# *S*-Methylation of *O,O*-Dialkyl Phosphorodithioic Acids: *O,O,S*-Trimethyl Phosphorodithioate and Phosphorothiolate as Metabolites of Dimethoate in Mice

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*O,O,S*-Trimethyl phosphorodithioate and phosphorothiolate [$(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$, respectively] are known from earlier studies to be impurities, delayed toxicants, and detoxication inhibitors in several major *O,O*-dimethyl phosphorodithioate insecticides. Our recent studies show extensive *S*-methylation of mono- and dithiocarbamic acids in mice, suggesting the possibility that phosphorodithioic acids such as $(MeO)_2P(S)SH$ might also undergo *S*-methylation. This possibility was examined in ip-treated mice with emphasis on the metabolites of dimethoate [$(MeO)_2P(S)SCH_2C(O)NHMe$], one of the most important organophosphorus insecticides. The urinary metabolites of dimethoate, which contains no P-SMe substituent, were found to include four compounds with P-SMe moieties identified by $^{31}P$ NMR spectroscopy as MeO(HS)P(O)SMe, MeO(HO)P(O)SMe, $(MeO)_2P(S)SMe$, and $(MeO)_2P(O)SMe$; the latter two compounds are also established by GC-MS as dimethoate metabolites in mouse urine, liver, kidney, and lung. Several approaches verified unequivocally that the previously unknown P-SMe metabolites in urine and tissues are due to in vivo *S*-methylation rather than to impurities. Studies with other *O,O*-dimethyl and *O,O*-diethyl phosphorodithioate insecticides established the analogous *S*-methylation pathway for ethion, malathion, phenthoate, phosalone, and phosmet in mice. Thus, metabolism of *O,O*-dialkyl phosphorodithioate insecticides in mammals is shown here for the first time to yield *S*-methyl phosphorodithioates and phosphorothiolates from in vivo *S*-methylation of the intermediate *O,O*-dialkyl phosphorodithioic acids.

## Introduction

The safety of several *O,O*-dimethyl phosphorodithioate insecticides is compromised when they contain small amounts of $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ because these impurities synergize the toxicity of the insecticide to mammals and are delayed toxicants in themselves (*1*−*5*). Thus, the rat oral $LD_{50}$ of malathion decreases from 12 500 to 4100 to 2900 mg/kg when $(MeO)_2P(O)SMe$ is present at 0%, 0.2%, and 1%, respectively (*2*, *5*). The oral $LD_{50}$ of $(MeO)_2P(O)SMe$ for rats is 20−60 mg/kg with deaths up to 22 days after treatment (*4, 5*); this delayed toxicity is antagonized or prevented by coadministration of small amounts of $(MeO)_2P(S)SMe$ or $(MeO)_3P(S)$ (*5*−*8*). To avoid toxicity problems, the manufacturing processes are designed to keep these *O,O,S*-trimethyl impurities at minimal levels.

The relationship of $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ as impurities and possible detoxication inhibitors in commercial *O,O*-dimethyl phosphorodithioate insecticides such as dimethoate and malathion is reexamined here in a new context that these *O,O,S*-trimethyl compounds might also be formed as metabolites by *S*-methylation. We recently reported that mono- and dithiocarbamic acid fungicides and herbicides or their metabolites are readily methylated in mice and that the *S*-methyl compounds are a contributing factor in their overall toxicology (*9*, *10*). The availability of $(MeO)_2P(S)SH$ as a metabolic precursor for *S*-methylation is well established since it is a urinary metabolite used for biological monitoring of human exposure to *O,O*-dimethyl phosphorodithioate insecticides, including dimethoate and malathion (*11*, *12*). The present study considers the possibility that the phosphorodithioic acid metabolites of *O,O*-dialkyl phosphorodithioate insecticides undergo in vivo *S*-methylation in mice to give *O,O*-dialkyl *S*-methyl phosphorodithioates and phosphorothiolates (Figure 1). It first compares the urinary metabolites in mice of dimethoate and $(MeO)_2P(S)SMe$ for common P-SMe products, indicating that dimethoate is cleaved and the P-SH metabolite(s) is methylated. It then examines the tissues for the presence and persistence of $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$.

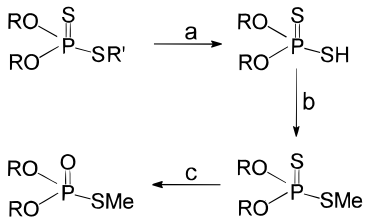


**Figure 1.** *S*-Methylation pathway for *O,O*-dialkyl phosphorodithioate insecticides. Reactions: (a) cleavage of S−R′ bond; (b) methylation of P-SH substituent; (c) oxidative desulfuration.

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## Experimental Procedures

**Caution.** *The OP compounds described herein include known or suspected acute and/or delayed neurotoxicants. Their use should be under careful containment conditions.*

**General**. $^1$H and $^{31}$P NMR spectra were recorded with a Bruker WM-300 spectrometer at 300 and 121.5 MHz, respectively. Chemical shifts are referenced to internal sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ in $D_2O$ or external trimethyl phosphate in $D_2O$ for $^1$H and $^{31}$P spectra, respectively. GC−chemical ionization−mass spectrometry (CI-MS)[1] with selected ion monitoring (SIM) involved the following: Hewlett Packard 5890 gas chromatograph coupled to a 5971A mass spectrometer; DB-5 fused-silica capillary column, 30 m × 0.25 mm i.d. (J and W Scientific, Folsom, CA); injection port temperature 250 °C; temperature program 70−250 °C over 18 min.

**Organophosphorus Compounds.** Chemicals designated by common name only are identified by structure in *The Pesticide Manual* (*13*). Dimethoate (>99% pure) was obtained from Chem Service (West Chester, PA), malathion (95% pure) and the dimethoate metabolite $(MeO)_2P(S)SCH_2C(O)OH$ were from American Cyanamid Co. (Wayne, NJ), and $MeO(NH_2)P(O)SMe$ and $MeO(NH_2)P(O)SNa$ were from Chevron Chemical Co. (Richmond, CA). The sample of dimethoate contained <0.05% $(MeO)_2P(S)SMe$ or $(MeO)_2P(O)SMe$ based on both $^{31}$P NMR and GC-CI-MS analysis. Malathion was purified by crystallization and four recrystallizations from methanol at −50 °C (*2*). Ethion, fenthion, phenthoate, phosalone, and phosmet (obtained from Chem Service) and the purified malathion contained <0.05% of P-SMe impurities analyzed by GC-CI-MS. $(MeO)_2P(S)SMe$ was prepared as described by Lin *et al*. (*14*) and $(MeO)_2P(O)SMe$ according to Norman *et al*. (*15*). $(EtO)_2P(S)SMe$ was synthesized from $(EtO)_2P(S)SK$ by reacting with excess MeI in refluxing methanol. Other organophosphorus compounds used were available from previous syntheses in this laboratory. The structure of each compound was verified by $^{31}$P NMR and GC-CI-MS (when appropriate).

Four candidate metabolites were prepared by *O*-demethylation of the appropriate *O,O,O*- or *O,O,S*-trimethyl derivatives. MeO(KS)P(O)SMe was obtained by treating $(MeO)_2P(S)SMe$ (10 mmol) with $(EtO)_2P(S)SK$ (10 mmol) in dry acetone at reflux for 4 h: mp 109−112 °C ($Et_2O$); $^1$H NMR $\delta$ 3.75 (3H, d, $J$ = 14.8 Hz, $OCH_3$), 2.31 (3H, d, $J$ = 13.8 Hz, $SCH_3$); $^{31}$P NMR $\delta$ 73.1. For characterization by GC/MS the salt was acidified and treated with diazomethane to give MeO(MeS)P(O)SMe ($MH^+$ = 173). For the preparation of MeO(NaO)P(O)SMe, $(MeO)_2P(O)SMe$ (10 mmol) was refluxed with NaI (15 mmol) in dry acetone for 4 h, resulting in precipitation of the desired product: $^1$H NMR $\delta$ 3.59 (3H, d, $J$ = 12.8 Hz, $OCH_3$), 2.13 (3H, d, $J$ = 13.8 Hz, $SCH_3$); $^{31}$P NMR $\delta$ 21.13. Esterification of the acidified product with diazomethane gave $(MeO)_2P(O)SMe$ characterized by GC/MS ($MH^+$ = 157). On a comparable basis, $(MeO)_2P(O)SH$ and $(MeO)_2P(O)OH$ were prepared by demethylation of $(MeO)_2P(S)OMe$ and $(MeO)_3P(O)$.

**Animals and Treatments.** Male albino Swiss-Webster mice (23−27 g) from Simonsen Laboratories (Gilroy, CA) were administered the test compounds ip or orally, at 100 mg/kg, using triethylene glycol monomethyl ether as the carrier (30 $\mu$L for each treatment); as exceptions, $(MeO)_2P(S)SMe$ was administered ip at 10 as well as 100 mg/kg in the same carrier and $MeO(NH_2)P(O)SNa$ ip at 500 mg/kg with water as the vehicle. At appropriate times the mice were sacrificed by cervical dislocation and dissected to obtain the liver, kidney, and lung which were analyzed immediately as indicated below.

**Extraction and Analysis of Tissues.** Fresh samples of liver (1 g), kidney (0.3−0.4 g), and lung (0.1 g) from individual mice were homogenized in methylene chloride (2 mL), and the homogenate was centrifuged at 5000*g* for 10 min. The separated organic layer was dried over sodium sulfate (anhydrous) and analyzed immediately by CI-MS-SIM, monitoring $MH^+$ ions. The analysis involved a 1-$\mu$L aliquot injected with the HP7673A automatic sample injector. Retention times (min) for the products were as follows: $(MeO)_2P(S)SMe$, 7.16; $(MeO)_2P(O)SMe$, 6.79; $(EtO)_2P(S)SMe$, 8.64; $(EtO)_2P(O)SMe$, 8.26. Quantitation of $(MeO)_2P(S)SMe$, $(MeO)_2P(O)SMe$, and dimethoate involved comparison to standard curves prepared with the authentic compounds using $(MeO)_3P(O)$ as the internal standard. Identification of $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ in liver extracts (concentrated by evaporation to 0.1 mL) utilized CI-MS in the scan mode, surveying all ions between 50 and 300 amu.

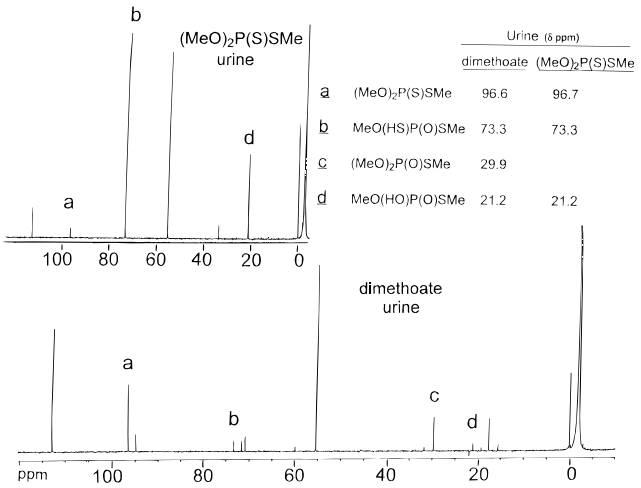


**Figure 2.** $^{31}$P NMR spectra of urinary metabolites of $(MeO)_2P(S)SMe$ and dimethoate. The 0−24 h urine is examined following an ip dose of 100 mg/kg. Metabolites with P-SMe substituents (a−d) as cleavage products of $(MeO)_2P(S)SMe$ and from methylation of dimethoate metabolites are identified by comparison with standards. Major metabolites of both $(MeO)_2P(S)SMe$ and dimethoate and their chemical shifts ($\delta$ ppm) are as follows: $(MeO)_2P(S)SH$ (112.7), $(MeO)_2P(O)SH$ (55.2), and $(MeO)_2P(O)OH$ (0.1). Additional metabolites of dimethoate are as follows: $(MeO)_2P(S)SCH_2C(O)OH$ (96.5) and $MeO(HS)P(O)SCH_2C(O)NHMe$ (70.8), assigned by comparison with standards; $MeO(HS)P(O)SCH_2C(O)OH$ (71.6) and $MeO(HO)P(O)SCH_2C(O)OH$ (17.6), tentatively proposed based on chemical shifts. Phosphoromonothioic acids are shown in the P-SH form.

**Urinary Metabolites.** The mice were treated ip with the test compounds, the urine was collected for 24 h, filtered (Millex-HV filter unit, 0.45 $\mu$m, nonsterilized, Millipore Products Division, Bedford, MA), and lyophilized by a Speed Vac concentrator (SVC 100, Savant Instruments, Farmington, NY), and the viscous liquid obtained was dissolved in $D_2O$ (0.5 mL). Phosphorus-containing urinary products were analyzed by $^{31}$P NMR. Organic extracts of the samples (1 mL of methylene chloride) were analyzed by CI-MS-SIM, monitoring $MH^+$ ions of the triesters.

**Enzymatic Methylation.** $(MeO)_2P(S)SH$ (0.12 mM final concentration) was incubated with *S*-adenosylmethionine (SAM) (Sigma Chemical Co., St. Louis, MO) (0.58 mM) and mouse liver microsomes (*10*) (1 mg of protein) in 100 mM sodium phosphate buffer (pH 7.4, 1 mL) for 60 min at 37 °C. For controls, SAM was not added or microsomes were denatured (85 °C, 15 min). Each reaction was partitioned with methylene chloride (1 mL) and analyzed by CI-MS-SIM as above.

## Results

**Urinary Metabolites of $(MeO)_2P(S)SMe$ and Dimethoate (Figure 2).** $(MeO)_2P(S)SMe$ treatment gives seven $^{31}$P NMR signals in the urine, six of which are identified by coincidence with those resulting from addition of the appropriate authentic standard. These compounds in order of decreasing peak heights are: MeO(HS)P(O)SMe, $(MeO)_2P(O)SH$, $(MeO)_2P(O)OH$, MeO(HO)P(O)SMe, $(MeO)_2P(S)SH$, and $(MeO)_2P(S)SMe$.

[1] Abbreviations: CI-MS, chemical ionization−mass spectrometry; SAM, *S*-adenosylmethionine; SIM, selected ion monitoring; Me, methyl; Et, ethyl.

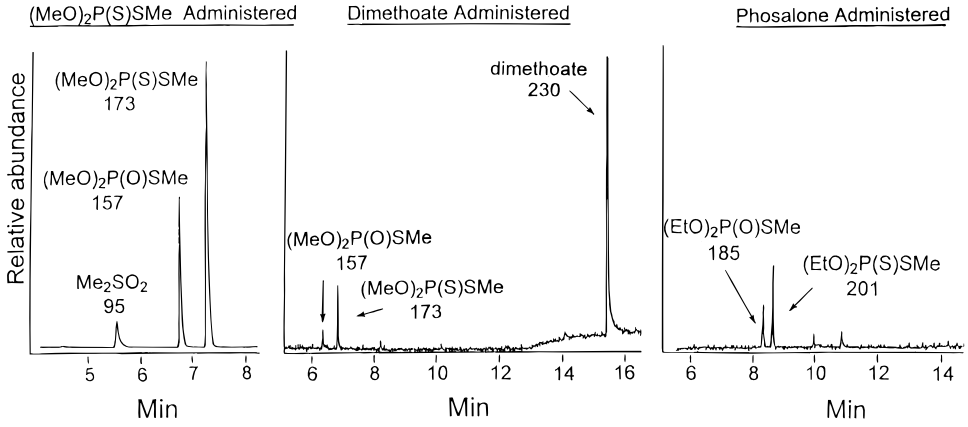


**Figure 3.** GC-CI-MS chromatograms showing the parent compound and two metabolites in the liver of mice 1 h after ip administration of $(MeO)_2P(S)SMe$, dimethoate, or phosalone. The relative sensitivity is 3 times greater for $(MeO)_2P(O)SMe$ than $(MeO)_2P(S)SMe$. Phosalone does not elute within 23 min under the standard conditions.

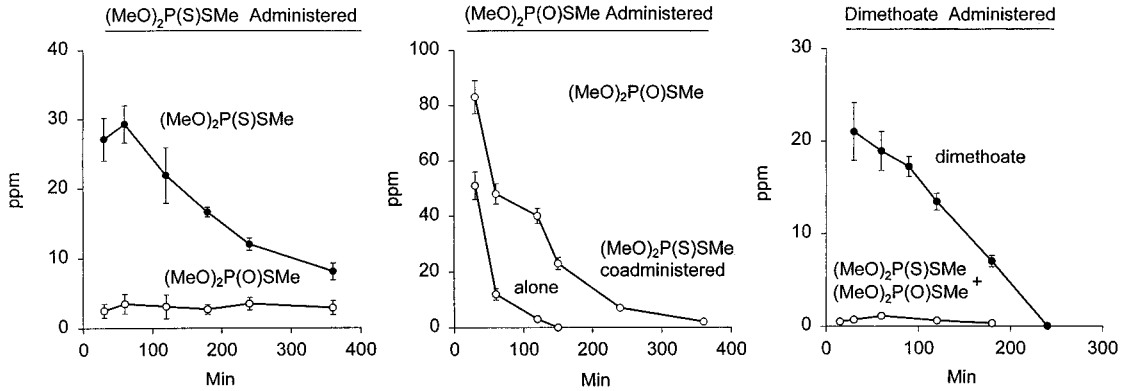


**Figure 4.** Metabolites and persistence of $(MeO)_2P(S)SMe$, $(MeO)_2P(O)SMe$, and dimethoate in the liver of mice 15−360 min after ip treatment at 100 mg/kg. The treatment with $(MeO)_2P(O)SMe$ was compared alone and with 5% $(MeO)_2P(S)SMe$.

Dimethoate gives four urinary metabolites with P-SMe substituents in decreasing $^{31}P$ NMR peak heights as follows: $(MeO)_2P(O)SMe$, MeO(HS)P(O)SMe, and MeO(HO)P(O)SMe plus $(MeO)_2P(S)SMe$ in a signal also containing $(MeO)_2P(S)SCH_2C(O)OH$. The presence of $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ as urinary metabolites of dimethoate is confirmed by GC-MS-SIM. Other identified metabolites are $(MeO)_2P(S)SCH_2C(O)OH$, MeO(HS)P(O)$SCH_2C(O)NHMe$, and three acids also obtained with $(MeO)_2P(S)SMe$, *i.e.*, $(MeO)_2P(O)SH$, $(MeO)_2P(O)OH$, and $(MeO)_2P(S)SH$ in decreasing peak heights. Tentatively-identified metabolites are the *O*-demethylated derivatives MeO(HS)P(O)$SCH_2C(O)OH$ and MeO(HO)P(O)$SCH_2C(O)OH$. These products are metabolites of dimethoate not impurities or degradation products formed during analysis as they are completely absent in two types of controls analyzed by $^{31}P$ NMR: the dimethoate sample used for this study; urine spiked with dimethoate for 24 h then the spectra recorded for a further 24 h at room temperature.

**Metabolites and Persistence of $(MeO)_2P(S)SMe$ in Liver (Figures 3 and 4).** The parent compound and two metabolites are detected in liver extracts of mice dosed at 100 mg/kg with $(MeO)_2P(S)SMe$, *i.e.*, $(MeO)_2P(O)SMe$ ($MH^+ = 157$) and $Me_2SO_2$ ($MH^+ = 95$). The level of parent compound peaks at ∼25−30 ppm at 30−60 min then progressively drops to 8 ppm at 360 min. The metabolite $(MeO)_2P(O)SMe$ remains at 2−4 ppm throughout the period of 30−360 min (but neither the parent compound nor the metabolite is detected at 14 h). In another study (not shown) with $(MeO)_2P(S)SMe$ treatment at 10 mg/kg and analysis 1 h after dosing, the parent compound is not detected in liver and $(MeO)_2P(O)SMe$ is found at 0.4 ppm whereas at a 5 mg/kg dose even the oxidized metabolite is not detected.

**Persistence of $(MeO)_2P(O)SMe$ in Liver (Figure 4).** The level of $(MeO)_2P(O)SMe$ in liver was examined as a function of time after an ip dose of 100 mg/kg of $(MeO)_2P(O)SMe$ alone or containing 5% $(MeO)_2P(S)SMe$ (*i.e.*, 5 mg/kg of the phosphorodithioate). The phosphorothiolate alone decreases from 50 to 10 to 3 ppm at 30, 60, and 120 min posttreatment and <0.05 ppm by 150 min, whereas with the small amount of dithioate present it is greatly stabilized with levels of >20 ppm persisting for 150 min and still with detectable levels at 360 min.

**Metabolites of Dimethoate in Tissues (Figures 3 and 4).** The dimethoate level in liver is maximal at 21 ppm at 30 min after ip treatment at 100 mg/kg. $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ are easily detected in the liver for 3 h after treatment at a ratio of ∼4:1, respectively, with the highest level of both appearing at 60 min (0.7 ppm in liver, 0.2 ppm in kidney, and <0.1 ppm in lung). The levels of $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ are similar following oral administration of dimethoate (60 min, 100 mg/kg, combined metabolites 0.6 ppm in liver), and at 60−120 min the amounts of the two metabolites combined are *ca.* 4% of the dimethoate level in liver.

***S*-Methylation of Other Phosphorodithioates.** $(MeO)_2P(S)SMe$ is detected in liver 60 min after a 100 mg/kg ip dose of the dimethyl phosphorodithioate insecticides dimethoate, phosmet, phenthoate, and malathion, decreasing in that order. In similar experiments with diethyl phosphorodithioate insecticides, $(EtO)_2P(S)SMe$

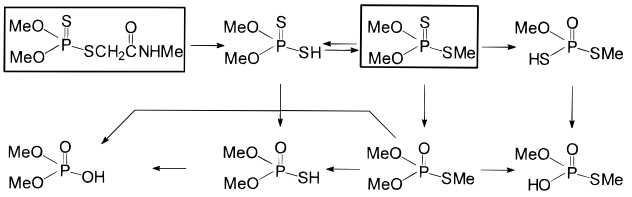

**Figure 5.** Partial metabolic pathways for dimethoate in mice with emphasis on *S*-methylation products and further metabolites of $(MeO)_2P(S)SMe$. All metabolites shown are detected in urine and the triesters also in liver.

is detected from phosalone (Figure 3 which also shows $(EtO)_2P(O)SMe$ as a metabolite), ethion, and the hydrolysis product $(EtO)_2P(S)SH$ in decreasing amounts. There is no detectable $(EtO)_2P(O)SMe$ with $(EtO)_2P(O)SR$ compounds when R = *n*-propyl, *sec*-butyl, and *tert*-butyl, nor with $(EtO)_2P(S)OEt$ or $(EtO)_2P(S)OC_6H_5$. There is also no detectable $(MeO)_2P(O)SMe$ formed via $(MeO)_2P(O)$-SH on metabolism of the $(MeO)_2P(S)O$-aryl insecticide fenthion. Finally, the urine of mice receiving an ip dose (500 mg/kg) of $MeO(NH_2)P(O)SNa$ does not contain $MeO(NH_2)P(O)SMe$ (methamidophos) detected by $^{31}P$ NMR examination nor is any toxicity observed.

**Enzymatic Methylation.** $(MeO)_2P(S)SMe$ is easily detected by CI-MS-SIM of incubation mixtures of $(MeO)_2P(S)SH$ in the enzymatic *S*-methylation system but not in two types of controls, *i.e.*, the peak height with microsomes and SAM is >17-fold greater than with incubations lacking this cofactor or when the microsomes are denatured (85 °C, 15 min).

## Discussion

Figure 5 shows the new *S*-methylation pathway for dialkyl phosphorodithioate insecticides as illustrated primarily with dimethoate giving rise to $(MeO)_2P(S)SMe$ and its further metabolites in mice including the toxicant $(MeO)_2P(O)SMe$ and its cleavage products. Identification of the four P-SMe urinary metabolites of dimethoate [which are not reported in earlier studies (refs 16−18 and references cited therein)] constitutes the strongest evidence for in vivo *S*-methylation, but GC-CI-SIM analysis also supports this observation. It is difficult to establish the overall yield of $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ from dimethoate since they are transient intermediates except for that portion removed from the metabolic pool by excretion. The P-SMe metabolites of dimethoate are not artifacts due to impurities based on rigorous controls and the identification of $(EtO)_2P(S)SMe$ from phosalone that in itself contains no methyl group, further validating the conclusion that *S*-methylation is metabolic and not artifactual. In comparing the $^{31}P$ NMR spectra of dimethoate metabolites in rat urine with those in mouse urine, we observe (data not shown) a preference for cleavage of the PS−C bond in mice and the C(O)−NHMe substituent in rats, consistent with known species differences in cleavage sites and rates (*19*) and perhaps with the higher toxicity of dimethoate to mice than to rats (*13*).

The *S*-methylation of metabolites from dimethoate and related compounds (ethion, malathion, phenthoate, phosalone, and phosmet) appears to be a general pathway for *O,O*-dialkyl phosphorodithioate pesticides with SAM a likely cofactor as demonstrated in vitro with liver and $(MeO)_2P(S)SH$. $(RO)_2P(S)SH$ is methylated, whereas the more acidic $(RO)_2P(O)SH$ is not observed as its methylated derivative, in contrast to $R_1R_2NC(S)SH$ and $R_1R_2$-$NC(O)SH$ both of which undergo *S*-methylation (*9, 10*). This conclusion extends to an important phosphoramidothiolate insecticide (*i.e.*, methamidophos) where the $MeO(NH_2)P(O)SH$ metabolite does not appear to be an acceptable substrate for methylation.

*O*- and *S*-demethylation are prominent metabolic reactions for $(MeO)_2P(O)SMe$ in mice, but it is not known if this involves sulfoxidation or is catalyzed by GSH *S*-transferase (*6*). However, the prolonged persistence in this study of $(MeO)_2P(O)SMe$ in the presence of a small amount of $(MeO)_2P(S)SMe$, as noted earlier with $(MeO)_3P(S)$ (*6*), is more easily attributed to inhibition of cytochrome P450. The mechanism of toxic action for $(MeO)_2P(O)SMe$ is unknown (*20*) but thought to involve a sulfoxide intermediate (*6, 21*) which covalently derivatizes tissues (*22*). $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ are poor inhibitors of cholinesterase (*4, 5*) and poor alkylating agents (*23, 24*). Several studies have focused on the lung as a primary site of toxicity for $(MeO)_2P(O)SMe$ (*7, 8, 25*−*27*). $(MeO)_2P(S)SMe$ is not a lung-specific toxin, and when administered to rats in small amounts, it prevents the lung damage caused by analogs of $(MeO)_2P(O)SMe$ (*8, 28*). $(MeO)_2P(O)SMe$ requires activation by the cytochrome P450 system before it becomes toxic to the lung (*28*), and $(MeO)_2P(S)SMe$ has been shown to be a specific inhibitor of pulmonary cytochrome P450 2B1 activity (*29*).

The contribution of metabolically-generated $(MeO)_2P(S)SMe$ and $(MeO)_2P(O)SMe$ to the mammalian toxicity of dimethoate, malathion, and related phosphorodithioate insecticides is unclear. The delayed neurotoxic poisoning symptoms after high doses of dimethoate in humans (*30*) are conceivably due in part to $(MeO)_2P(O)SMe$ as a metabolite, a possibility that has not been examined. This study demonstrates that *O,O,S*-trimethyl phosphorodithioate and phosphorothiolate are not only impurities in technical phosphorodithioate insecticides but also potentially toxic metabolites formed in tissues by a previously unrecognized pathway for mammals.

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