Metabolism of the Insecticidal Carbamate Methyl *N*-[[[[[(1,1-Dimethylethyl)(5,5-dimethyl-2-thioxo-1,3,2-dioxaphosphorinan-2-yl)-amino]thio]methylamino]carbonyl]oxy]ethanimidothioate in Rats

Prem S. Jaglan* and T. S. Arnold

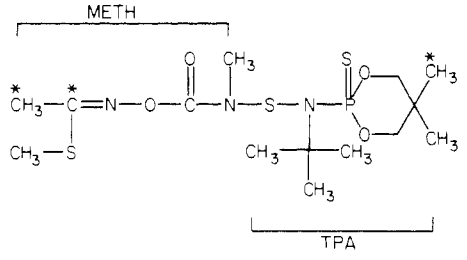
The metabolism of methyl *N*-[[[[[1,1-dimethylethyl)(5,5-dimethyl-2-thioxo-1,3,2-dioxaphosphorinan-2-yl)amino]thio]methylamino]carbonyl]oxy]ethanimidothioate (I) in the Sprague-Dawley rats administered a single oral dose labeled either in the methomyl part (^{14}C–^{14}C=N, METH label) or on the thiophosphoramide part (^{14}C-*gem*-dimethyl, TPA label) showed striking differences in their metabolites, indicating that the N–S–N bond of the molecule was split. Metabolism of the METH label gave rise to radioactive carbon dioxide and acetonitrile as major metabolites in the respired air. The metabolism of TPA label involved the desulfuration of thiophosphoramide to oxyphosphoramide. Hydroxyoxyphosphoramide and its glucuronide conjugate were the major urinary metabolites. Hydroxylation occurred at the equitorial methyl of oxyphosphoramide.

Methyl *N*-[[[[[(1,1-dimethylethyl)(5,5-dimethyl-2-thioxo-1,3,2-dioxaphosphorinan-2-yl)amino]thio]methylamino]carbonyl]oxy]ethanimidothioate (I) is efficacious for the control of lepidopterous larvae on many agronomic and horticultural crops. It is a phosphoramidothio derivative of the carbamate insecticide methomyl (II), *S*-methyl *N*-[[methylcarbamoyl]oxy]thioacetimidate, and is safer to mammals over many existing insecticides (LD_{50} in the adult rats fasted overnight ~5000 mg/kg). The mechanism of safety of derivatized carbamates to mammals has recently been proposed (Fukuto and Fahmy, 1981).

In the present study, the absorption, excretion, and metabolism of ^{14}C-labeled I was studied in the rat as part of a program to aid toxicologists to evaluate the safety of this insecticide.

EXPERIMENTAL SECTION

Materials. *[^{14}C]I.* In order to study the metabolism of I, both methomyl (METH) and thiophosphoramide (TPA) parts of the molecule were labeled as shown. Both



I

[^{14}C]I METH and [^{14}C]I TPA labeled with specific activities of 31.5 and 24.8 μCi/mg, respectively, were synthesized by Dr. D. B. Johnson (The Upjohn Co., Kalamazoo, MI). Purity was found to be greater than 98% by TLC on silica gel GF in the solvent system hexane–ethyl acetate (1:1). The labeled compounds were diluted with nonlabeled I to give the desired specific activities in the various experiments described below.

Other Compounds. Other compounds used and discussed are described in Table I, along with their R_f values in two solvent systems.

Instrumental Analysis. Mass spectra of V and VI (Table I) were recorded by using chemical ionization (CI) mass spectrometry on the CH7-A (Varian) and by using isobutane as the reagent gas. The mass spectrum of VII was obtained by fast atom bombardment (FAB) on a VG-ZAB-2F double-focusing mass spectrometer using argon as the ionization gas. The sample was dissolved in methanol and added to a redistilled glycerol matrix. The mass spectrum was obtained from 1 to 1500 daltons at 8-kV accelerating voltage.

Proton nuclear magnetic resonance spectra (NMR) of the standard oxyphosphoramide (V) and radioactive metabolite (VI) were recorded on a Varian XL-200 Fourier transform spectrometer. The samples were dissolved in deuteriochloroform, and the spectra were expressed in parts per million from tetramethylsilane internal standard. Homonuclear decoupling was employed to confirm coupling patterns.

Methods. *Treatment of Rats and Sample Collection.* Individual Sprague-Dawley (Charles River) rats of each sex, about 8 weeks old, were acclimated for 3–4 days in special metabolism cages where urine was collected separately from feces (Maryland Plastic, Inc., Model No. E-1100, Federalsburg, MD). Water and food were provided ad libitum. One rat of each sex was used at each dose level. The selected dose (5 or 40 mg/kg) of [^{14}C]I (METH or TPA label) was prepared in a 2-mL mixture of 0.5 g/mL Vehicle No. 122 (0.25% methylcellulose, The Upjohn Co.) and 0.005 g/mL Tween-20 [poly(oxyethylene) sorbitan monolaurate, Atlas Chemicals, Inc.]. A 5 mg/kg dose was evaluated to be a nontoxic dose from chronic feeding studies whereas 40 mg/kg was considered toxic. The rats were orally dosed by gastric intubation and immediately put into the metabolism cages. The cages were placed into enclosed polyethylene chambers (19 in. length × 11 in. width × 12.5 in. height) immediately. The top of the chambers was sealed with glass on a silicon rubber gasket. The chambers had an inlet and outlet for the collection of respired air. The outlets were connected to three 250-mL gas collection cylinders fitted with fine-frit filters in the inlet tubes. All cylinders were connected in series with polyethylene tubing. The last cylinder was connected to a vacuum line through a suction flask to catch the accidental overflow of liquid. The first cylinder attached to the cage contained 200 mL of methanol, the second contained 100 mL of methanol cooled in dry ice–ethanol, and the third cylinder contained 100 mL of 4 N sodium hydroxide. The flow rate through the traps was adjusted to 400 mL/min. The traps were changed at 12, 24, and 48 h posttreatment (no traps were used beyond 48 h).

Urine and feces were collected at 48 and 72 h. Each cage was washed with methanol and water at the end of the study.

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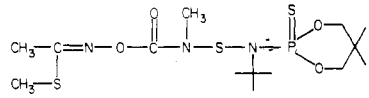
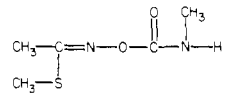
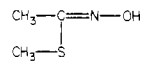
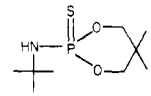
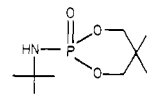
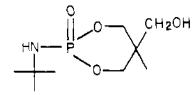
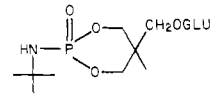
Table I. Number, Name, and Structure of Compounds Described in the Text and Their R_f Value in Two Solvent Systems

no.	trivial name	structure	R_f in solvent systems[a] A	B
I[b]	U-56,295		0.50	1.00
II[b]	methomyl		0.28	1.00
III[b]	methomyl oxime		0.65	1.00
IV[b]	thiophosphoramide		0.95	1.00
V[b]	oxyphosphoramide		0.05	0.65
VI[c]	hydroxyoxyphosphoramide		0.00	0.30
VII[c]	glucuronide of VI		0.00	0.05

[a] Silica gel GF 250 μm thick, developed 10 cm in (A) hexane–EtOAc (1:1) or (B) EtOAc–MeOH (96:4). [b] The Upjohn Co., Kalamazoo, MI.

The rats treated with 40 and 5 mg/kg doses were sacrificed after 2 and 3 days posttreatment, respectively, and blood, GI tract, and tissues were harvested for the tissue residue determination. The remainder of the carcass was homogenized in water for the radioactivity determination.

In addition, four rats were treated with high doses (~1500 mg/kg) of [^{14}C]I TPA label in order to characterize the urinary metabolites.

Radioactive Determination. Radioactivity in samples of urine, feces, cage washes, and tissues was determined by standard procedures (Jaglan et al., 1976). Aliquots from traps were counted similar to those from urine.

Nature of Volatile Metabolites. Aliquots from the sodium hydroxide traps were treated with an equal volume of saturated barium chloride solution and then centrifuged at 2000 rpm for 20 min. Aliquots of the supernatant were counted for radioactivity by liquid scintillation counting (LSC).

Aliquots of the methanol traps were refluxed with 10% sodium hydroxide for 16 h, and the methanol was evaporated off at 60 °C. The pH was adjusted to 3 with concentrated HCl and the samples were extracted in ether. The radioactivity from the ether was partitioned into 5% sodium bicarbonate. After neutralization of the sodium bicarbonate solution with concentrated HCl, aliquots were analyzed on a 4 ft × 3 mm id. Porapak Q (Water Associates) column operated at 140 °C with flow of argon–methane (95:5) of 40 mL/min with a 5700 Hewlet-Packard GC hooked to a Panax radioactive monitor (RAM). The split ratio of column effluents for hydrogen flame and RAM was 75:25.

Characterization of Fecal Metabolites. The feces were extracted 3 times with 5 volumes of methylene chloride followed by methanol. Aliquots of the methylene chloride and methanol extracts were examined on TLC silica gel GF 250 μm thick plates. The plates were developed 10 cm in both hexane–ethyl acetate (1:1) and EtOAc–MeOH (96:4). Standard compounds (Table I) were cochromatographed, and the amounts of metabolites based on the R_f of standard compounds were calculated by counting the scraping of silica gel.

Characterization of Urinary Metabolites. (*1*) *METH Label.* Aliquots of urine were hydrolyzed in 10% sodium hydroxide, and the radioactive compounds were extracted with ether at pH 3 and analyzed on a Porapak Q column as described under Nature of Volatile Metabolites.

(*2*) *TPA Label.* Aliquots of urine were charged onto an XAD-2 column (45 × 1.9 cm), and the radioactive metabolites were eluted with 1 L of water, followed by 1 L of methanol, and finally with 1 L of methanol–HCl (1%). The methanol elutes, which comprised most of the radioactivity, were evaporated to dryness and examined by TLC (silica gel GF 250 μm developed 10 cm in ethyl-acetate–methanol, 96:4). Histograms of the scraping of silica in 5-mm increments, using a Camag Zonel TLC plate scraper, were prepared. Radioactive bands from TLC plates were eluted with methanol and were analyzed by mass spectrometry and NMR.

RESULTS AND DISCUSSION

Disposition of Radioactive Dose. No significant differences in the disposition of I, either the METH or TPA label, were observed between male and female rats at both dose levels, and the data in Tables II and III show average values. As expected, the ratio of dose excreted (Table II) in urine to feces was higher from low dose (5 mg/kg) as compared to high dose (40 mg/kg). Most of the dose was excreted in urine and feces during the first 24 h posttreatment, and excretion of radioactivity was essentially complete by 48 h posttreatment.

The fate of the METH label was markedly different than that of the TPA label. At both dose levels of the METH label, about 39% of the dose was expired as radioactive carbon dioxide and acetonitrile (Table II). On the other hand, very little dose was expired from the TPA label. These data therefore suggest that I was probably

Table II. Excretion and Respiration of [^{14}C]I, METH and TPA Label (Percent of Dose)

	dose level			
	40 mg/kg		5 mg/kg	
	METH	TPA	METH	TPA
expired				
CO_2	7.4	0.9	11.8	2.1
CH_3CN	30.4	0.0	28.0	0.1
total	37.8	0.9	39.8	2.2
excreted				
urine	10.1	35.6	19.5	79.7
feces	31.8	59.9	14.3	19.4
cage wash	0.5	1.5	1.0	1.5
total	42.4	97.0	34.8	100.6
retained				
GI tract	1.7	0.1	1.6	0.1
tissues	7.8	1.1	9.0	1.4
total	9.5	1.2	10.6	1.5
total accounted	89.7	99.1	86.2	104.3

Table III. Residues of I (ppm ^{14}C equiv) from Oral Treatment of Rats

	dose level			
	40 mg/kg label		5 mg/kg label	
tissues	METH	TPA	METH	TPA
blood	7.4	0.1	3.3	0.0
liver	6.2	2.9	1.1	0.8
kidney	4.7	1.0	1.2	0.4
heart	3.0	0.9	0.8	0.2
lung	3.6	0.8	0.8	0.5
brain	1.2	0.2	0.3	0.0
spleen	4.1	0.5	1.2	0.1
fat	2.0	0.1	0.4	0.1
muscle	1.5	0.2	0.3	0.1
hide	5.2	0.3	0.6	0.1

metabolized to methomyl (II) and thiophosphoramide (IV) by splitting off the CH_3-N-S- bond.

Residues in the tissues from treatment with the [^{14}C]I METH label were considerably higher as compared to those of the TPA label (Table III). Similar high residues were also observed from the treatment of rats with [^{14}C]methomyl (Harvey et al., 1973) and with radioactive [^{14}C]acetonitrile (Jaglan and Arnold, 1981), a metabolite identified from the treatment of rats with the METH label (see below).

Characterization of Volatile Metabolites. About 10% of the dose trapped in sodium hydroxide following treatment with the [^{14}C]I METH label was characterized as CO_2 by precipitation with saturated barium chloride solution. The radioactive metabolite trapped in methanol was characterized as acetonitrile. This metabolite was lost when methanol solutions were evaporated to dryness at 40 °C similar to standard radioactive [^{14}C]acetonitrile. Fractional distillation of methanol solutions and analysis of distillate on the Porapak Q column on a GC/radioactive monitor showed that it was [^{14}C]acetonitrile since the peak was radioactive and had an identical retention time to that of standard acetonitrile. After hydrolysis of the radioactive metabolites with base, the pH was adjusted to 3 with concentrated HCl and the radioactive compound was extracted into ether. It was analyzed by the GC/radioactive monitor on Porapak Q. The radioactive metabolite derivative was acetic acid. This provided confirming evidence for acetonitrile as the neutral radioactive metabolite.

Harvey et al. (1973) also observed carbon dioxide and acetonitrile as radioactive metabolites of methomyl identical with that observed in this study. The mechanism of formation of acetonitrile from *anti*-methomyl, which undoubtedly arises from isomerization of *syn*-methomyl, has already been postulated (Huhtanen and Dorough, 1976).

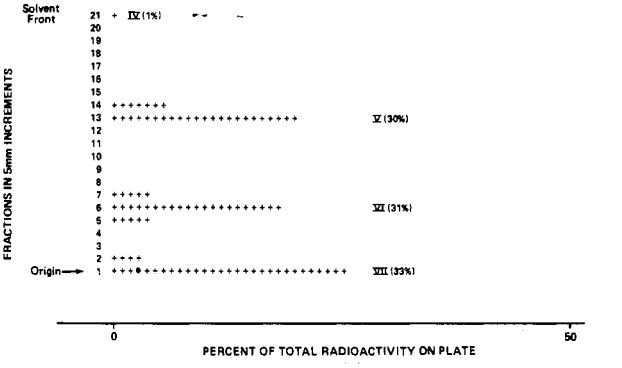


Figure 1. Histogram of urinary radioactive metabolites from the [^{14}C]I TPA label. Silica gel GF 250 μm thick plate developed 10 cm from origin in EtOAc–MeOH (96:4).

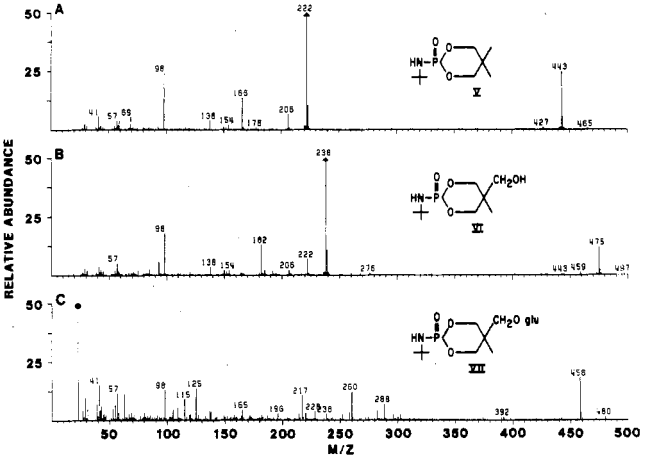


Figure 2. Chemical ionization mass spectra of V (A) and VI (B). FAB mass spectrum of VII (C).

Characterization of Metabolites in Feces and Urine. The nature of metabolites in urine and feces appeared similar at both dose levels.

Nature of Fecal Metabolites. From the METH label, only trace amounts of methomyl (II) were present, and no methomyl oxime (III) was found. The metabolites were polar and stayed at the origin of the plate. From the TPA label, about 50 and 10% of the fecal radioactivity were characterized as thiophosphoramide (IV) and oxyphosphoramide (V), respectively. Other metabolites were polar and stayed at the origin. At a high dose level (40 mg/kg), considerable amounts of unchanged I were present. At the highest dose tested (~1500 mg/kg), more than 90% of fecal metabolites was comprised of unchanged I.

Nature of Urinary Metabolites. All urinary metabolites from the METH label were highly polar. There was no I, methomyl (II), and methomyl oxime (III), and the metabolites could not be hydrolyzed with glusulase, indicating that the glucuronide or sulfate conjugates were not present. Harvey et al. (1973) also observed similar behavior of the urinary metabolite of methomyl in rats. However, when the conjugates were hydrolyzed with base and the radioactivity was extracted with ether at pH 3, radioactive acetic acid was characterized by the GC/radioactive monitor.

Several urinary metabolites frm the [^{14}C]I TPA label were separated by TLC (Figure 1). Thiophosphoramide (IV) was present in trace amounts. Chemical ionization mass spectrometry of the TLC eluate confirmed the structure of V and VI (parts A and B of Figure 2). Mebatolite V gave ions at m/z 222 $(M + H)^+$ and m/z 443

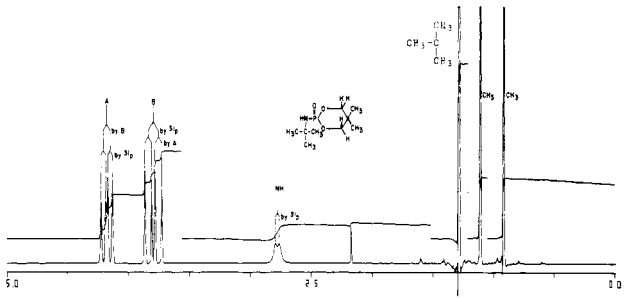


Figure 3. NMR spectrum of V in deuteriochloroform (abscissa in ppm with respect to tetramethylsilane standard at 0 ppm).

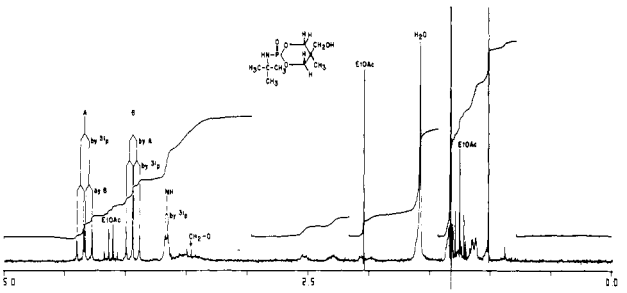


Figure 4. NMR spectrum of VI in deuteriochloroform (abscissa in ppm with respect to tetramethylsilane standard at 0 ppm).

$(2M + H)^+$ for oxyphosphoramide, whereas VI gave ions at m/z 238 $(M + H)^+$ and 475 $(2M + H)^+$ for hydroxyoxyphosphoramide. The nature of the polar metabolite VII was deduced by FAB/MS as being the glucuronide conjugate of VI as a disodium salt, m/z 458 (Figure 2C).

Because hydroxylation can occur at several positions of the molecule, NMR of V was compared with that of VI.

In the case of V (Figure 3), the axial *gem*-dimethyl showed a singlet with three protons at 0.92 ppm and the equitorial methyl gave a singlet with three protons at 1.10 ppm. There was a singlet with nine protons at 1.29 ppm due to the *tert*-butyl group and a doublet at 2.78 ppm due to NH with $J_{H,P}$ of 6 Hz. The two axial protons of the two methylene groups are also equivalent and appear as a doublet of a doublet at 3.80 ppm with $J_{H,P}$ of 16 Hz and geminal coupling of 11 Hz. The two equitorial protons of the two methylene groups are also equivalent and appear as a doublet of a doublet at 4.18 ppm with $J_{H,P}$ of 8 Hz and geminal coupling of 11 Hz.

VI (Figure 4) showed a singlet with three protons at 1.03 ppm similar to the axial proton of *gem*-dimethyl of oxyphosphoramide (V). There was also a singlet with nine protons at 1.34 ppm due to the *tert*-butyl group and a doublet at 3.20 ppm due to NH with $J_{H,P}$ of 6 Hz. Similar to oxyphosphoramide, two axial protons of two methylenes show a doublet of a doublet at 3.95 ppm with $J_{H,P}$ of 10 Hz and geminal coupling of 12 Hz. Two equitorial protons of two methylene groups also show a doublet of a doublet at 4.35 ppm with $J_{H,P}$ of 14 Hz and geminal coupling of 12 Hz.

There was no absorption due to the equitorial methyl of the *gem*-dimethyl and a new absorption of O-CH_2 protons appeared as an A and a B pattern centered at 3.48 ppm, which showed that hydroxylation took place at the equitorial methyl.

Although hydroxylation of the *tert*-butyl group has been observed in the metabolism of tebuthiuron (Morton and Hoffman, 1976) and buthidazole (Attallah et al., 1980) in animals, it was not observed in the present study.

CONCLUSION

I is relatively safe (LD_{50} ~ 5000 mg/kg in rats), and extensive research in Dr. Fukuto's laboratories has shown

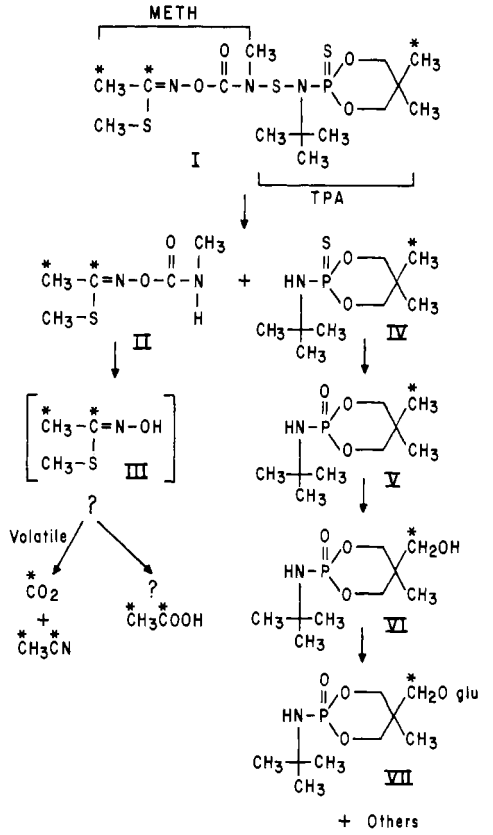


Figure 5. Metabolism scheme of I in the rats.

that derivatization of carbamate insecticides invariably results in increased mammalian safety. Sulfenylated derivatives of toxic methyl carbamate esters were metabolized to nontoxic products before intoxication can take place by splitting of the N–S–N bond (Fukuto and Fahmy, 1981). Splitting of CH_3–N–S– bond appears to be the primary pathway for the metabolism of I (Figure 5) to methomyl (II) and thiophosphoramide (IV). Although Norman et al. (1974), DeMatteis (1977), and Obrehska et al. (1980) have suggested that the singlet sulfur atoms released by metabolic desulfuration are powerful electrophile-like nitrenes and carbenes and bind covalently to macromolecules to elicit toxicity, I is safe, indicating that the sulfur atoms released are incorporated in the general sulfur pool, probably as inorganic sulfate (Trennery and Waring, 1983). However, the ultimate fate of sulfur in the moiety is unknown. Further metabolism of the METH label was similar to that of methomyl (II). Radioactive CO_2 and CH_3CN were the major metabolites. Metabolism of the TPA label followed well-established pathways, i.e., desulfuration of IV to V. Hydroxylation of the methyl group of V gave rise to VI, which was conjugated with glucuronic acid.

ACKNOWLEDGMENT

The technical help of Dr. L. Baczynskyj, Dr. R. E. Hornish, S. A. Mizsak, R. E. Gosline, and Paul Skinner is appreciated. Drs. A. W. Neff and E. G. Gemrich provided valuable suggestions.

Registry No. I, 72542-56-4; II, 16752-77-5; IV, 72542-64-4; V, 944-23-0; VI, 89399-24-6; VII, 89399-25-7; acetonitrile, 75-05-8; carbon dioxide, 124-38-9.

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Trace Analysis for Organothiophosphate Agricultural Chemicals by High-Performance Liquid Chromatography–Photolysis–Electrochemical Detection

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Organic thiophosphate agricultural chemicals, such as malathion, parathion, and others, can be satisfactorily analyzed by the newer method of high-performance liquid chromatography (HPLC) with on-line photolysis (hv), followed by electrochemical detection (EC) using single- or dual-electrode approaches for the species generated. This approach, HPLC–hv–EC, has been applied to about 20 different thiophosphates, most of which are widely used agriculturally and for which trace residue levels are routinely monitored. Dual-electrode response ratios have been determined for all of these analytes, along with minimum detection limits (MDLs) in many cases. These approaches can also be used for the quality control evaluation of commercial formulations by flow injection analysis (FIA) with hv–EC and no HPLC separations. Wheat middling extracts have been analyzed by the commonly used gas chromatography (GC) flame photometric detection (FPD) method of residue analysis, as well as by HPLC–hv–EC. These comparative studies indicate that the newer method is reproducible, accurate, precise, and entirely reliable. Standard additions have been applied to wheat middling extracts, and the quantitative results are compared with the external standard method.

Many government regulatory or private service laboratories still monitor trace residue levels of various agricultural chemicals (Das, 1981). Although many routine-type analyses still utilize thin-layer chromatography (TLC) or gas chromatography (GC), several pesticide-, herbicide-, or fungicide-type analyses are now using high-performance liquid chromatography (HPLC) (Moye, 1975; Lawrence et al., 1980; Lawrence, 1984; Papadopoulou-Mourkidou et al., 1981; Harvey and Zweig, 1980). Nevertheless, most government agencies today still routinely utilize involved sample preparation and workup together with GC-selective detection. Organic thiophosphates represent a very large class of agricultural chemicals in widespread use, and there remains a need for improved, specific HPLC approaches for their detection. Although many of these thiophosphates are aromatic derived and chromophoric, a large number of them are not. Thus, HPLC with either ultraviolet (UV) or fluorescence (FL) detectors is not a satisfactory detection method. Derivatization for improved HPLC–UV/FL detection of pesticides is always a real possibility, though this requires additional sample handling, treatment, and workup and provides additional room for error (Frei and Lawrence, 1981). Ideally, derivatization should be continuous, on-line, in real-time, postcolumn, and very selective for the particular analyte of interest (Xie et al., 1983). For those organic thiophosphates that are not UV or FL active, there remains electrochemical detection (EC), assuming that such materials respond oxidatively and/or reductively. Several organic thiophosphate agricultural chemicals can be analyzed by reductive LCEC (liquid chromatography–electrochemical detection) (Shoup, 1982). Reductive LCEC, however, has its own operational difficulties, and it generally requires more expertise and experience than oxidative LCEC (Krull et al., 1983). EC detection approaches, especially with the dual-electrode transducers (series or parallel), can provide improved analyte specificity over single-electrode methods. Dual-electrode LCEC can also provide minimum detection limits (MDLs) equal to or better than single-electrode LCEC, especially in the series (upstream–downstream) mode.

It occurred to us that oxidative dual-electrode LCEC could provide an easy-to-use, sensitive, and highly selective approach to all organic thiophosphates, but only if the original analytes could be easily and reproducibly converted into derivatives, on-line, pre- or postcolumn, that were then suitable for oxidative EC detection. This particular class of thiophosphates is not suitable for direct oxidative EC. Most derivatization approaches in LCEC have used off-line, precolumn techniques (Shoup, 1982; Krull et al., 1983). Though continuous, postcolumn, on-line photochemistry has been used in HPLC–UV and HPLC–FL, very little has yet been described in LCEC (Krull and

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