mg of ^{32}P-phoxim was placed in 10 ml ethanol containing 2 pellets of potassium hydroxide and allowed to stand at room temperature for several days. After thin-layer chromatographic analysis showed that hydrolysis was completed the solution was concentrated in a warm air jet to less than 1 ml. The acid was purified by thin-

TABLE 1

Physical properties of phoxim and analogs.



R_1	R_2	X	bp °C (mm)[a]	n_D^{25}	Analysis Calcd C	Calcd H	Found C	Found H
CH_3O	CH_3O	S	—	1.5448	44.44	4.07	44.30	4.45
C_2H_5O	C_2H_5O	S	—	1.5410	48.32	5.03	48.47	5.33
i-C_3H_7O	i-C_3H_7O	S	—	1.5210	51.53	5.82	51.76	5.98
C_2H_5	C_2H_5O	S	—	—[b]	51.06	5.35	50.66	5.38
C_2H_5	C_2H_5	S	—	1.5818	54.13	5.63	54.33	5.87
CH_3O	CH_3O	0	150 (0.025)	1.5260	47.24	4.32	47.34	4.30
C_2H_5O	C_2H_5O	0	150 (0.05)	1.5082	51.06	5.35	51.19	5.43
i-C_3H_7O	i-C_3H_7O	0	150 (0.05)	1.4992	54.19	6.12	54.28	6.33
C_2H_5	C_2H_5O	0	100 (0.025)	1.5244	54.13	5.63	53.94	5.59
C_2H_5	C_2H_5	0	130 (0.05)	1.5582	57.60	6.00	57.20	6.18

[a] All distillations carried out in a falling-film still. Temperatures reported represent wall temperatures.
[b] This sample was darkly colored and a refractive index could not be obtained.

layer chromatography using isopropanol: water:concentrated ammonium hydroxide (75:24:1) as described in the methods on metabolism. Sections containing pure *O,O*-diethyl phosphorothioic acid were scraped from the thin-layer plates, extracted with distilled water and concentrated to 0.15 ml for oral treatment of mice.

Bioassay. Toxicity to two strains of house flies (*Musca domestica* L.), the NAIDM-48 susceptible strain (S_{NAIDM}) and the Stauffer chlorthion resistant strain (R_{SC}), and to the German cockroach (*Blatella germanica*) was determined by the topical application technique described by March and Metcalf (11). Mortalities were determined 24 hr after treatment and LD_{50} values were estimated from plots on logarithm-probit paper. Mammalian toxicity was determined orally on 3- to 6-month-old Swiss white mice reared from a strain originally purchased from Curd's Caviary, La Puente, Calif. The test compound was dissolved in olive oil and applied as previously described by Hollingworth et al. (12).

The technique for the preparation of fly-head cholinesterase and the determination of anticholinesterase activity (I_{50} and k_e values) have been described previously (13, 14). I_{50} values were determined after 15 min incubation with cholinesterase and estimated from the best eye-fitted lines after plotting log A_0/A, where A_0 is the activity of the enzyme without inhibitor and A is the activity with inhibitor, against the molar concentration of the test compound. Purified bovine erythrocyte cholinesterase was obtained from the Sigma Chemical Co.

Hydrolysis rates. Pseudo first-order hydrolysis constants (k_b) of the PO esters of phoxim and its analogs in 0.1 *M* phosphate buffer (pH 10.6) were determined spectrophotometrically at 30° by estimating the amount of hydrolysis product (oxime) in a Unicam SP-800 spectrophotometer at 280 mμ. Spectra of the PO esters and phenylglyoxylonitrile oxime were determined prior to kinetic studies to show that overlaps in pertinent absorption peaks were not present. A plot was made of log $A_f - A_0/A_f - A_t$

against time, where A_f is absorbance after complete hydrolysis, A_0 is absorbance at zero time, and A_t is absorbance at time t. The best line was fitted by eye and k_b was calculated according to the equation:

$$k_b = \frac{2.303 \log A_f - A_0/A_f - A_t}{t}$$

Metabolism. Male Swiss mice were treated orally with ^{32}P-phoxim in olive oil and placed in individual metabolism chambers which allowed the separate collection of urine and feces (15). Urine samples were collected (when urine was present) at 6, 24, 48, 72, 120, and 140 hr after treatment and frozen immediately until analysis. Feces were collected after 72, 120, and 140 hr. Urine and feces samples represented a composite from 2 or 3 mice. Urinary metabolites were separated by anion exchange chromatography using Dowex 1-X8 (100–200 mesh) according to procedures described by Plapp and Casida (16). Aliquots of urine samples also were partitioned beween water and chloroform to quantify organic- and water-soluble ^{32}P materials. Radioactivity was determined in steel planchets by gas-flow counting.

The identification of the metabolic products was accomplished by comparing their behavior on the anion exchange column with pure model compounds and by thin-layer chromatography. Silica gel thin-layer plates (0.25 cm) were prepared from Absorbosil 1 (Applied Science Laboratories) and the two solvent systems chloroform: ethyl acetate (9:1 by volume) and isopropanol: water: concentrated ammonium hydroxide (75:24:1) were used. The compounds were located on thin-layer plates by the method of Hanes and Isherwood (17) and by using a specially prepared solution obtained from EM Reagents Division of Brinkmann Instruments for the detection of phosphorus. Thin-layer plates were also developed with 2,6-dibromoquinone-4-chloroimine (18). R_f values for the various metabolites in the two solvent systems are given in Table 2.

TABLE 2

R_f values for thin-layer chromatography of phoxim and its metabolites

Compound	Solvent system	
	$CHCl_3$-EtOAc	i-C_3H_7OH-H_2O-NH_3
phoxim	0.86	1.00
$(C_2H_5O)_2P(O)ON{=}C(CN){-}C_6H_5$	0.62	1.00
$(C_2H_5O)_2P(S)ON{=}C(COOH){-}C_6H_5$	0.0	—
$(C_2H_5O)_2P(S)(OH)$	0.0	0.71
$(C_2H_5O)_2P(O)(OH)$	0.0	0.54
H_3PO_4	0.0	0.04

Phoxim carboxylic acid was tentatively identified by analysis of infrared spectrum and by its conversion to a salt. Phoxim carboxylic acid leaves the anion-exchange column at the same position as phoxim, but the two compounds are readily separable on the basis of their solubilities in organic solvents and by thin-layer chromatography. The identity of phoxim carboxylic acid by infrared spectrum was based on the presence of a strong carbonyl absorption at 1690 cm^{-1} and by weak OH absorption between 2500–3000 cm^{-1}. Further, the definitive CN absorption found in phoxim at 2200–2300 cm^{-1} was absent. The carboxylic acid was shown to be a PS ester by the absence of the characteristic PO absorption near 1250 cm^{-1}. As additional supporting evidence, a small amount of the purified metabolite was partitioned between water and chloroform, resulting in approximately 10% of the radioactivity in the chloroform phase. Partitioning between aqueous sodium bicarbonate and chloroform resulted in total radioactivity in the aqueous phase, indicating the presence of a carboxylic acid moiety.

For the estimation of radioactivity in

mice tissue, various organs were removed from one freshly sacrificed animal, washed with distilled water, and homogenized in a ground-glass homogenizer. The homogenate was diluted to a known volume with distilled water and 5-ml aliquots were partitioned against chloroform. The aqueous and chloroform phases were analyzed for radioactivity in the usual manner.

^{32}P-Phoxim in acetone was applied topically to the thoraces of 3-day-old S_{NAIDM} and R_{SC} houseflies. Acetone solutions were radioassayed to determine the amount of material applied to each insect. Usually, several hundred houseflies were treated in each experiment. After treatment, flies were placed in 500-ml wide-mouth jars which were closed with cheesecloth and placed in a 60°F constant temperature room without food or water. The flies were sacrificed at ½, 1, 2, and 4 hours after treatment by placing the holding container in a dry ice cabinet for about 15 min. The procedures used to determine penetration rates and for the extraction of metabolites was essentially the same as that described in detail by Hollingworth et al. (19) and Camp et al. (20).

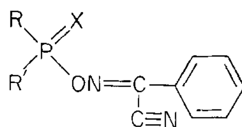
RESULTS

Toxicity. The toxicity data for phoxim and its analogs against susceptible (S_{NAIDM}) and resistant (R_{SC}) houseflies, German cockroaches, and the white mouse are summarized in Table 3.

With the exception of the diethylphosphinothioate (IX) and phosphinate (X) analogs, all of the compounds were quite toxic to susceptible flies with LD_{50} values between 2.1–16.0 μg/g. Toxicity, however, varied widely against resistant flies with LD_{50} values ranging from 21.5–>2,500 μg/g. The highest level of resistance by the latter strain was found with the dimethyl phosphorothioate (III), and dimethyl (IV) and diethyl (V) phosphate esters and the lowest was with the ethylphosphonothioate (VII) and diisopropyl phosphate (VI) esters. All

TABLE 3

Summary of toxicity data for phoxim and its analogs to houseflies (S_{NAIDM} and R_{SC}), German cockroaches, and white mice.



Analog	R	R′	X	LD_{50} Values: Insects (μg/g) S_{NAIDM} flies	R_{SC} flies	German cockroach	White mice (mg/kg)
I	C_2H_5O	C_2H_5O	S	2.1	215	6.1	>2,000
II	C_2H_5O	C_2H_5O	0	3.4	>2,500	5.5	1,000
III	CH_3O	CH_3O	S	3.3	>2,500	7.8	>2,000
IV	CH_3O	CH_3O	0	5.0	>2,500	7.6	>1,000
V	i-C_3H_7O	i-C_3H_7O	S	16.0	220	209	>1,500
VI	i-C_3H_7O	i-C_3H_7O	0	5.0	53.5	12.5	1,250
VII	C_2H_5O	C_2H_5	S	5.8	21.5	9.7	>500
VIII	C_2H_5O	C_2H_5	0	5.9	150	5.5	70–73
IX	C_2H_5	C_2H_5	S	125	>2,500	—	>500
X	C_2H_5	C_2H_5	0	410	>2,500	—	>500

of the test compounds [except the diisopropyl phosphorothioate (V)] showed high toxicity to the German cockroach with LD_{50} values ranging from 5.0–12.5 μg/g.

As Table 3 indicates, the toxicity of phoxim and its analogs was generally very low to the white mouse and only the ethylphosphonate ester (VIII) showed appreciable toxicity to mice (LD_{50} 70–73 mg/kg).

Cholinesterase inhibition. Inhibition studies using cholinesterase (ChE) from heads of susceptible and resistant houseflies, bovine erythrocytes, and to a limited extent mouse brain were carried out with the various PO analogs (Table 4). In the case of flies, I_{50} and k_e values at 37.5°C are given only for S_{NAIDM} cholinesterase since the results were essentially the same as those obtained from R_{SC}.

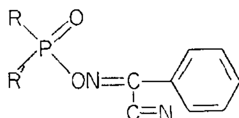
Against fly-head ChE only small differences in anticholinesterase activity were found between the diethyl (II), dimethyl (IV), diisopropyl phosphate (VI), and ethylphosphonate (VIII) esters and the phosphinate (X) was approximately tenfold less active than the other PO esters. Compounds II, IV, VI, and VIII were all extremely potent inhibitors of fly-head ChE with I_{50} values in the neighborhood of $10^{-9}M$.

Bovine-erythrocyte ChE also was inactivated by the PO esters but at slower rates compared to fly-head ChE. I_{50} and k_e values against bovine-erythrocyte ChE were similar for II, IV, and VI, but VIII was about tenfold more effective and the phosphinate (X) was approximately 100-fold less effective as an anticholinesterase than the preceding compounds. Mouse brain ChE, although studied on a limited scale, was more susceptible to inhibition by PO phoxim (II) compared to bovine-erythrocyte ChE with I_{50} and k_e values of $6.0 \times 10^{-8}M$ and $4.9 \times 10^5 M^{-1}$ min^{-1}, respectively.

The data in Table 4 show that there are substantial differences in the sensitivity of insect and mammalian ChE to inhibition by the various PO esters. The magnitude of these differences is represented in Table 4 by the ratios of inhibition of the two cholinesterases, i.e., k_e (fly-head ChE)/k_e (bovine-erythrocyte ChE) defined as the selective inhibition ratio. The largest ratio was found with X, the diethylphosphinate analog of phoxim. However, in spite of the large difference in inhibition rates, X was nontoxic to both the house fly and the mouse probably because of its high susceptibility to hydrolysis. Compounds II, IV, and VI all showed large selective inhibition ratios

TABLE 4

Cholinesterase inhibition data for phoxim and its analogs at 37.5°



Analog	R	R′	S_{NAIDM} fly-head ChE		Bovine-erythrocyte ChE		k_e (fly)/k_e (bovine)	k_b (min^{-1}) 30°
			I_{50} (M)	k_e (M^{-1}, min^{-1})	I_{50} (M)	k_e (M^{-1}, min^{-1})		
II	C_2H_5O	C_2H_5O	1.9×10^{-9}	8.4×10^7	2.2×10^{-7}	3.1×10^5	270	5.9×10^{-2}
IV	CH_3O	CH_3O	1.1×10^{-9}	1.3×10^8	3.6×10^{-7}	1.7×10^5	730	1.4×10^{-1}
VI	i-C_3H_7O	i-C_3H_7O	7.8×10^{-10}	8.1×10^7	4.4×10^{-7}	1.1×10^5	756	2.7×10^{-2}
VIII	C_2H_5O	C_2H_5	2.5×10^{-9}	5.9×10^7	5.1×10^{-8}	1.2×10^6	49	3.9×10^{-1}
X	C_2H_5	C_2H_5	1.2×10^{-8}	1.9×10^6	3.8×10^{-5}	8.3×10^2	2 330	7.1×10^{-1}

and all these compounds were substantially more toxic to houseflies than to mice. The phosphonate analog VIII, the compound most toxic to the mouse, showed the lowest inhibition ratio of 49.

Pseudo first-order hydrolysis constants in phosphate buffer (pH 10.6) at 30° also are given in Table 4. As might be predicted from their high anticholinesterase activities, the various PO esters were quite susceptible to alkaline hydrolysis. Hydrolysis rates were in the order predictable on the basis of polar and steric effects. The phosphinate analog (X) hydrolyzed faster than the phosphonate (VIII) which in turn hydrolyzed faster than the phosphates. The phosphate analogs hydrolyzed in the order: dimethyl (IV) > diethyl (II) > diisopropyl (VI).

Metabolism studies in the white mouse. Figure 1 shows the rates of appearance of radioactive metabolites in mouse urine and feces after oral administration of ^{32}P-labeled phoxim at 10.5, 114, and 955 mg/kg. At all three dosage levels, the ultimate recovery of administered radioactivity in the urine and feces was in the region of 73–82%. However, the radioactivity appeared in the urine and feces at a much lower than expected rate in light of the low mammalian toxicity of phoxim. For example, 24 hr after oral treatment of mice at 10.5 and 114 mg/kg of radioactive phoxim only 43% and 22%, respectively, of the administered radioactivity was excreted in the urine. At 955 mg/kg, only 17% of the administered radioactivity was excreted in the urine after 30 hr.

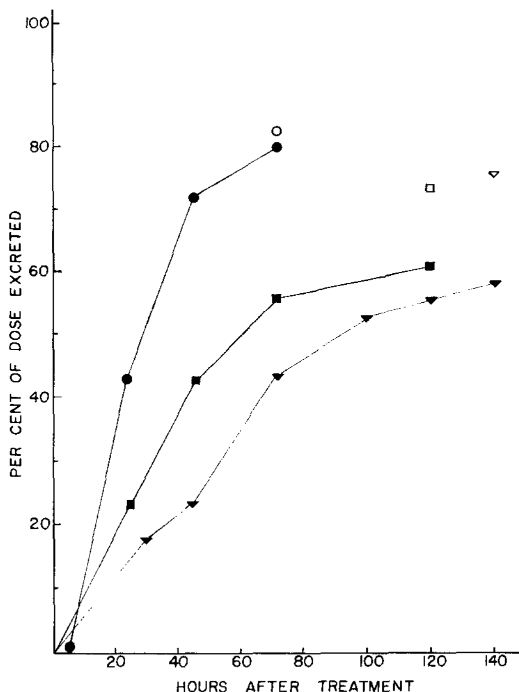


Fig. 1. *Exretion of radioactivity in the urine of mice treated with phoxim at three dosages;* ● *10.5 mg/kg,* ■ *114 mg/kg,* ▲ *955 mg/kg. Open figures represent amounts for urine plus feces.*

Because of the problem presented by the slow rate of excretion of phoxim, an autopsy was performed on a white mouse treated with 114 mg/kg of phoxim after 48 hr to determine the internal fate of the administered dose. At this time approximately 43% of the dose had been excreted in the urine. The results of the autopsy are presented in Table 5 and indicate that virtually all of the radioactivity was found in either the gut or urinary bladder. The amounts of radioactivity found in other organs such as the brain and heart were minor and the amounts of organic-soluble material (which could include the strong anticholinesterase PO phoxim) were essentially insignificant. Therefore, even though the rates of excretion of ^{32}P-phoxim and its metabolites were slower than expected, this apparently was not due to any lack of ability on the part of the mouse to metabolize phoxim. The autopsy data indicate that nearly all of the internal dose of phoxim remaining 48 hr after treatment was metabolized to water-soluble compounds and that the slower than expected excretion rates were due to retention or storage of the metabolites in the urinary bladder of the mouse. The reason for the apparent storage of phoxim and its metabolites by mice after treatment with phoxim is not clear.

Table 6 summarizes the data concerning the identity and relative amounts of the various metabolites obtained at 114 mg/kg and 955 mg/kg. The results also are shown graphically for the higher dosage in Fig. 2.

TABLE 5

Summary of autopsy data on a white mouse 48 hr after oral treatment with 114 mg/kg of phoxim.

Organ	% Recovered internal radioactivity		
	Organic-soluble	Water-soluble	Sum of organic- + water-soluble
Brain	0.07	0.14	0.21
Thymus gland	0.01	0.05	0.06
Hind leg muscle	0.00	0.54	0.54
Heart	0.01	0.23	0.24
Kidney	0.03	0.13	0.16
Liver	0.05	1.60	1.65
Gut	0.17	8.60	8.77
Urinary bladder	2.10	86.30	88.40
Total	2.44	97.59	100.00

Radioactive urine from white mice after oral treatment with ^{32}P-phoxim was analyzed by ion-exchange and thin-layer chromatography. Urine from mice treated with 10.5 mg/kg of phoxim was not subjected to any further analysis beyond that already discussed.

Five metabolites were recovered after treatment with phoxim at both treatment levels studied. The identity of the metabolites was established by one or more of the methods previously discussed and were identified as (1) diethyl phosphoric acid, (2) phoxim, (3) phoxim carboxylic acid, (4) *O,O*-diethyl phosphorothioic acid, and (5) either desethyl phoxim or desethyl PO phoxim.

The actual amounts of *O,O*-diethyl phosphorothioic acid found in the urine from white mice treated with 114 mg/kg and 955 mg/kg of phoxim were 0.21 mg and 0.97 mg, respectively (expressed in terms of phoxim equivalents and based on the aver-

TABLE 6

Metabolites found in urine from white mice 24 hr after treatment with phoxim at 114 mg/kg and after 30 hr at 955 mg/kg.

Metabolite	Anion exchange tube numbers	114 mg/kg dose		955 mg/kg dose	
		%[a]	mg[b]	%	mg
O ‖ 1. $(C_2H_5O)_2P$-OH	21–35	58.9	0.62	43.1	2.36
2. phoxim	47–57	1.1	0.01	2.1	0.12
S COOH ‖ | 3. $(C_2H_5O)_2PON{=}C\text{-}C_6H_5$	47–57	2.8	0.03	23.6	1.29
S ‖ 4. $(C_2H_5O)_2POH$	59–64	20.0	0.21	17.7	0.97
S(O) ‖ 5. (C_2H_5O) $(HO)PON{=}C\text{-}C_6H_5$ | CN	84–93	6.2	0.07	5.0	0.27
Radioactivity not in peaks		4.3	0.05	5.4	0.30
Unrecoverable radioactivity		6.7	0.07	3.1	0.17

[a] % of radioactivity in urine applied to column.

[b] Values expressed in terms of phoxim equivalents based on average dose given to each mouse.

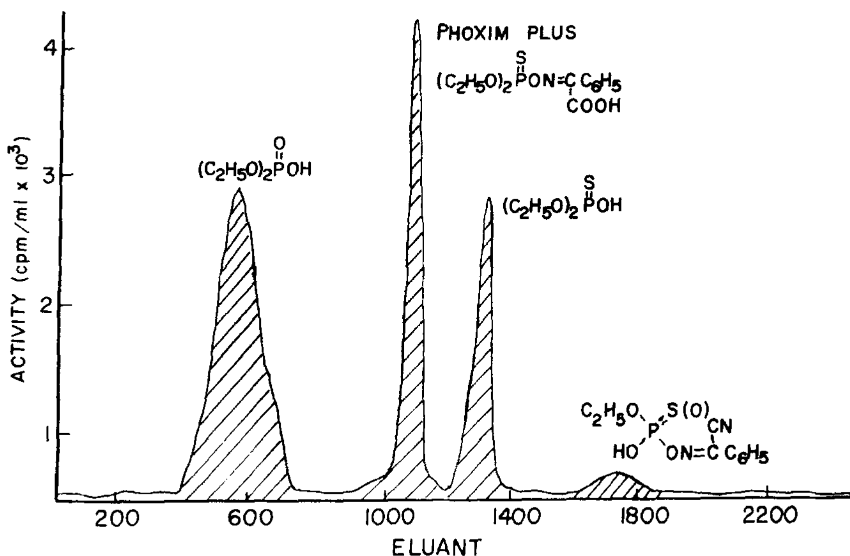


FIG. 2. *Anion-Exchange column chromatograph of mouse urine 30 hr after oral treatment with 955 mg/kg of phoxim.*

age dose given to each mouse, i.e., 4.5 mg at 114 mg/kg and 31.5 mg at 955 mg/kg). The amounts of desethyl phoxim or desethyl PO phoxim found in the urine were 0.07 mg and 0.27 mg at the two respective dosages. It should be pointed out that pure desethyl phoxim and desethyl PO phoxim were not available and the identification of intact desethyl ester is based on analogy in elution patterns obtained in this study and those obtained by Hollingworth et al. (15), Camp et al. (20), and Nakatsugawa et al. (21) with related organophosphorus esters. Identification of the desethyl ester, therefore, is tentative. Although the actual amounts of *O,O*-diethyl phosphorothioic acid and desethyl ester, as expected, increased substantially upon raising the dose, the relative percentages of these metabolites changed very little. Thus, *O,O*-diethyl phosphorothioic acid represented 20.0 and 17.7% and desethyl ester represented 6.2 and 5.0% of the radioactivity in the urine at the two dosages. The relatively small amounts of desethyl ester at both dosage levels indicates that *O*-deethylation is not an important avenue for detoxication of phoxim in the mouse. The major metabolite found at both dosage levels was diethyl phosphoric acid which at 114 mg/kg constituted 58.9% of the radioactivity in urine and 43.1% at 955 mg/kg.

Owing to the large amounts of diethyl phosphoric acid present in urine, ^{32}P-*O,O*-diethyl phosphorothioic acid was given orally to the mouse to determine whether it served as a precursor to diethyl phosphoric acid. The results of this study showed that greater than 95% of an administered dose of pure *O,O*-diethyl phosphorothioic acid was excreted (in the 24- and 48-hour post-treatment urine) as the unchanged thioic acid. Thus, this experiment tentatively indicates that little, if any, diethyl phosphoric acid is formed from desulfuration of *O,O*-diethyl phosphorothioic acid which agrees with the findings of Nakatsugawa et al. (21) who studied parathion metabolism in the white rat. Although these results strongly suggest that diethyl phosphoric acid is formed from the hydrolysis of the PO analog of phoxim, they are not entirely conclusive. It is still

possible that the water-soluble thioic acid administered orally to the white mouse is rapidly excreted unchanged in the urine, conceivably bypassing the active sites in the liver or other tissues known to be capable of desulfuration reactions.

In addition to the above mentioned routes for the formation of diethyl phosphoric acid, a third possible route exists for the formation of this acid. This route involves desulfuration of the PS phoxim carboxylic acid to the PO carboxylic acid and subsequent hydrolysis of the PO carboxylic acid to diethyl phosphoric acid. Although no direct evidence for this pathway was found in the present study, it is mentioned as a possible additional source of diethyl phosphoric acid.

The most striking change in the relative amounts of any metabolite upon increasing the dose from 114 mg/kg to 955 mg/kg occurred with phoxim carboxylic acid. The amounts of this metabolite in the urine at the two dosages were 0.03 mg (2.8%) and 1.29 mg (23.6%), respectively. Thus, in terms of actual amounts of material present in the urine, phoxim carboxylic acid was 43 times more abundant at the higher than at the lower dosage. These results point out the possible importance of this metabolic reaction in making phoxim safe to mammals.

The hydrolysis of the —C≡N moiety in phoxim to the COOH group of phoxim carboxylic acid probably involves amidase-type action. The mechanism should involve initial hydrolysis of the C≡N group to an amide [C(O)—NH_2] and then hydrolysis to the carboxylic acid (COOH). The hydrolysis of the C(O)—NH_2 group to COOH in phoxim may involve specific carboxyamidases analogous to those catalyzing the hydrolysis of the C(O)—$NHCH_3$ group of dimethoate (22, 23). In addition, according to infrared analysis of the metabolite the apparent absence of the intermediate phoxim amide indicates that cleavage of the amide to phoxim carboxylic acid must be very fast, thus preventing the accumulation of the phoxim amide intermediate.

The final compound found in the urine of phoxim-treated mice was unchanged phoxim itself. Intact phoxim was found in smaller amounts than any of the metabolites. Only 0.01 mg (1.1%) and 0.12 mg (2.1%) of phoxim were found in the urine from mice treated with 114 mg/kg and 955 mg/kg of phoxim, respectively.

Metabolism studies in susceptible and resistant houseflies. The results of metabolism studies performed in susceptible (S_{NAIDM}) and resistant (R_{SC}) houseflies are presented in Tables 7–10.

The general distribution of radioactivity in the various fractions ½, 1, 2, and 4 hr after treatment with phoxim is presented in Table 7. As this table indicates, excellent recovery of the total applied radioactivity was obtained from both fly strains after treatment with 1.75 μg/g of phoxim. Generally, the percentages of the applied dosage recovered were greater than 90%.

Penetration as measured by the rate of surface loss (fly wash) was rapid in both susceptible and resistant house flies at 1.75 μg/g (90% penetration and 80% penetration, respectively, after 30 min). Although penetration of phoxim is quite rapid in both housefly strains at 1.75 μg/g, the fly wash data in Table 7 indicate a 10–15% lag in penetration rates with the resistant strain compared to the susceptible strain at each post-treatment time interval and this difference may be a factor contributing to the resistance of the R_{SC} strain to phoxim.

The results indicate conclusively that differences in the rates of detoxication of phoxim exist between susceptible and resistant flies at the 1.75 μg/g dosage and there are considerably larger amounts of hydrolytic products in the resistant strain at all times. Table 8 gives data showing the percentage of the penetrated dosage hydrolyzed (determined from the summation of radio-

TABLE 7

Distribution of phoxim and its metabolites at different time intervals after topical treatment of susceptible (S_{NAIDM}) and resistant (R_{SC}) houseflies.

Fly strain	Dose (μg/g)	Fly wash		Jar wash		Fly extract		Unextractable material	% Recovery of applied radioactivity
		aqueous μg/g[a]	organic μg/g	aqueous μg/g	organic μg/g	aqueous μg/g	organic μg/g		
½ Hour after treatment									
S_{NAIDM}	1.75	0.02	0.16	0.01	0.01	0.49	0.86 (PS 0.76) (PO 0.10)	0.21	100.00
R_{SC}	1.75	0.03	0.32	0.01	0.03	0.73	0.53 (PS 0.46) (PO 0.07)	0.04	96.4
R_{SC}	150	1.4	67.8	0.3	1.9	7.2	28.2 (PS 27.6) (PO 0.6)	6.8	75.6
1 Hour after treatment									
S_{NAIDM}	1.75	0.07	0.09	0.01	0 01	0.41	0.74 (PS 0.61) (PO 0.13)	0.11	82.6
R_{SC}	1.75	0.07	0.18	0.03	0.02	0.85	0.35 (PS 0.26) (PO 0.09)	0.06	88.9
R_{SC}	150	2.1	53.3	0.6	3.5	14.8	29.8 (PS 28.7) (PO 1.1)	3.3	71.6
2 Hours after treatment									
S_{NAIDM}	1.75	0.05	0.08	0.01	0.01	0.86	0.55 (PS 0.44) (PO 0.11)	0.18	99.3
R_{SC}	1.75	0.15	0.09	0.16	0.04	1.12	0.11 (PS 0.08) (PO 0.03)	0.04	96.5
R_{SC}	150	5.9	46.5	1.3	3.6	21.0	21.6 (PS 20.4) (PO 1.2)	7.8	71.9
4 Hours after treatment									
S_{NAIDM}	1.75	0.02	0.01	0.06	0.01	1.02	0.35 (PS 0.24) (PO 0.11)	0.13	91.2
R_{SC}	1.75	0.3	0.04	0.24	0.04	1.13	0.02 (PS 0.01) (PO 0.01)	0.06	103.8
R_{SC}	150	6.9	38.4	1.8	3.1	32.1	21.1 (PS 19.6) (PO 1.5)	1.5	69.8

[a] Values expressed in terms of phoxim equivalents.

activity in the aqueous-soluble parts of the FLY WASH, JAR WASH, and FLY-EXTRACT fractions) at various time intervals after treatment. At ½, 1, 2, and 4 hr after treatment with 1.75 μg/g of phoxim in the susceptible strain, 38, 40, 62, and 76% respectively, of the applied dosage was hydrolyzed while in the resistant strain, 59, 73, 93, and 98% was hydrolyzed.

The 2-hr FLY-EXTRACT fraction from susceptible and resistant house flies treated at 1.75 μg/g was analyzed by ion-exchange and thin-layer chromatography. Results concerning the nature and distribution of the metabolites are presented in Tables 9 and 10. Five metabolites were found in the FLY-EXTRACT fraction from susceptible house flies and six metabolites were found in resistant houseflies. Phoxim, PO phoxim, *O,O*-diethyl phosphorothioic acid, diethyl phosphoric acid, and phosphoric acid were found in both strains. A metabolite tentatively identified as ethyl phosphoric acid was found only in resistant flies. Pure model ethyl phosphoric acid was not available and, therefore, the identification of this metabolite was based on analogy in elution patterns with the work of other investigators using similar organophosphorus compounds (15, 20, 21).

Although the nature of the metabolites in both fly strains treated with 1.75 μg/g of phoxim are similar (except for ethyl phosphoric acid), the amounts and distribution of the metabolites in the two strains are quite different. Table 9 indicates that much smaller amounts of intact phoxim and its PO analog were found in the EXTRACT fraction from resistant compared to susceptible house flies. Over six times more phoxim ester and almost four times more PO phoxim ester were found in the susceptible strain 2 hr after treatment with 1.75 μg/g of phoxim. The greatly reduced amounts of phoxim and PO phoxim in the resistant strain must be the result of more rapid metabolic degradation resulting in significantly greater amounts of PO hydrolysis products, i.e., diethyl phosphoric and ethyl phosphoric acid in the resistant strain compared to the susceptible strain.

TABLE 8

Percentage of penetrated dose hydrolyzed by susceptible and resistant houseflies at various times after topical application of phoxim.

Hours after treatment	SNAIDM	RSC	
	1.75 μg/g dose	1.75 μg/g dose	150 μg/g dose
½	38	59	24
1	40	73	37
2	62	93	57
4	76	98	66

In previous discussion concerning phoxim metabolism in the white mouse, it was concluded that diethyl phosphoric acid and other PO hydrolysis products found in mouse urine were most likely formed from the hydrolysis of the PO analog of phoxim. Similarly, the larger amounts of PO hydrolysis products noted in resistant houseflies probably also are formed from the hydrolysis of PO phoxim. Accordingly, the relative rates of desulfuration of phoxim to its PO analog must be faster in the resistant strain. This increase in formation of the PO anticholinesterase is offset, however, by very efficient P(O)O-aryl hydrolysis.

The data shown in Table 9 is summarized in Table 10 in which the differences in rates of metabolism between the two strains is more readily apparent at the 1.75 μg/g dosage. For example, the higher rate of desulfuration in the resistant compared to the susceptible strain is reflected in the relative percentages of total PO containing compounds (58.1% in the resistant against 37.4% in the susceptible strain). Also, the much greater rates of hydrolysis of the PO containing compounds in the resistant strain is reflected in the large amounts of PO hydrolysis products, e.g., 55.7% in the resist-

TABLE 9

Metabolites in EXTRACT fraction from susceptible and resistant houseflies 2 hr after topical application of phoxim

Metabolite		S_{NAIDM}	R_{SC}	
		1.75 μg/g dose	1.75 μg/g dose	150 μg/g dose
H_3PO_4	%	3.1	3.2	0.9
	μg/g	0.04	0.04	0.38
$(C_2H_5O)(HO)P(O)OH$ (?)	%	—	9.2	—
	μg/g	—	0.11	—
$(C_2H_5O)_2P(O)OH$	%	26.9	43.3	11.9
	μg/g	0.38	0.53	5.1
$(C_2H_5O)_2P(O)ON{=}C(C{\equiv}N)C_6H_5$	%	7.4	2.4	2.9
	μg/g	0.11	0.03	1.2
$(C_2H_5O)_2P(S)ON{=}C(C{\equiv}N)C_6H_5$	%	30.3	5.9	47.9
	μg/g	0.43	0.07	20.4
$(C_2H_5O)_2P(S)OH$	%	27.6	30.6	34.4
	μg/g	0.39	0.38	14.7

ant compared to 30.0% in the susceptible strain.

Metabolism in resistant house flies was also studied at 150 μg/g. At this level, roughly equal symptomology and mortalities were observed in the resistant compared to the susceptible strain at 1.75 μg/g. The first effect which became obvious upon raising the dosage level in resistant houseflies was saturation of the penetration mechanism. For example, in susceptible and resistant houseflies treated at 1.75 μg/g, 80–90% of the recovered radioactivity had penetrated within 30 min while at 150 μg/g, only 40% had penetrated after 30 min. These results indicate that at the dosage level of 150 μg/g, decreased penetration of phoxim in resistant houseflies may be a contributing factor for their resistance to phoxim.

However, in spite of greatly decreased penetration rates at the higher dosage, Tables 7 and 9 show that much larger amounts of intact PS and PO esters are present in resistant flies at 150 μg/g than in susceptible flies at 1.75 μg/g. For example, after 2 hr at the 150 μg/g dosage, 1.2 μg/g and 20.4 μg/g of the PO and PS esters were found compared to 0.11 μg/g and 0.43 μg/g of the same ester in susceptible flies at the lower dosage. Thus, approximately 11-fold more PO phoxim (presumably the active anticholinesterase) was found in resistant flies at the higher dosage than in susceptible flies at the lower dosage. The results present an interesting paradox which is difficult to

TABLE 10

Various forms of the EXTRACT fraction from susceptible and resistant houseflies 2 hr after topical application of phoxim

	S_{NAIDM}		R_{SC}			
	1.75 μg/g dose		1.75 μg/g dose		150 μg/g dose	
	%	μg/g	%	μg/g	%	μg/g
Total PO containing compounds	37.4	0.53	58.1	0.71	15.7	6.7
Total PS containing compounds	57.9	0.82	36.5	0.45	82.3	35.1
Hydrolysis products containing PS	27.6	0.39	30.6	0.38	34.4	14.7
Hydrolysis products containing PO	30.0	0.42	55.7	0.68	12.8	5.5

explain since symptomology and 24-hr mortalities in the two strains at the lower and higher dosages are similar and, therefore, approximately equal amounts of PO phoxim would be expected. Perhaps the desulfuration of phoxim to the PO anticholinesterase occurs at a site quite removed from the target center and, therefore, the total amount of PO phoxim found in the EXTRACT does not represent the actual amount of the anticholinesterase at the target.

The relative percentages of *O,O*-diethyl phosphorothioic acid in the EXTRACT fraction at 1.75 and 150 μg/g in resistant flies are similar, indicating that the process for P(S)O-aryl cleavage is probably not saturated at 150 μg/g. On the other hand, desulfuration of phoxim to its PO analog and also P(O)O-aryl hydrolysis appear to be saturated at 150 μg/g. This is reflected in the lower percentages of PO phoxim relative to PS phoxim, and in the lower percentages of PO hydrolysis products from resistant flies at 150 μg/g. Thus, the results of increasing the dosage level from 1.75 μg/g to 150 μg/g can be summarized as follows:

Saturated by higher dosage: Penetration, desulfuration, —P(O)O-aryl hydrolysis.

Unsaturated by higher dosage: P(S)O-aryl cleavage.

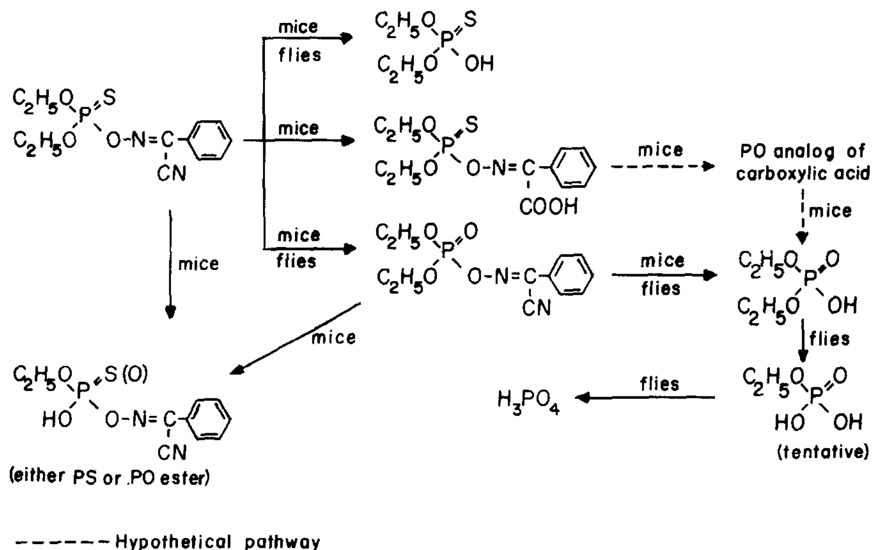
DISCUSSION

Phoxim and all of its analogs, except for the phosphinate esters, have been shown to be highly insecticidal. Further, these compounds were generally nontoxic to the white mouse although there was wide variation in the groups attached to the phenylglyoxylonitrile oxime moiety.

Most of the PO analogs of phoxim were found to be strong inhibitors of both insect and mammalian cholinesterase. House fly-head cholinesterase, however, was significantly more susceptible to inhibition by the PO esters than bovine-erythrocyte and mouse-brain cholinesterase, suggesting that differences in sensitivity to inhibition are, at least in part, responsible for the selective action of phoxim and its derivatives toward insects. However, in spite of the large differences in their ability to inhibit insect and mammalian cholinesterase, it is difficult to ignore the fact that these compounds are still relatively strong inhibitors of mammalian cholinesterase, e.g., the I_{50} value of phenylglyoxylonitrile oxime diethyl phosphate (II) for mouse-brain cholinesterase is $6.0 \times 10^{-8} M$. The high rates of inhibition of mammalian cholinesterase are somewhat inconsistent with their extremely low mammalian toxicity, indicating that other factors besides target enzyme inhibition must be considered to explain the remarkable selective toxicity of phoxim and its analogs.

Our results indicate that metabolic factors play an important role in the biocidal activity of phoxim to susceptible and resistant insects, and mammals. The equations

below show the proposed scheme for the metabolism of phoxim in the white mouse and in houseflies.



phosphorothioic acid and to phoxim carboxylic acid. The hydrolysis of phoxim to O,O-diethyl phosphorothioic acid appears to be relatively constant with respect to the administered dose. Hydrolysis to phoxim carboxylic acid, on the other hand, appears to increase with increase in administered dosage, and evidently becomes an important detoxication pathway at high dosages. The net result is that PO phoxim, in spite of its strong anticholinesterase properties, is not allowed to reach a level critical in the mouse.

The basis for the extreme safety of phoxim to mice is readily apparent from the metabolism data. Only very small amounts of phoxim were found in mouse urine 24 hr after treatment and in no case was any PO phoxim detected. Further, only insignificant amounts of intact ester were noted in the internal organs of the mouse autopsied 48 hr after treatment with 114 mg/kg phoxim. The evidence clearly indicates that phoxim is peculiarly suited for metabolic detoxication by the white mouse. Owing to the large amounts of diethyl phosphoric acid found in mouse urine it is evident that phoxim is desulfurated to PO phoxim which in turn must be hydrolyzed with remarkable rapidity to diethyl phosphoric acid and at no time does PO phoxim reach a sufficiently high concentration to intoxicate the mouse. Support for rapid degradation of PO phoxim is found in the fact that PO phoxim itself is quite nontoxic with an LD_{50} value of 1000 mg/kg. In addition to the above pathway for detoxication, the mouse also is capable of degrading phoxim directly to O,O-diethyl

The basis for the ineffectiveness of phoxim to resistant house flies, however, is not quite as clear-cut. The metabolism of phoxim in resistant house flies at the lower dosage of 1.75 μg/g is similar to that of the white mouse in that degradation of phoxim and PO phoxim is rapid and complete. The rate of desulfuration of phoxim apparently is fast, but as in the mouse, the PO compound is hydrolyzed very efficiently to form eventually several PO hydrolysis products. Cleavage of phoxim to O,O-diethyl phosphorothioic acid also is an important detoxication pathway in resistant flies but the pathway to phoxim carboxylic acid found in mice evidently is absent in resistant flies. The rela-

tionship between the internal levels of PO phoxim and intoxication becomes evident when metabolism in susceptible houseflies and resistant flies at 150 μg/g is compared. Considering the data for 2 hr after treatment, relatively large amounts of intact PS and PO ester were present internally in susceptible flies compared to resistant flies at the same dose. Although the rate of desulfuration of phoxim to PO phoxim appears to be slower in susceptible flies, at the same time hydrolysis of the PO ester to detoxified products also occurs at a much slower rate and, as a consequence, the level of PO phoxim remains relatively high in susceptible flies. Thus, a direct comparison of susceptible and resistant flies at the same 1.75 μg/g dose shows significantly larger amounts of PO phoxim (and also phoxim) in the susceptible strain. At the higher 150 μg/g dose, the resistant flies contained relatively large amounts of intact PS and PO phoxim internally. In fact approximately 46- and 11-fold more PS and PO phoxim were found in resistant flies treated at 150 μg/g than in susceptible flies treated at 1.75 μg/g. As discussed earlier, a correlation between symptomology and levels of PO phoxim found internally cannot be made. At the higher dosage, the efficient P-O-aryl hydrolysis system operating in resistant flies apparently has become saturated, allowing the build-up of more than lethal levels of PO phoxim. The results and conclusions of the present study on the metabolism of phoxim in susceptible and resistant houseflies closely parallel those reported by Hollingworth et al. (19) using the same insect strains and methodology but with the compounds methyl parathion and Sumithion®.

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