studies support this speculation. Under the conditions of our experiments where no degradation of [^{14}C]2,4-D was observed in the water, all the radioactivity in the fish was present as the unchanged herbicide.

ACKNOWLEDGMENT

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Metabolism of Cytrolane Systemic Insecticide (Mephosfolan), Propylene (Diethoxyphosphinyl)dithioimidocarbonate, in Cotton Plants

Jack Zulalian* and Roger C. Blinn

Cytrolane Systemic Insecticide (mephosfolan), labeled with carbon-14 at the imino carbon atom, was applied foliarly to cotton plants in the greenhouse. After 42 days, 58.2% of the applied dose was recovered from the cotton plants. Most of the radioactivity, 53% of the applied dose, was recovered from the treated leaves while small amounts of radioactivity were recovered from the untreated leaves and the stems (including the petioles and roots), 1.1 and 4.1%, respectively. Of the applied radioactivity that was recovered from the treated leaves, 41.5% was in the ethanol extract and 11.5% was in the plant marc. The major residue that was found on the leaves, 60.9% of the radioactivity in the extract after 42 days (25.3% of the applied dose), was identified as mephosfolan. Thiocyanate ion and the glucose conjugates of (hydroxymethyl)ethylene (diethoxyphosphinyl)dithioimidocarbonate and (hydroxymethyl)vinylene (diethoxyphosphinyl)dithioimidocarbonate were identified as metabolites of mephosfolan. These metabolites accounted for 0.2, 2.1, and 4.6% of the applied dose, respectively. Two additional metabolites were found, accounting for 1.9 and 0.9% of the applied dose, which had two-dimensional TLC properties that were similar to the two metabolites derived from the carbon-14 labeled iminodithiolane intermediate, propylene dithioimidocarbonate hydrochloride. Therefore, these two metabolites may not retain the diethoxyphosphinyl moiety of mephosfolan.

Mephosfolan (I) [propylene (diethoxyphosphinyl)dithioimidocarbonate], the active ingredient in Cytrolane (trademark of American Cyanamid Co.) Systemic Insecticide, is an organophosphate insecticide effective for the control of both sucking and chewing insects that attack crops of economic importance. Field evaluation of mephosfolan on cotton grown in Egypt revealed excellent control of the cotton leafworm *Spodoptera littoralis* (Zeid et al., 1968; Kamel and Mitri, 1970), the pink bollworm *Pectinophora* spp., and the spiny bollworm *Earias insulana*. The compound is also effective against several major pests of beets, carrots, celery, corn, rice, and sugarcane.

A metabolism study of carbon-14 labeled mephosfolan was conducted with cotton plants (Delta pine smooth leaf variety) in a greenhouse in order to determine the nature and toxicity of the plant metabolites. This paper describes the isolation, identification, and synthesis of the cotton plant metabolites of mephosfolan.

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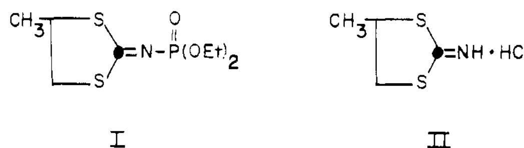
MATERIALS AND METHODS

Radiochemicals. Carbon-14 labeled mephosfolan was synthesized from [^{14}C]cyanogen chloride and 1,2-dimercaptopropane by New England Nuclear Corp. (Boston, Mass.) following a procedure developed by Addor (1964, 1965). The radiopurity of the preparation was 99% as ascertained by two-dimensional TLC in chloroform–ethyl acetate–dioxane (150:30:30) vs. chloroform–acetone–acetic acid (129:15:6). The sp. act. was 5.38 mCi/mmol (20 μCi/mg, 44.4 × 10^3 dpm/μg). The radiolabeled synthesis intermediate, propylene [^{14}C]dithioimidocarbonate hydrochloride (II), was obtained with a sp. act. of 5.38 mCi/mmol (32 μCi/mg).



Synthesis of Metabolites. The 1,2-dimercaptopropane, 2,3-dimercaptopropanol, diethyl phosphoro-

chloridate, and ethyl phosphorodichloridate that were used for the synthesis of potential metabolites of mephosfolan are all available from the Aldrich Chemical Co. All of the compounds synthesized as potential metabolites were authenticated where possible by NMR, infrared analysis, mass spectral analysis, and elemental analysis.

(Hydroxymethyl)ethylene Dithioimidocarbonate Hydrochloride. Dry hydrogen chloride was passed into a mixture of 50 mL of toluene and 0.1 mL of ethanol for about 1 min at 13 °C. After adding 10.0 g of 2,3-dimercaptopropanol, 5.0 g of gaseous cyanogen chloride was added over 2 h with the temperature held below 22 °C. After stirring the mixture overnight, the toluene was decanted and the solid was triturated twice with 50 mL of acetone and once with 100 mL of 1-butanol (with heat). The solid was collected and after vacuum drying amounted to 2.6 g (mp 142–155 °C dec). The infrared spectrum showed strong OH absorption at 3200 cm^{-1} and no SH absorption at 2500 cm^{-1}. An analytical sample of (hydroxymethyl)ethylene dithioimidocarbonate hydrochloride had mp 159–161 °C. Anal. Calcd for $C_4H_8S_2ONCl$: C, 25.87; H, 4.34; N, 7.54. Found: C, 25.34; H, 4.43; N, 7.97.

(Hydroxymethyl)ethylene (Diethoxyphosphinyl)-dithioimidocarbonate (Aglycone 14a). To a well-stirred mixture of 2.5 g (0.013 mol) of (hydroxymethyl)ethylene dithioimidocarbonate hydrochloride in 50 mL of methylene chloride and 5 mL of water was added 2.6 g (0.014 mol) of diethyl phosphorochloridate over several minutes. After 1 h, the organic phase was separated, washed with saturated potassium carbonate, and filtered through sodium sulfate. The oil (3.7 g) obtained after removal of solvent was purified by chromatography on 20 × 20 cm 2.5-mm thick silica gel plates using 9:1 ethanol–ethyl acetate. The major component, corresponding to an R_f ca. 0.4, was recovered and amounted to 1.2 g. TLC on silica gel showed a single spot at R_f 0.23 using 9:1 diethyl ether–ethanol and at R_f 0.39 using 8:2 ethyl acetate–ethanol. The infrared spectrum showed strong bands at 3300 (OH), 1560 (C=N), 1230 (P=O), and 1040 cm^{-1} (P–O–C_2H_5). NMR ($CDCl_3$) δ 2.68 (t, 6 H, CH_3), 4.4–5.5 (m, 9 H, OCH_2's and ring C–H's); mass spectrum (methane CI) 286 (M + 1), 196 [$(C_2H_5O)_2PONCS$] (M + 1).

(Hydroxymethyl)vinylene (Diethoxyphosphinyl)-dithioimidocarbonate (Aglycone 14b). To 5.34 g (0.04 mol) of methylvinylene (diethoxyphosphinyl)dithioimidocarbonate (Addor, 1968), dissolved in 100 mL of dry carbon tetrachloride, was added *N*-bromosuccinimide (3.6 g, 0.02 mol) and 0.1 g of 2,2-azobis(2-methylpropionitrile). The mixture was stirred mechanically and irradiated for 3 h with a 150-W incandescent bulb. The solution was then filtered and the solvent evaporated to afford a light brown oil. The chemical ionization–mass spectrum of the product gave an ion at *m/e* 346, the NMR spectrum showed a signal at 4.36 ppm (CH_2Br) and a shift of the vinyl proton from 6.30 to 6.88 ppm. These data were consistent for the (bromomethyl)vinylene (diethoxyphosphinyl)dithioimidocarbonate. One gram of the product was hydrolyzed with potassium carbonate (0.2 g) at 80 °C and the chemical ionization–mass spectrum of the purified hydrolysis product gave an ion at *m/e* 284. The infrared spectrum showed a broad band at 3400 (OH), 1510 (C=N), 1210 (P=O), and 1010 cm^{-1} (P–O–C_2H_5). These data were consistent for (hydroxymethyl)vinylene (diethoxyphosphinyl)dithioimidocarbonate (Kapoor, 1974).

***S,S'*-(Hydroxymethyl)ethylene Dithiocarbonate.** One gram of (hydroxymethyl)ethylene dithioimidocarbonate hydrochloride was dissolved in 40 mL of water and heated to reflux for 4 h. The solution was cooled and extracted with chloroform, and the solvent was evaporated to afford 0.6 g of an oil. Chemical ionization–mass spectral analysis gave an ion *m/e* 150. The infrared spectrum had bands at 3380 (OH) and 1630 cm^{-1} (C=O).

2-Imino-1,3-dithiolane-4-carboxylic Acid Hydrochloride. One gram of methyl-2-imino-1,3-dithiolane-4-carboxylate (Addor, 1964) was stirred overnight with 15 mL of 10% hydrochloric acid. The water was evaporated to afford 0.9 g of a solid, mp 177–179 °C. Anal. Calcd for $C_4H_6NS_2O_2Cl$: C, 24.06; H, 3.03; N, 7.01; S, 32.12; Cl, 17.75. Found: C, 23.41; H, 3.31; N, 6.80; S, 30.52; Cl, 19.47.

Sodium Salt of Propylene (Ethoxyhydroxyphosphinyl)dithioimidocarbonate. Sodium bicarbonate (5 g) was added to 10 g of propylene dithioimidocarbonate hydrochloride (Addor, 1964) dissolved in 50 mL of water and the solution extracted three times with chloroform. The chloroform extract was dried (anhydrous sodium sulfate) and the solvent evaporated to afford 7.1 g of propylene dithioimidocarbonate.

Ethyl phosphorodichloridate (4.2 g, 0.026 mol) in 50 mL of toluene was added over a period of 0.5 h to propylene dithioimidocarbonate (7.1 g, 0.053 mol) dissolved in 50 mL of toluene. The reaction mixture was cooled and stirred in an ice bath during the addition. After 3 h, the reaction mixture was filtered to recover propylene dithioimidocarbonate hydrochloride. The solid was washed with benzene and the wash combined with the filtrate. The solvent was evaporated to give 6.2 g of an oil. NMR analysis of the crude product showed a phosphorus–*O*-ethyl coupling and integration of the protons gave a 1:1 ratio for the protons for the ethyl and methyl groups which was consistent for propylene (ethoxyphosphorochloridate)dithioimidocarbonate. The crude product was dissolved in 50 mL of benzene and mixed with sodium carbonate (2.1 g, 0.025 mol) in 50 mL of water. After 2 h, the two phases were separated and the aqueous phase extracted three times with chloroform. The water was evaporated to give 3.7 g of solid. NMR analysis of the solid showed a phosphorus–*O*-ethyl coupling and the 1:1 ratio of methyl and ethyl protons upon integration was consistent for the product. Further proof of structure for the sodium salt of propylene (ethoxyhydroxyphosphinyl)dithioimidocarbonate was obtained by treating the sodium salt with ethyl iodide to yield a compound that had IR and NMR spectra identical with propylene (diethoxyphosphinyl)dithioimidocarbonate (Addor, 1965).

Disodium Salt of Propylene (Dihydroxyphosphinyl)dithioimidocarbonate. A mixture of propylene dithioimidocarbonate hydrochloride (10.0 g, 0.058 mol) and phosphorous oxytrichloride (10.0 g, 0.70 mol) in 75 mL of toluene was heated to reflux for 6 h with mechanical stirring. The mixture was filtered and the solvent evaporated to afford 12.0 g of an oil. NMR analysis of the product was consistent for the assigned structure, propylene (phosphorodichloridate)dithioimidocarbonate. The crude product was dissolved in 50 mL of benzene, cooled in an ice bath, and stirred magnetically while sodium carbonate (11 g, 0.10 mol) dissolved in 50 mL of water was added dropwise. After 3 h, the two phases were separated, the aqueous phase was extracted three times with chloroform and evaporated in vacuo on a rotary evaporator to afford a yellow solid. The solid was digested twice with acetone and 11.9 g of a white solid was recovered. NMR analysis of the product was consistent for the disodium salt of propylene (dihydroxyphosphinyl)dithioimidocarbonate.

Plants and Treatment. Cotton plants were grown from seed (Delta pine smooth leaf variety) and the seedlings were transferred to individual pots after 4 weeks. In

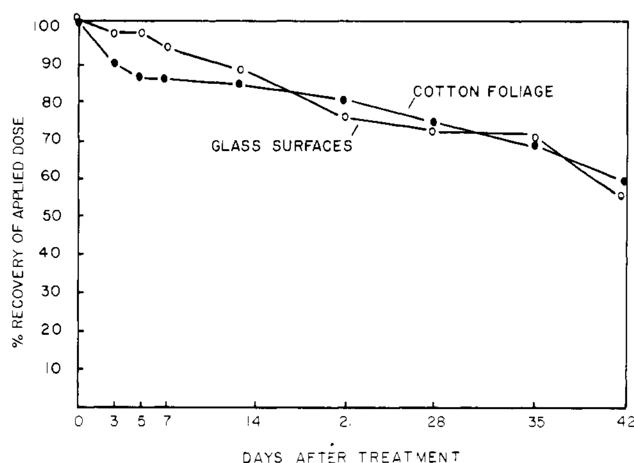


Figure 1. Disappearance of radioactivity from cotton foliage and glass surfaces treated with carbon-14 labeled mephosfolan.

experiment I, conducted on July 31, 1970, [^{14}C]mephosfolan (62.5 mg, 1.25 mCi) was dissolved in 25 mL of 50% aqueous acetone containing 50 mg of Alyrodyne 315, a nonionic emulsifier (Ciba-Geigy Chemical Co.). Twenty-eight 6-week-old cotton plants, containing four to six leaves, were treated foliary with the radioactive insecticide and maintained in the greenhouse. Each cotton leaf, four leaves per plant, was treated with approximately 11.1 × 10^6 dpm (250 μg, 5 μCi) by applying 100 μL of the microformulation onto the leaf with a pipet.

In experiment II, designed to assist the identification of the metabolites of mephosfolan, conducted in the greenhouse on July 30, 1971, four 9-week-old cotton plants were treated foliarly with 29 μCi of propylene [^{14}C]dithioimidocarbonate hydrochloride (II). A separate cotton plant was treated with 14 μCi of [^{14}C]mephosfolan in order to provide a metabolism control.

Experiment III was conducted on Aug 10, 1971, in order to isolate metabolites for instrumental characterization. Sixty 10-week-old cotton plants were sprayed with 2 L of a 50% aqueous acetone solution containing nonlabeled mephosfolan (2.1 g) and the nonionic emulsifier (2.5 g). The solution was applied in two equal portions over a 3-day period. Six additional cotton plants were treated foliarly with 100 μCi (5 mg) of mephosfolan in order to monitor the isolation of the metabolites.

Harvest and Extraction. In experiment I, one cotton plant was harvested immediately after treatment for a zero-day sample, four plants were harvested at 3, 5, and 7 days, two plants at 14, 21, and 28 days, and one plant at 35 and 42 days after treatment as indicated in Figure 1. After 60 days, only one treated cotton leaf was left on the remaining plants, and this was collected for analysis. As indicated in Table I, the treated leaves, untreated leaves, and stems (including the petioles and roots) were separated for the analysis of the radioactive residues. In experiment II, cotton plants treated with the nonphosphorylated dithiolane (II) were harvested at 3, 5, and 7 days and leaves from the one plant treated with mephosfolan were harvested at 5 and 7 days after treatment. In experiment III, all the plants were harvested 8 days after treatment.

The plant material was weighed and homogenized in a blender with 80% ethanol (4 mL/g of plant material) and filtered through a medium porosity fritted glass Buchner funnel. After a second extraction with more ethanol, the marc was dried, weighed, and ground to a powder in a mill.

The ethanol extracts of the treated leaves were concentrated on a rotary evaporator at 40 °C to remove the solvent. In experiments I and III the radioactive residue

Table I. Distribution of Radioactivity in Cotton Plants after Foliar Application of Carbon-14 Labeled Mephosfolan

Plant part	% of applied dose, Days after treatment: 7[a]	21[b]	42[c]
Treated leaf			
Ethanol extract	81.2	67.5	41.5
Marc	5.1	8.0	11.5
	86.3	75.5	53.0
Untreated leaf			
Ethanol extract	0.1	0.7	0.5
Marc	0.2	0.6	0.6
	0.3	1.3	1.1
Root-stem-petioles			
Ethanol extract	0.4	1.2	1.7
Marc	0.6	1.8	2.4
	1.0	3.0	4.1
Total	87.6	79.8	58.2

[a] Average of four plants. [b] Average of two plants. [c] One plant only.

from the treated leaves was mixed with 50–100 mL of water and extracted four times with 100-mL portions of chloroform and four times with 100-mL portions of ethyl acetate to separate the organosoluble and water-soluble metabolites.

Persistence of Mephosfolan on Glass Slides. In experiment I, [^{14}C]mephosfolan was also applied to 24 × 54 mm glass microscope slides (Dow Corning Co.). Ten pairs of glass slides, each pair being treated with 11.1 × 10^6 dpm (5 μCi), were placed in petri dishes and the dishes placed adjacent to the cotton plants in the greenhouse. A pair of glass slides was taken at the time intervals paralleling the plant harvest, as indicated in Figure 1. The slides were placed into 2-oz. glass bottles and rinsed with 25 mL of ethanol.

Determination of Radioactivity. The radioactivity in the extracts of the untreated leaves, roots-stems-petioles, and glass slides was determined by counting aliquots of the extracts in DAM-611 liquid scintillation cocktail (Davidson and Feigelson, 1957). Radioactivity in the treated leaf extracts and the plant marc was determined by a modified Schöniger flask combustion technique (Kelly et al., 1961) and liquid scintillation counting of the $^{14}CO_2$ trapped in the absorbent scintillator. Radioactive spots on thin-layer chromatograms were located by means of autoradiography using Royal Pan professional film (Eastman Kodak). After an exposure time of 2–3 weeks, the films were developed using Baumann's Diafine two-bath film developer. For purposes of quantitation of metabolites, each radioactive spot on the TLC plate was marked. Each spot was scraped and the silica gel placed into a scintillation vial. The silica gel was suspended in the DAM-611 containing 5% Cab-O-Sil thixotropic gel and counted directly. All radioactive measurements were made in a Packard Tri-Carb liquid scintillation spectrometer (Model 4322) using [^{14}C]toluene (New England Nuclear Corp.) as the internal standard to determine the counting efficiency.

Chromatographic Procedures. Two-dimensional TLC was performed on 20 × 20 cm silica gel precoated plates (E. M. Laboratories, Inc.) with the plates scribed for a 15-cm solvent development in each direction. System I employed ethyl acetate–1-propanol–formic acid–water (120:200:20:60) vs. ethyl acetate–1-propanol–ammonium hydroxide–water (120:200:20:60) and was used for the

Table II. Relative Concentration and Characteristics of Mephosfolan and the Metabolites Found on Cotton Foliage 42 Days after Treatment with Carbon-14 Labeled Mephosfolan

					Sensitivity to		
Compound	TLC spot	TLC coordinates[a]	% of extract	Organo- or hydrophilic[c]	β glucosidase	Acid hydrolysis	Diazomethane
Unknown	1	0.90-0.90	1.0	O		Yes	
Mephosfolan	3	0.75-0.80	60.9 (70.7)[c]	O	No	Yes	
Thiocyanate	8	0.68-0.60	0.4 (7.9)[c]	H	No	No	
Unknown	9	0.65-0.50	1.1	O		Yes	
Aglycone 14a			5.1	O		Yes	
	14	0.60-0.40	16.3 (7.6)[c]	H	Yes	Yes	
Aglycone 14b			11.2	O		Yes	
Unknown[d]	15	0.55-0.32	4.5	H	No	Yes	
Unknown	16	0.55-0.25	5.0 (4.9)[c]	H	No	Yes	Yes
Unknown	18	0.45-0.32	2.4	H	No	Yes	Yes
Unknown	19	0.42-0.28	3.3	H	No	Yes	Yes
UnKnown[d]	22	0.68-0.00	2.1 (2.4)[c]	H	No		
			Total 97.0				

[a] Expressed as the grid coordinates on the TLC plate after two-dimensional chromatography in system I. [b] O = chloroform or ethyl acetate soluble; H = water soluble. [c] Data for one leaf only after 60 days. [d] Metabolism of carbon-14 labeled propylene dithioimidocarbonate hydrochloride (II) yielded metabolites which were similar chromatographically.

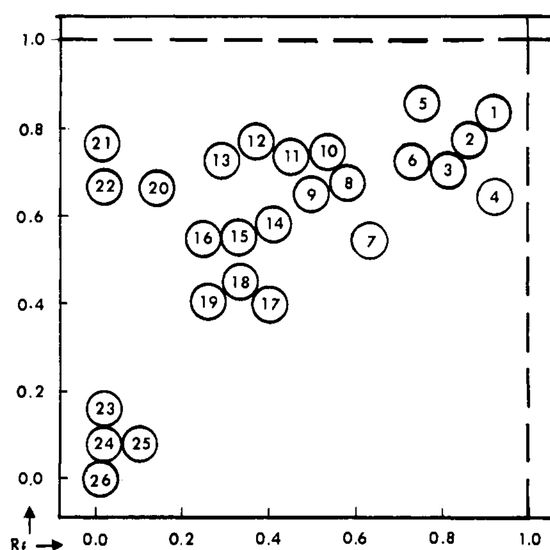


Figure 2. Two-dimensional TLC of mephosfolan and its metabolites.

analysis of the plant extracts as indicated in Figure 2 and Table II. System II employed chloroform–methanol–water (225:66:9) vs. diethyl ether–methanol (270:30) and was used for the analysis of the aglycones. As an aid to the identification of metabolites, the unknowns were subjected to TLC in mixture with the nonlabeled authentic standards. Coincident spots were detected by autoradiography, followed by visualization of the standards with an ultraviolet lamp (254 nm) or with a spray reagent (1% palladium chloride).

The radioactivity in the chloroform extract was chromatographed on a 2.5 × 60 cm column of Sephadex LH-20 (Pharmacia Fine Chemicals) with a 1:1 mixture of ethanol–ethyl acetate. The water-soluble radioactivity was chromatographed on a 2.5 × 30 cm column of Sephadex G-10 (Pharmacia Fine Chemicals) with water and on a 1.25 × 90 cm column of Dowex-1 anion-exchange resin in the acetate form with a gradient from water to 2.0 N acetic acid to 8.0 N formic acid. Fractions from these columns were collected in an LKB 7000A UltroRac fraction collector (LKB-Produkter) and monitored for radioactivity by a Packard Tri-Carb flow analyzer (Model 3041). Radioactive fractions comprising each peak were analyzed by TLC in system I.

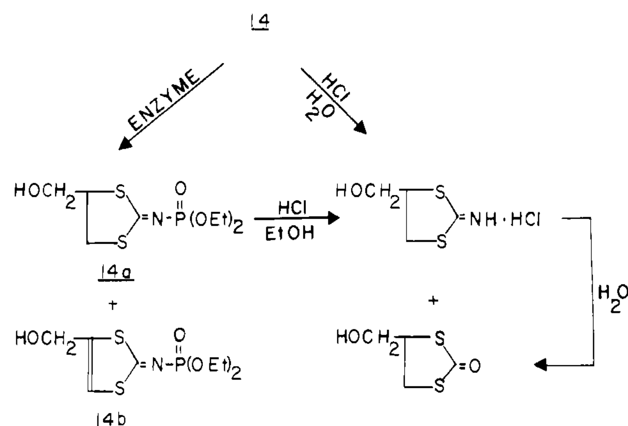


Figure 3. Hydrolysis of metabolite 14.

Diagnostic Reactions. The two water-soluble radioactive fractions from the G-10 column were taken up in 5 mL of 0.1 M sodium acetate buffer (pH 5.0). A mixture of 1 mg each of β-glucosidase (almonds, Sigma Chemicals, Inc.) and hemicellulase (Nutritional Biochem Co.) was added and incubated at 37 °C for 15 h in a Dubnoff mechanical shaker. The water-soluble metabolite mixture was hydrolyzed at 60 °C with 1 N hydrochloric acid and the aglycones from the enzyme hydrolysis were hydrolyzed with 1.2 N ethanolic hydrogen chloride in benzene at 80 °C. The water-soluble metabolite mixture and the acidic metabolites from the ion-exchange column were treated with diazomethane prepared from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Inc.) by the procedure of Shafik and Enos (1969). TLC in systems I and II of the reaction mixture was used to evaluate the effects of these diagnostic reactions. The results of these diagnostic reactions are summarized in Table II and Figure 3.

Isolation and Purification of Aglycones. A mixture of the two unknown aglycones (14a and 14b) was obtained by enzymatic hydrolysis of the mass-isolated, water-soluble cotton metabolites (experiment III). The aglycones were extracted with chloroform and separated on 1-mm thick layer plates, 20 × 20 cm, obtained from Quantum Industries (Type PQ1F), using diethyl ether–methanol (270:30). The aglycones, located as radioactive bands at R_f ca. 0.53 and 0.47 (15 cm solvent development) by autoradiography, were scraped from the plate and the gel

Table III. Comparative Toxicities to Female Albino Mice of the Identified Metabolites and Mephosfolan

Chemical name	LD_{50}, mg/kg
Propylene (diethoxyphosphinyl)-dithioimidocarbonate (Mephosfolan)	11.7
(Hydroxymethyl)ethylene (diethoxyphosphinyl)dithioimidocarbonate (Aglycone 14a)	28
(Hydroxymethyl)vinylene (diethoxyphosphinyl)dithioimidocarbonate (Aglycone 14b)	162
Potassium thiocyanate	884

extracted with methanol. The major aglycone (14b), having R_f ca. 0.53, was rechromatographed using chloroform–methanol–water (225:66:9), recovered from the gel, and purified for instrumental analysis by liquid chromatography on a du Pont analytical liquid chromatograph (Model 830) using two 0.25-m silica gel microsphere columns (Zorbax-Sil, du Pont) connected in series and methylene chloride–isopropanol (950:50) as the mobile phase.

Instrumental Analysis. The infrared spectra of the model compounds were recorded on a Perkin-Elmer Infracord spectrophotometer (Model 137B). A Perkin-Elmer spectrophotometer (Model 421) with a multiple-reflectance attachment (Wilks Scientific Co.) and a 2-mm KRS-5 plate was used to record the spectrum of the metabolites. Chemical ionization–mass spectrometry was performed on a Finnigan mass spectrometer (Model 1015C). Nuclear magnetic resonance spectra were recorded on a Varian A-60 spectrometer. Microanalyses were performed by the Microanalytical Group of the Organic Chemicals Division, American Cyanamid Co. (Pearl River, N.Y.). An F & M Model 402 gas chromatograph equipped with a cesium bromide detector (Steller and Pasarela, 1972) was used for the GLC analysis of the chloroform-soluble radioactivity. The following GLC column and conditions were used: column, 1 m, 4-mm i.d. Pyrex glass U-tube packed with 11.2% DC-200–0.01% Versamid 900 on Gas-Chrom Q 60–80 mesh (Applied Science Laboratories, Inc.); column temperature, 200 °C; injection port temperature, 250 °C; detector temperature, 210 °C; carrier (helium) flow, 100 mL/min; air flow, 215 mL/min; and hydrogen flow, 30 mL/min. The retention time for mephosfolan was 2.6 min. An extract of a control plant showed no peaks which eluted with the retention time identical with that of the insecticide. Analysis of a plant extract fortified with mephosfolan and of a treated leaf extract both gave a peak with a retention time identical with mephosfolan.

Toxicity of Mephosfolan and Its Metabolites. Oral LD_{50} values were determined in female albino mice. The compounds were fed via stomach tube as corn oil suspensions in volumes of 1 mL/mouse. In the LD_{50} region, five replicates were used. The mortality counts were taken daily up to 14 days after treatment. The LD_{50} values that are given in Table III were calculated by the method of moving averages using the tables constructed by Weil (1952).

RESULTS AND DISCUSSION

When [^{14}C]mephosfolan was applied to the surfaces of cotton leaves and glass slides, the rate of disappearance of radioactivity (Figure 1) from leaves due to volatility was similar to the rate from glass slides. A major portion of the radioactivity was recovered from the treated leaves and very little radioactivity, either as the unchanged insecticide or its metabolites, had translocated to the other parts of the plant (Table I). The radioactivity recovered from the glass slides corresponded to the insecticide by thin-layer chromatography. The chromatographic profile of the radioactivity recovered from the treated leaves differed considerably (Figure 2). A minimum of 26 radiospots was detected using System I. Table II shows the characteristics and the approximate contribution of the ten most predominant radiospots, accounting for 97% of the treated leaf extract (40.3% of the applied dose). Only one treated cotton leaf was available for analysis after 60 days and only radiospots 3, 8, 14, 16, and 22 accounted for the radioactive residue that was found. Of all the radiospots that were detected, those which individually accounted for more than 5% of the total radioactive residue recovered in the ethanol extract (3, 8, 14, and 16) were considered to be quantitatively significant. When considered on the basis of their individual contribution to the total residue, including the radioactivity in the plant marc, only radiospot 3, accounting for 25.3% of the applied dose, was significant. The other radiospots were less than 5% each. The very small amounts of radioactivity recovered in the untreated leaves and the stems, petioles, and roots (Table I) and the radioactivity in the marc of the treated leaves were not identified.

Chloroform-Soluble Radioactivity. About 53 to 64% of the radioactivity of the treated leaf extract (experiment I) was chloroform soluble. Radiospot 3, accounting for about 60% of the radioactivity of the treated leaf extract after 42 days, was found in the chloroform phase along with several minor metabolites. This compound was identified as mephosfolan by thin-layer chromatography, gas chromatography, and mass spectral analysis. Chromatography of the chloroform-soluble radioactivity on Sephadex LH-20 resolved the radioactivity into eight fractions with 86% of the recovered radioactivity corresponding to the unchanged insecticide. The remainder of the radioactivity was distributed more or less evenly over the other seven fractions; no one fraction accounted for more than 3% of the radioactivity. No attempts were made to identify these minor compounds other than to show that each fraction was composed of at least two compounds. Thin-layer chromatography of the ethyl acetate soluble radioactivity, comprising between 2 and 6% of the treated leaf extract, showed that this was also composed of metabolites that were found in the chloroform extracts. These were not examined further.

Water-Soluble Radioactivity. About 31 to 49% of the radioactivity of the treated leaf extract was water soluble. Metabolite 14, by far the most significant metabolite in the ethanol extract, was partially resolved by thin-layer chromatography (Table II). Chromatography on Sephadex G-10 was partially successful in separating metabolites 15, 16, 18, and 19 (fraction 1) from metabolites 14, 17, and 20 (fraction 2). Chromatography on a Dowex-1 anion-exchange resin in the acetate form, using a gradient elution from water to 2.0 N acetic acid to 8.0 N formic acid, resolved the water-soluble radiometabolite mixture into 14 fractions. Metabolite 14, isolated from the Sephadex G-10, was hydrolyzed with β-glucosidase to two aglycones (14a and 14b) which could be partitioned into chloroform. These aglycones were not chromatographically separable in system I and were similar to mephosfolan (Figure 2). Mephosfolan and the aglycones were separable by TLC in system II. The other metabolites in the G-10 fractions and mephosfolan were unaffected by the enzymes. This suggested that the aglycones released from metabolite 14 were glycoside conjugates. Acid hydrolysis of metabolite 14 and the glucosidase-produced aglycones gave the same

two hydrolysis products. By TLC in system I, propylene dithioimidocarbonate hydrochloride (II) had chromatographic coordinates (0.40–0.60 in Figure 2) which were similar to those of one of the acid hydrolysis products while mephosfolan had chromatographic coordinates (0.75–0.80) which were similar to the other hydrolysis product. On the basis of these data, one of the glucosidase-produced aglycones was postulated to be (hydroxymethyl)ethylene (diethoxyphosphinyl)dithioimidocarbonate. The postulated compound was prepared and by TLC in system II it was identical with the aglycone 14a. This was the minor aglycone of the pair and accounted for 5.1% of the leaf extract (2.1% of the applied dose) after 42 days. In order to support the TLC identification of aglycone 14a, a mixture of radioaglycone and the synthetic compound 14a was hydrolyzed with ethanolic hydrogen chloride in benzene, and the hydrolysis product, (hydroxymethyl)-ethylene dithioimidocarbonate hydrochloride, was isolated and recrystallized to a constant specific activity. This compound had chromatographic properties similar to the iminodithiolane hydrochloride (II). When this compound was hydrolyzed it gave *S,S'*-(hydroxymethyl)ethylene dithiocarbonate which by TLC was identified as one of the other acid hydrolysis products of metabolite 14 and 14a. It is considered a secondary hydrolysis product. These results are summarized in Figure 3.

The major aglycone (14b, Table II) was isolated from experiment III for mass spectral and infrared analysis. The chemical ionization mass spectrum showed an ion at m/e 283 which was 14 amu higher than mephosfolan and 2 amu lower than the compound identified as the aglycone 14a. The relative intensities of the ion at m/e 283 and its (P + 2) isotope peak (m/e 285) indicated that there were two sulfur atoms in the aglycone. When the mass spectrum of the aglycone was generated using deuterium oxide as the reagent gas, ions at m/e 285 and 265 were obtained. This indicated that the aglycone had one exchangeable proton. The m/e 265 ion corresponded to a loss of deuterium oxide from the m/e 285 ion. Ions at m/e 287 and 267 were noted when the mass spectrum of the synthetic compound corresponding to the aglycone 14a was generated using deuterium oxide also. The infrared spectrum of the major aglycone showed absorption bands at 3350, 1625, and 820 cm^{-1}. Based on this data, the major aglycone was considered to be (hydroxymethyl)vinylene (diethoxyphosphinyl)dithioimidocarbonate. The postulated compound was prepared and by TLC in system II it was identical with aglycone 14b. The infrared spectrum of the synthetic compound contained all the absorption bands that were found in the spectrum of the aglycone.

In experiment I, metabolite 8 accounted for 7.9% of the radioactivity in the extract of one treated leaf after 60 days, while in experiment II this metabolite accounted for 36% of the radioactivity derived from mephosfolan. This metabolite was tentatively identified as thiocyanate ion by cochromatography in system I, followed by autoradiography and visualization of the carrier thiocyanate as a brown spot after spraying with 1% palladium chloride solution. In order to confirm the identification, carrier nonradioactive potassium thiocyanate was added to the water-soluble radiometabolite mixture (5-day plant extract). The mixture was treated with 6-phenyl-1-aziridineethanol and carried through a sequence of reaction steps to eventually form the derivative of thiocyanate ion, 6-phenyl-2,3,5,6-tetrahydroimidazo[2,1*b*]thiazole (Spicer et al., 1968). The derivative was isolated and recrystallized as its hydrochloride salt from acetone–ethanol to a constant specific activity.

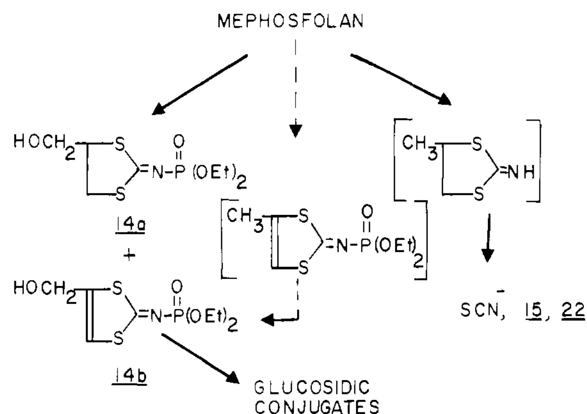


Figure 4. A proposed pathway for metabolism of mephosfolan in cotton.

Metabolite 16, accounting for 5.0% of the radioactivity in the extract, was unaffected by treatment with β-glucosidase. It could be hydrolyzed with acid and was converted to a compound more polar than the metabolite. This metabolite was eluted from the anion-exchange resin with the 2.0 N acetic acid to 8.0 N formic acid gradient and was considered to be an acid based on this behavior and its reactivity to diazomethane. The postulated structures, 2-imino-1,3-dithiolane-4-carboxylic acid and methyl 2-[(diethoxyphosphinyl)imino]-1,3-dithiolane-4-carboxylate (Addor, 1964), did not correspond to the respective hydrolysis product or the diazomethane product of metabolite 16. The sodium salts of the desethyl and didesethyl derivatives of mephosfolan were prepared as potential metabolites; however, these compounds did not correspond to metabolite 16 or to any of the other metabolites. Metabolite 16 may possibly be a glutathione or cysteine conjugate (Hutson, 1976).

In experiment II, the metabolism of nonphosphorylated iminodithiolane (II) produced three water-soluble metabolites, accounting for 84% of the radioactivity in the extract of the treated leaf. Thiocyanate ion was identified as a major metabolite while the other two metabolites chromatographically resembled metabolites 15 and 22 (Figure 2). These mephosfolan metabolites, accounting for 4.5 and 2.1% of the leaf extract (Table II), 1.9 and 0.9% of the applied dose, may not retain the diethoxyphosphinyl moiety of mephosfolan and would, therefore, be considered to much less toxicological significance.

CONCLUSION

Based on the findings of this study, pathways for the metabolism of mephosfolan by cotton plants involve oxidation, hydrolysis, and conjugation with glucose (Figure 4). The methylvinylene (diethoxyphosphinyl)dithioimidocarbonate, a postulated intermediate to aglycone 14b, was not found. Since thiocyanate and the two aglycones have acute toxicities that are less than mephosfolan (Table III) and since they all are present at levels an order of magnitude smaller than that of mephosfolan (Table II), an analytical residue method responding only to mephosfolan provides a realistic means of evaluating the levels of toxicologically significant residues which may result from its field use. While the radioactive residue in the cotton plant marc was not identified, the results from the metabolism study of [^{14}C]mephosfolan in rice plants (Ku, 1976) showed that the imino carbon atom was incorporated into the glucose, into the cellulose and possibly into the lignin of the rice straw. A similar explanation may account for the radioactivity found in the cotton plant marc. Metabolism and tissue residue studies were conducted with

[^{14}C]mephosfolan in rats (Kapoor et al., 1976) and thiocyanate was identified as a major urinary and tissue metabolite.

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Phenylpyrazole Amide Herbicides

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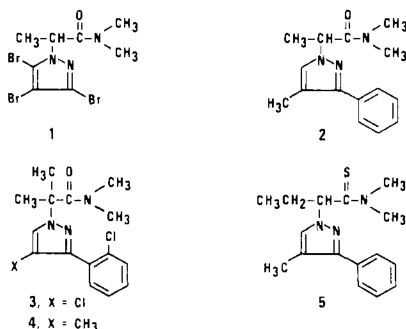
N,*N*,α,4-Tetramethyl-3-phenylpyrazole-1-acetamide (**2**) and a series of related phenylpyrazole amides have been found active as preemergence herbicides. The compounds showed activity at low rates on many broadleaf and grassy weeds and were exceptionally phytotoxic to yellow nutsedge. In greenhouse tests incorporated applications of the more active compounds controlled nutsedge at rates near 0.01 lb/acre. The effects of chemical placement, simulated rainfall, and application method on activity of the compounds are described. Information on soil mobility of representative compounds is presented. Results of field tests are presented showing weed control for several of the compounds at rates less than 1 lb/acre.

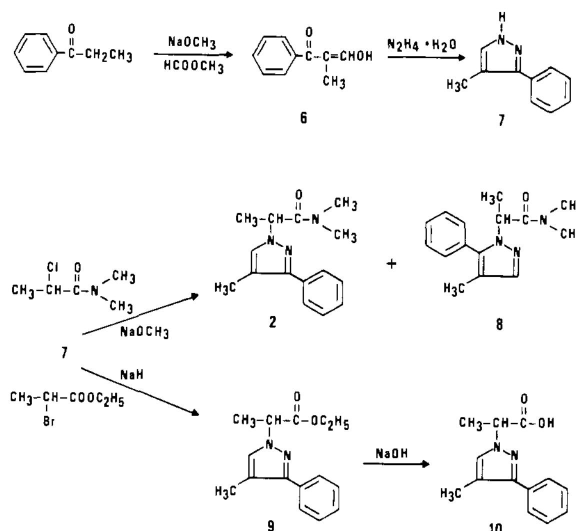
A report from our laboratories has described the herbicidal activity of **1** and other bromopyrazoles (Chambers et al., 1972). In related work we have found *N*,*N*,α,4-tetramethyl-3-phenylpyrazole-1-acetamide (**2**) to be active as a preemergence herbicide. The compound controlled many annual broadleaf and grassy weeds and was exceptionally active on yellow nutsedge (*Cyperus esculentus*



L.) and purple nutsedge (*Cyperus rotundus* L.). Many analogues of **2** have been prepared (Moon and Kornis,

The Upjohn Company, Kalamazoo, Michigan 49004.

Scheme I. Synthesis of *N*,*N*,α,4-Tetramethyl-3-phenylpyrazole-1-acetamide



1976) and compounds more active (e.g., **3** and **4**) and more selective (e.g., **5**) than **2** have been found. Activities of this