Pentachloronitrobenzene Metabolism in Peanut. 1. Mass Spectral Characterization of Seven Glutathione-Related Conjugates Produced in Vivo or in Vitro

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(^{14}C)Pentachloronitrobenzene was rapidly absorbed through the roots of hydroponically grown peanuts (*Arachis hypogae* L. cv. Spanish); however, only 4–7% of the absorbed radioactivity was translocated to the shoots. Thirty-seven days following treatment, 6% of the radioactivity in the roots was soluble in chloroform, 24% was soluble in ethyl ether, 33% was soluble in water, and 37% was insoluble in 80% methanol. The following polar metabolites were isolated from root tissue and characterized by synthesis and/or mass spectrometry: *S*-(pentachlorophenyl)glutathione, *S*-(*ar*-tetrachloronitrophenyl)glutathione, *S*-(pentachlorophenyl)cysteine, *S,S'*-(*ar*-tetrachlorophenylene)dicysteine, *S*-(pentachlorophenyl)-*N*-malonylcysteine, and *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine. In addition, the presence of two isomeric forms of *S,S'*-(*ar*-tetrachlorophenylene)diglutathione was indicated by in vitro and in vivo experiments. *S*-(Pentachlorophenyl)cysteine and pentachloroaniline were among the precursors of the insoluble residue. Glutathione *S*-transferase enzymes that utilized PCNB as a substrate were isolated from pea (*Pisum sativum* L. cv. Little Marvel) and peanut. Products from the reaction catalyzed by the pea enzyme were identical with products isolated from peanut roots.

INTRODUCTION

Pentachloronitrobenzene (PCNB) is marketed worldwide as a fungicide for the control of a wide variety of plant pathogens. In the United States, PCNB is used primarily as a soil fungicide to control *Rhizoctonia* in cotton (*Gossypium hirsutum* L.) and *Sclerotium* in peanut (*Arachis hypogaea* L.) (EPA, 1976).

Soil metabolism and residues of nonradioactive PCNB have been studied by a number of researchers. Pentachloroaniline (PCA), pentachlorothioanisole (PCTA), and PCNB were consistently reported as the most common residues (Bristow et al., 1973; Dejonckheere et al., 1976; DeVos et al., 1974; Wang and Broadbent, 1973; Ko and Farley, 1969; Beck and Hansen, 1974; EPA, 1976). These residues are quite stable and may persist in the soil for 2–3 years following treatment (Beck and Hansen, 1974). Other halogenated aromatic residues have also been reported in PCNB-treated soil. Some of these residues were probably impurities of PCNB present in the formulation while others appear to have been true metabolites (Beck and Hansen, 1974; Dejonckheere et al., 1976). In 1978, Murthy and Kaufman reported that (^{14}C)PCNB was converted to PCA and PCTA in soil under anaerobic conditions. They also reported the loss of ^{14}C due to volatilization. Crosby and Hamadmad (1971) found PCNB to be quite stable to sunlight and concluded that photolysis did not play a major role in the loss of PCNB from the environment.

In animals, PCNB appears to be metabolized by several different routes. Betts et al. (1955) reported that pentachlorophenylmercapturic acid and PCA were metabolites of PCNB in the rabbit. St. John et al. (1965) showed that PCA was a major urinary excretion product in the dairy cow, and Kuchar et al. (1969) demonstrated that PCA and PCTA were major fecal excretion products in beagle dogs. Dunn et al. (1978) found no appreciable bioaccumulation of PCNB in white leghorn cockerels and showed that PCA and PCTA were minor metabolites. A complex pattern of (^{14}C)PCNB metabolism was demonstrated recently in the Rhesus monkey by Kogel et al. (1979).

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In plants, studies of PCNB metabolism have dealt only with the nonpolar products, and most studies have been conducted with nonradioactive PCNB. Residues in the below ground parts of plants grown in PCNB-treated soil are common (Bristow et al., 1973; Gorbach and Wagner, 1967), but it is unclear whether PCNB is readily translocated to the foliar tissue. Low residues of PCNB were reported in lettuce grown in PCNB-containing soil (EPA, 1976; Dejonckheere et al., 1976) whereas high residues have been reported in the foliar tissue of cotton grown in soil containing 300-ppm PCNB (Kuchar et al., 1969). Bristow et al. (1973), however, reported that PCNB was not readily translocated in bean (*Phaseolus vulgaris* L.). PCNB was detected in the cotyledons, but no PCNB was found in the true leaves. Pentachloroaniline and PCTA have been reported as primary metabolites of PCNB in higher plants. Minor residues of other halogenated aromatic compounds have also been reported (Kuchar et al., 1969). A recent study on (^{14}C)PCNB metabolism in onion (Begum et al., 1979) indicated the presence of a substantial residue of ^{14}C in the foliar tissue and showed that nonextractable residue accounted for a significant percent of the ^{14}C in the plant. Many nonpolar metabolites, including PCA and PCTA, were identified. Pentachloroaniline and PCTA have also been reported as the primary metabolites of PCNB in a resistant fungi (Nakanishi and Oku, 1969).

Previous studies on PCNB metabolism in plants did not clearly establish whether PCNB was translocated from the roots to the foliar tissue. Residues detected in the foliar tissue could have been due to volatilization from the soil or exposure of plant parts to contaminated soil. Also, in most previous plant metabolism studies (^{14}C)PCNB was not used or the extraction and analytical techniques precluded the detection and/or identification of polar metabolites. The purpose of this study was to determine if PCNB or its metabolites were readily translocated from the roots to the foliar tissue and if polar metabolites played an important role in PCNB metabolism in higher plants. A preliminary report was presented by Lamoureux and Rusness (1976).

MATERIALS AND METHODS

Chemicals. Pentachloronitrobenzene-*UL*-^{14}*C*, specific activity 1.76 mCi/mmol, was synthesized by Kadunce and Lamoureux (1976). Radiochemical purity was estimated

to be at least 99.5% by gas chromatography (GC) and thin-layer chromatography (TLC). The major radiochemical impurity was shown by mass spectral analysis to be *ar*-bromotetrachloronitrobenzene. Nonradioactive pentachloronitrobenzene (98% pure) was obtained from K and K Laboratories, glutathione (reduced form, A grade) from Calbiochem-Behring Corp., and L-cysteine from Sigma Chemical Co. Pentachloroaniline-*UL*-^{14}C was synthesized in vivo and purified as described by Rusness and Lamoureux (1980).

Derivatization Procedures. Metabolites and standards were esterified in dry alcohol and 3 N hydrogen chloride at 90 °C for 30 min. Excess alcohol and hydrogen chloride were removed under vacuum. Acylation reactions were run in sealed vials at 90 °C for 30 min using a 3:1 (v/v) ratio of methylene chloride to acetic anhydride or trifluoroacetic anhydride. Aromatic nitro compounds were detected on thin-layer plates with a modified Bratton–Marshall test by using procedures D126 and D121 described by Hais and Macek (1963). It was necessary to heat the thin-layer chromatograms to 80 °C for 15 min after step D126.

Mass Spectrometry. Mass spectra were obtained at 70 eV with a Varian MAT CH-5 DF mass spectrometer equipped with a high-resolution peak-matching unit. Samples were introduced into the spectrometer with a solid sample inlet probe.

Radioactive Analyses. Radioactivity in plant extracts, etc., was determined by liquid scintillation counting by using external standardization techniques, and radioactivity in insoluble plant residues was determined by liquid scintillation counting after the samples were combusted to $^{14}CO_2$ in a Model 306 Packard Tricarb sample oxidizer (Lamoureux and Stafford, 1977). Radioactive products eluted from gas chromatographs were detected with a Model 894 Packard gas proportional counter and radioactive products eluted from ion-exchange columns were detected with a Model 317 Packard Tricarb flow monitor using an anthracene-filled flow cell. Radioactivity from high-pressure LC chromatograms was detected by liquid scintillation counting of aliquots from fractions. Radioactivity on thin-layer chromatograms was detected by radiochromatogram scanning or autoradiography and was quantitated by liquid scintillation counting of silica gel zones scrapped from the chromatograms.

Sample Concentration. Plant extracts and other solutions were usually concentrated to dryness by using rotary vacuum evaporators operated at temperatures consistent with the volatility and stability of the sample. In special cases, samples were allowed to air-dry at ambient temperature without forced air.

Chromatographic Methods. Glass plates used in TLC were coated with either a 250-μm or a 500-μm layer of silica gel HF_{254} (Merck). Chloroform-soluble products were spotted on the thin-layer plates without the use of forced air. Chromatograms were developed in one of the following solvents: (A) 1-butanol–glacial acetic acid–water (12:3:5), (B) ethanol–water (7:3), (C) benzene–ethyl acetate (19:1), (D) benzene–ethyl acetate (9:1), (E) hexane–chloroform–glacial acetic acid (10:10:1), (F) benzene–acetone (6:1), (G) hexane–chloroform–glacial acetic acid (15:4:1), (H) chloroform–methanol–water (13:7:1), and (I) chloroform–absolute ethanol (20:1). Chromatography tanks were paper lined when solvent systems A, B, and H were used. Where multiple solvent development was necessary, chromatograms were removed from the tanks when the solvent reached the desired point. The chromatograms were air-dried and redeveloped in the same direction. This was repeated for the indicated number of times. When a product was isolated from a chromatogram, the gel was scraped from the plate and eluted with the appropriate solvents. In some cases (metabolites I and II) 10 mM HCl in aqueous 80% methanol was used as the solvent.

Gas chromatography was conducted with a Varian Model 3700 Gas Chromatograph equipped with a flame-ionization detector and a Model 894 Packard gas proportional counter. For the analysis of *N*-trifluoroacetyl butyl esters of amino acids, a 2 mm × 1.82 m column of 0.65% ethylene glycol adipate on 60/80 mesh Chromosorb WAW was used under conditions similar to those described by Fischer Scientific Co. All other GC analyses were performed with a 2 mm × 1.82 mm column of 3% SP-2100 on 80/100 mesh Supelcoport. Conditions were similar to those described by Kadunce and Lamoureux (1976); however, carrier gas flow rates were reduced to approximately 20 mL/min.

High-pressure liquid chromatography was performed on 3.9 mm × 30 cm columns of μBondapak C/18 (Waters Associates) with an Altex Model 312 HPLC System. A flow rate of 2.0 mL/min was used, ultraviolet absorption was monitored at 254 nm, and fractions were collected and assayed for radioactivity. With system A, the column was eluted for 8 min with aqueous 20% acetonitrile and 1% acetic acid, then for 22 min with aqueous 30% acetonitrile and 1% acetic acid, and finally with 75% acetonitrile. With system B, the column was eluted isocratically with aqueous 33% acetonitrile and 1% acetic acid.

Low-pressure column chromatography with 200/400 mesh AG 50W-X2 resin (Bio-Rad Laboratories) was performed in a manner similar to that described by Schroeder et al. (1962) and Lamoureux et al. (1972). Chromatography buffers were prepared in the manner indicated with glass distilled water and the highest purity reagents available: 0.2 N, pH 3.1, buffer (64.5 mL of pyridine, 1114 mL of glacial acetic acid per 4 L) and 2.0 N, pH 5.0, buffer (645 mL of pyridine, 573 mL of glacial acetic acid per 4 L). A pH 2.1 sample application buffer was prepared by titration of the pH 3.1 buffer with 6 N HCl. The chromatograms were developed at ambient temperature on 1 × 60 cm columns of AG 50W-X2 resin preequilibrated with pH 3.1 buffer. Samples were applied with a minimum volume of pH 2.1 buffer, the columns were eluted at 0.50 mL/min, and 4-min fractions were collected. With system A, a buffer gradient was developed from three chambers of a gradient device (Varigrad, Buchler Instruments). The first chamber contained 150 mL of pH 3.1 buffer and the second and third chambers each contained 150 mL of pH 5.0 buffer. In system B, the column was eluted with 120 mL of pH 3.1 buffer before the above-mentioned gradient was used.

Syntheses. *S*-(Pentachlorophenyl)cysteine and *S,S'*-(*ar*-tetrachlorophenylene)dicysteine were synthesized by reaction of PCNB with L-cysteine. PCNB (12.5 μmol in 75 μL of acetone), (^{14}C)PCNB (0.177 μmol in 25 μL of acetone), L-cysteine (25 μmol in 250 μL of methanol), and sodium hydroxide (230 μmol in 60 μL of water) were reacted in a sealed vial at 40 °C. After 2 h, the reaction mixture was cooled, acidified with 1 N HCl, concentrated to dryness, dissolved in water, and purified by high-pressure LC system A. Recovery of ^{14}C from the column was quantitative. *S,S'*-(*ar*-Tetrachlorophenylene)dicysteine eluted after 8.6 min (9.4%), *S*-(pentachlorophenyl)cysteine after 21.4 min (47.7%), and an unidentified product that was probably *S*-(*ar*-tetrachloronitrophenyl)cysteine after 18.3 min (6.2%). The remainder of the radioactivity eluted in other minor peaks (5.0%) or in the 75% acetonitrile

wash (31.7%). *S,S'*-(*ar*-Tetrachlorophenylene)dicysteine and *S*-(pentachlorophenyl)cysteine were esterified with methanol, acylated with trifluoroacetic anhydride, and purified by TLC system G (two solvent developments). The derivatives migrated to R_f 0.15 and 0.45, respectively. The mass spectrum of the derivative of *S,S'*-(*ar*-tetrachlorophenylene)dicysteine was characterized by a molecular ion at *m/e* 672 and will be discussed in detail in relationship to metabolite V. The mass spectrum of the derivative of *S*-(pentachlorophenyl)cysteine was characterized by a molecular ion at *m/e* 477 and intense ion clusters at *m/e* 364 ($M - NH_2C(O)CF_3$), 293 ($C_6Cl_5SCH_2$), 279 (C_6Cl_5S), and other highly characteristic ions. Isotope abundance clusters clearly showed that the product contained five chlorines.

S-(Pentachlorophenyl)glutathione, *S*-(*ar*-tetrachloronitrophenyl)glutathione, and *S,S'*-(*ar*-tetrachlorophenylene)diglutathione were synthesized by reaction of PCNB with reduced glutathione (GSH). Pentachloronitrobenzene-*UL*-^{14}C (3.38 μmol, 0.477 mCi/mmol), 30 μmol of GSH, and 300 μmol of potassium hydroxide were reacted at 40 °C in ca. 8 mL of water–methanol–acetone (43:40:17). After 2 h, the reaction mixture was adjusted to pH 2.5 and concentrated. The resulting syrup was dissolved in 25 mL of 0.01 N HCl, washed twice with methylene chloride, concentrated to dryness, and purified by high-pressure LC system B. Four radioactive products were detected: IIIa at 21 mL (11%), IIIb at 25 mL (10%), II at 47 mL (3%), and I at 51 mL (46%). Aliquots of these products were esterified with various alcohols, acetylated with acetic anhydride, and subjected to mass spectral analysis. The mass spectra indicated that IIIa and IIIb were isomeric forms of *S,S'*-(*ar*-tetrachlorophenylene)diglutathione while the spectra of I and II clearly showed these products to be *S*-(pentachlorophenyl)glutathione and *S*-(*ar*-tetrachloronitrophenyl)glutathione, respectively. The spectra of these compounds are discussed in detail in relationship to the identification of metabolites I, II, and III.

Enzyme Isolation and Assay. Glutathione *S*-transferase enzyme activity was isolated from pea epicotyl by the method of Frear and Swanson (1973) (enzyme A) and also by the method of Rusness and Still (1977) (enzyme B). Enzyme A was purified through the Sephadex G-25 step and enzyme B was purified through the 60–80% ammonium sulfate precipitation step and then dialyzed. Enzyme reactions were run at 30 °C in 0.5–50-mL reaction volumes containing (^{14}C)PCNB, GSH, buffer, and *tert*-butyl alcohol. The reactions were stopped by extracting the radioactive substrate with methylene chloride and quantitated by assaying the radioactivity in the water-soluble fraction. The reactions were assayed for nitrite liberation by the methods of Snell and Snell (1949) and Habig et al. (1974). Protein concentrations were determined by UV absorbance as described by Layne (1957). Radioactive products were verified by TLC system A. For mass spectral identification of products, the following 50-mL enzyme reaction mixture was incubated for 9 h: 0.75 mg of enzyme A/mL of reaction mixture, 65 μM (^{14}C)PCNB, 10 mM GSH, 50 mM, pH 7.9, Tris-HCl buffer, and 15% *tert*-butyl alcohol. The reaction was terminated by adjusting the pH to 1.5 and the denatured protein was removed by centrifugation at 10 000*g* for 20 min at 4 °C. The protein pellet was washed in 10 mM HCl and recentrifuged. The aqueous supernatant and wash were combined and extracted with methylene chloride to remove residual (^{14}C)PCNB. The aqueous phase was then concentrated and chromatographed on XAD-2 as described for the purification of the in vivo water-soluble metabolites. The water eluate contained ca. 2% of the applied radioactivity and the methanol eluates ca. 91%. The methanolic eluates were combined, concentrated, dissolved in pH 2 buffer, and chromatographed on AG 50W-X2 system A. The remaining XAD-2 methanolic eluate was used directly for purification by high-pressure LC system A and for comparative analyses in TLC systems A and H.

Plant Materials and Treatments. Peanuts (*Arachis hypogaea* L. cv. Spanish) were grown in a controlled environment room with a 14-h photoperiod at 172 klux light intensity, day–night temperatures of 30 and 25 °C, and a constant relative humidity of 40%. Peanut seeds were planted in vermiculite moistened with one-third strength Hoagland's solution. After 4 weeks, seedlings were transferred to direct culture in aerated one-third strength Hoagland's solution (Blankendaal et al., 1972). Six weeks after planting, 75 seedlings were transferred to test tubes (2–3 seedlings/tube) containing 50 mL of one-third strength Hoagland's solution per tube. The tubes were shielded from light to inhibit the growth of algae and widely spaced to promote plant transpiration. During the first two photoperiods following transfer, each tube of plants was dosed eight times at 3.5-h intervals with 50-μL aliquots of an acetone solution of 3 mM (^{14}C)PCNB (specific activity 0.477 mCi/mmol). Water was added periodically to make up for transpiration and evaporation losses until 84 h after the first dose. The plants were harvested 96 h after the first dose. Because of the volatility of PCNB, the nutrient solution was not aerated during or following treatment with (^{14}C)PCNB. This treatment was replicated once.

In a separate experiment, 12 peanut plants were treated individually with 60-mL aliquots of a supersaturated solution of (^{14}C)PCNB (486 ppm, specific activity 1.76 mCi/mol) in one-third strength Hoagland's solution. Exposure of foliar tissue to (^{14}C)PCNB vapors was prevented by wrapping the top of each treating tube and the stem of each plant with a sheet of parafilm. Water was added as needed to make up for transpiration losses. After 48 h, six plants were harvested and the remaining six were transferred to fresh one-third strength Hoagland's solution and harvested 96 h later. The nutrient solution was not aerated during or following treatment. At each harvest, three seedlings were pressed, freeze-dried, and placed on single emulsion X-ray film for 1 month at 4 °C. The roots and shoots of the remaining seedlings were extracted as described below.

For a time-course study, twelve 1-month old plants in individual containers of one-third strength Hoagland's solution (50 mL) were individually treated with single doses of (^{14}C)PCNB (1.78 μmol, 1.76 mCi/mmol in 250 μL of acetone). Water was added as needed to make up for transpiration losses. Four days after treatment, three plants were harvested, and the remaining plants were transferred to fresh nutrient solution. Aeration of fresh nutrient solution was resumed, and the remaining plants were harvested after 21 and 37 days. The percent chloroform-soluble products in the 80% methanol extracts was determined immediately after extraction by TLC system G. Plants treated with (^{14}C)PCA (35 μM, specific activity 1.76 mCi/mmol) or *S*-[(^{14}C)pentachlorophenyl]cysteine (12 μM, specific activity 0.0246 mCi/mmol) were handled in a similar manner. These plants were harvested 21 days following treatment.

Extraction and Fractionation of Metabolites. The methods of extraction, fractionation, and derivatization of the metabolites are outlined in Figure 1. The roots and shoots of the plants were diced with scissors, pulverized

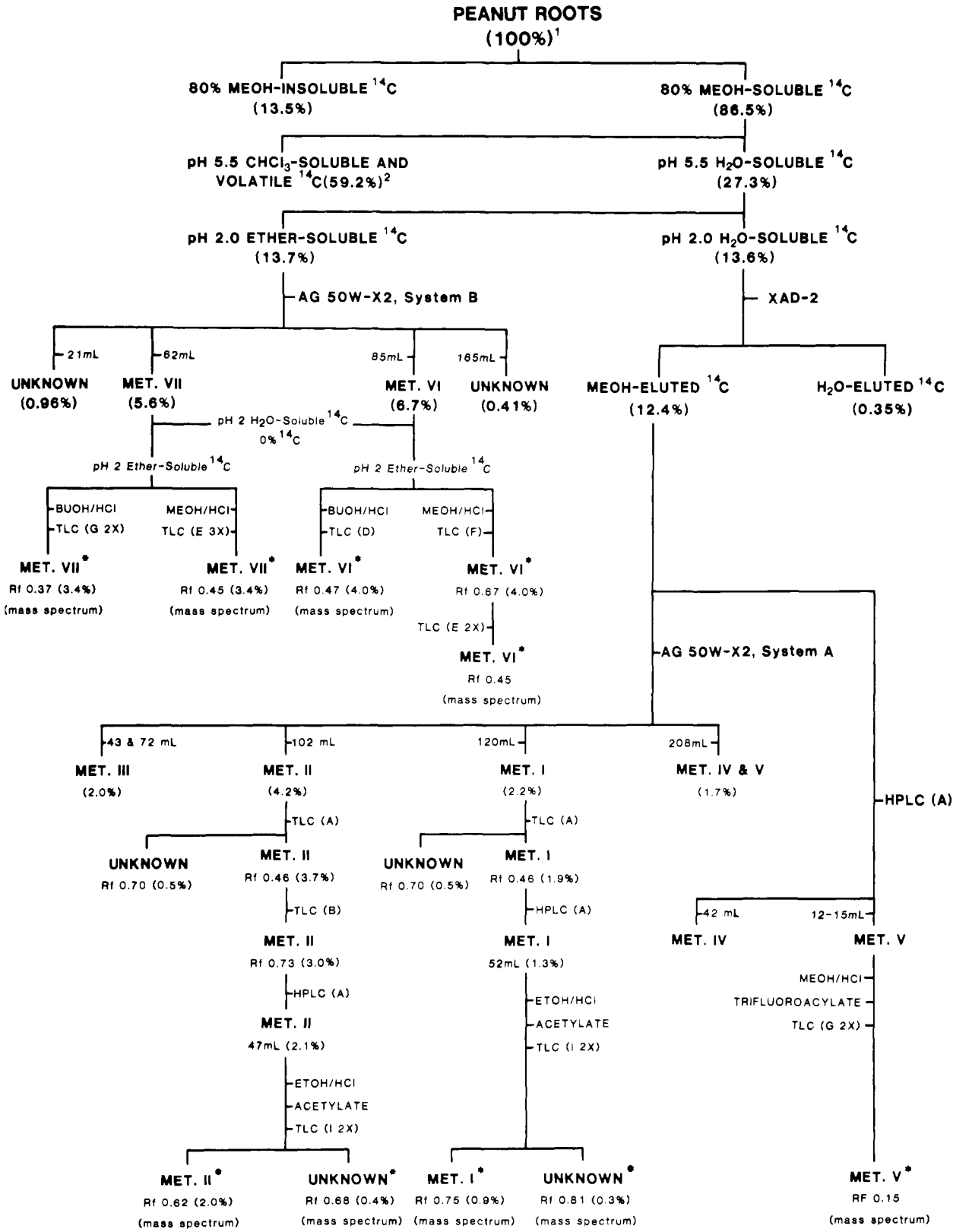

Figure 1. Extraction, purification, and derivatization of (^{14}C)PCNB metabolites from peanut roots. (1) The roots contained 88.8% of the ^{14}C used to treat the plants. (2) Radioactivity in these fractions is the subject of a separate report (Rusness and Lamoureux, 1980). (*) Indicates a derivative of the metabolite.

with a mortar and pestle under liquid nitrogen, and homogenized in an omnimixer (Sorvall, Inc.) in the presence of 2 volumes of cold aqueous 80% methanol. The plant debris was separated from the extracts by centrifugation at 10 000*g* for 20 min and reextracted three additional times with 80% methanol. The radioactivity in the combined methanolic extracts was quantitated, examined by TLC systems A and C, and concentrated in vacuo at 30 °C. The concentrates were diluted to 400 mL with water and extracted three times with 2-volume portions of chloroform at ca. pH 5.5. The combined chloroform extracts were used for metabolite identification in a parallel study (Rusness and Lamoureux, 1980). The 80% methanol-insoluble plant residues were pulverized and analyzed

Table I. Treatment of Peanut Plants with (^{14}C)PCNB, (^{14}C)PCA, and *S*-[(^{14}C)Pentachlorophenyl]cysteine

expt	treatment method	harvest time	% ^{14}C left in trt soln	% ^{14}C left in post-trt soln	% of ^{14}C in shoots		% of ^{14}C in roots		total % recovery
					soluble	insoluble	soluble	insoluble	
1	PCNB, 486 ppm, 2 days	immediate	12	NA	6.3	0.7	64.7	3.6	87.3
		4 days post-trt	12	3.1	3.0	0.47	44.6	11.7	74.9
2	PCNB, 8 × 17.6 ppm, 2 days	2 days post-trt	1.2	NA	6.1	1.1	76.8	12.1	97.2
3	PCNB, 10.4 ppm, 4 days	immediate	5.3	NA	not assayed		77.1	11.5	94
		17 days post-trt	5.3	trace	not assayed		42.5	21.9	
		33 days post-trt	5.3	trace	3.4	1.5	38.5	22.4	71.1
4	PCA, 9 ppm, 3 days	17 days post-trt	1.1	2.6	9.4	2.4	38.0	18.2	71.7
5	PCP-S-Cys, 4.5 ppm, 3 days	17 days post-trt	14.1	18.0	4.1	1.6	22.4	25.4	85.6

quantitatively for ^{14}C. The chloroform extracted aqueous phases were adjusted to pH 2 with 3 N HCl and extracted three times with 2-volume portions of ethyl ether. The ethyl ether and pH 2 aqueous fractions were concentrated to dryness and used for subsequent metabolite identification.

Replicate pH 2 aqueous extracts equivalent to ca. 2.85 mmol of PCNB were applied in 50 mL of water to 1.5 × 30 cm columns of Amberlite XAD-2 resin (Mallinckrodt Chemical Works) water-jacketed at 4 °C. The 50-mL aqueous effluents obtained during sample application to the XAD-2 columns contained some radioactivity and were reapplied to the columns. The columns were then eluted with 60 mL of water, 60 mL of methanol, and 30 mL of aqueous 80% methanol. The water eluates contained ca. 3% of the applied radioactivity and the methanol eluates ca. 91%. The methanol eluates were concentrated to dryness, dissolved in pH 2.1 buffer, and chromatographed on AG 50W-X2 system A. Replicate chromatograms yielded four radioactive fractions with an overall recovery of 94 ± 2% of the applied radioactivity. The radioactive fractions were concentrated to dryness and stored frozen until they could be subjected to further purification, derivatization, and mass spectral analysis as outlined in Figure 1.

Replicate pH 2 ether-soluble extracts were dissolved in a minimal volume of pH 2 buffer and applied to 1 × 60 cm columns of AG 50W-X2 and chromatographed by procedure B. Four radioactive fractions were eluted from the column in an overall recovery of 100% (Figure 1). Metabolite fractions VI and VII were adjusted to pH 2, extracted into ethyl ether, esterified with methanol or 1-butanol, purified by additional TLC, and subjected to mass spectral analysis as further outlined (Figure 1).

An alternative method of purification of the ether extract involved chromatography of 250 000-dpm aliquots of the crude ether extract on high-pressure LC system B. Major radioactive products were eluted after 37 mL (metabolite VII) and 45 mL (metabolite VI). Metabolite VII was rechromatographed (system B), and then metabolites VI and VII were esterified with 1-butanol and purified by TLC system G (two times). Metabolites VI and VII yielded 70 and 67% conversion to products with R_f values of 0.40 and 0.37, respectively.

RESULTS AND DISCUSSION

The results summarized in Table I clearly showed that (^{14}C)PCNB is rapidly absorbed by the roots of peanut plants grown in nutrient culture. Plants treated with eight successive 17.6-ppm doses of (^{14}C)PCNB in a 2-day period absorbed all but 1.2% of the ^{14}C. The concentration of (^{14}C)PCNB in the nutrient solution or the exact manner of treatment did not greatly affect the translocation pattern (Table I). In all cases, only 3.5–7.2% of the added ^{14}C was found in the shoots, while 56.6–88.9% of the added ^{14}C was found in the roots. When a parafilm barrier was used to prevent contamination of the shoots by (^{14}C)PCNB vapors, the results were nearly the same as when no barrier was used, suggesting that the ^{14}C in the shoots was translocated. Radioautograms made immediately after plants were exposed to 486-ppm (^{14}C)PCNB for 48 h verified that the ^{14}C was concentrated in the roots and showed that the ^{14}C in the leaves was evenly distributed. Radioautograms made 4 days after the plants were transferred to ^{14}C-free nutrient solution showed that the leaves that developed during the post-treatment period contained little or no radioactivity. It appears that translocation of PCNB and its metabolites occurs soon after PCNB enters the roots. The recovery of ^{14}C from (^{14}C)PCNB-treated plants and nutrient solutions decreased as a function of the post-treatment harvest time. In experiment 1 (Table I), the recovery of ^{14}C from the system decreased 12% in 4 days. This decrease corresponded to a loss of 12% of the ^{14}C in the roots. In experiment 3 (Table I), a 22.3% decrease in the recovery of ^{14}C was observed between plants harvested immediately after treatment and those harvested 33-days post-treatment. This corresponded to a 27.7% decrease of ^{14}C in the roots. In the roots, 80% methanol-insoluble ^{14}C increased as a function of time. This increase was essentially complete by 21 days.

Pentachloroaniline and *S*-(pentachlorophenyl)cysteine, metabolites of PCNB, were also readily absorbed by peanut roots. Uptake of *S*-(pentachlorophenyl)cysteine (4.7 ppm) from the nutrient solution was 50% complete after 5 h and 68% complete after 24 h. Uptake of PCA (9 ppm) was nearly quantitative after 24 h. Translocation of PCA and *S*-(pentachlorophenyl)cysteine from the roots to the shoots was very restricted; most of the ^{14}C remained in the roots (Table I). Both metabolites gave rise to insoluble residues in comparable amounts.

In Vitro Enzyme Studies. A glutathione *S*-transferase enzyme system that utilized PCNB as a substrate was isolated from pea epicotyl by the methods of Frear and Swanson (1973) (enzyme A) and Rusness and Still (1977) (enzyme B). A similar enzyme was also isolated from peanut by the methods described by Shuey–Burkholder (1977), but the enzyme from peanut was not isolated in

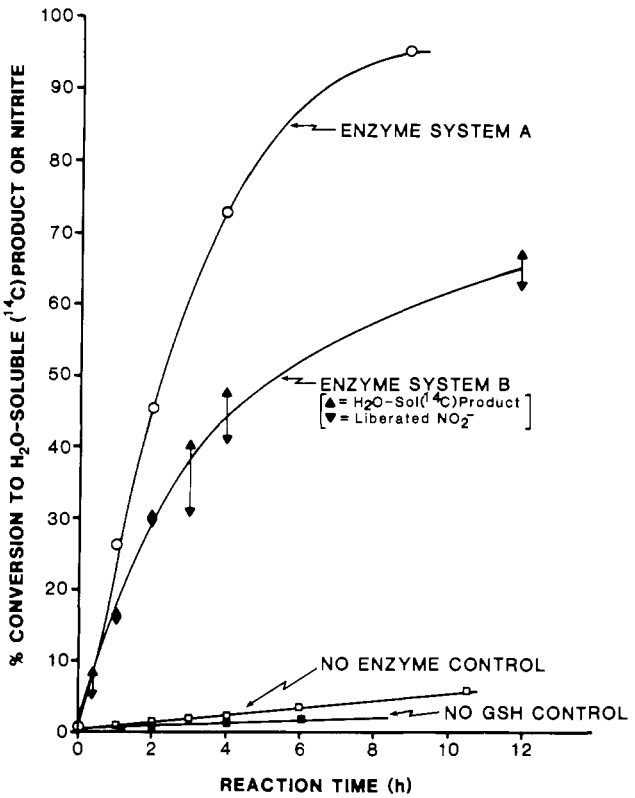

Figure 2. Enzymatic formation of glutathione conjugates. Enzyme A (0.75 mg of protein/mL reaction mixture), 65 μM (^{14}C)PCNB, 10 mM GSH, 15% *tert*-butyl alcohol, and 50 mM, pH 7.9, Tris-HCl buffer were assayed for water-soluble ^{14}C (○). Enzyme B (0.26 mg/mL reaction mixture), 30 μM (^{14}C)PCNB, 1 mM GSH, 15% *tert*-butyl alcohol, and 100 mM, pH 7.0, potassium phosphate buffer were assayed for water-soluble ^{14}C (▲) and liberated nitrite (▼). The no-GSH control contained 65 μM (^{14}C)PCNB, 15% *tert*-butyl alcohol, 100 mM, pH 7.9, potassium phosphate buffer, and enzyme B (0.64 mg/mL). It was assayed for water-soluble ^{14}C (■). The no-enzyme control mixture was identical with the enzyme B reaction mixture except for the absence of enzyme. It was assayed for water-soluble ^{14}C products (□).

a consistently active form. For this reason, the enzyme from pea epicotyl was used for these studies. The reactions catalyzed by the pea epicotyl enzymes were linear for about 2 h and were dependent upon the presence of both GSH and enzyme (Figure 2). Nitrite and water-soluble ^{14}C-labeled products were formed in a nearly 1:1 ratio throughout the course of the reaction, suggesting that the primary reaction involved nucleophilic displacement of the nitro group. The specific activities of enzyme preparations A and B were 20.3 and 19.0 nmol of product per mg of protein per h, respectively. Because of the insolubility of PCNB in aqueous systems, it was necessary to add organic solvents to the enzyme assay mixtures. *tert*-Butyl alcohol at 14–16% (v/v) increased the amount of PCNB that could be suspended or kept in solution during the assays and also increased the reaction rate. *tert*-Butyl alcohol has been reported to stimulate other enzyme activities (Tan and Lovrien, 1972). In the presence of 15% *tert*-butyl alcohol, glutathione *S*-transferase activity was 3.2-fold greater at pH 7.9 than at pH 6.5. The enzyme system appeared to be specific for glutathione; cysteine could not be substituted for glutathione at either pH 6.5 or 7.9. Analysis of the enzyme A reaction mixture by high-pressure LC system A indicated that four products were produced (Figure 3). The minor products, accounting for 24% of the radioactivity in the chromatogram, appeared to be artifacts formed from storage of product I. When PCNB reacted with GSH in 0.04 N potassium hydroxide under mild conditions, the same products were formed in approximately the same yield as was observed in the enzymatic reaction (Figure 3). The products from these reactions were esterified, acylated, and further purified. Products IIIa and IIIb were tentatively characterized as isomeric forms of *S*,*S*′-(*ar*-tetrachlorophenylene)diglutathione, and I and II were conclusively identified as *S*-(pentachlorophenyl)glutathione and *S*-(*ar*-tetrachloronitrophenyl)glutathione. The mass spectra of these products and their interpretation are discussed in detail in conjunction with the identification of the in vivo metabolites.

Isolation and Identification of Metabolite I, *S*-(Pentachlorophenyl)glutathione, and Metabolite II, *S*-(*ar*-Tetrachloronitrophenyl)glutathione. Metabolites I and II were isolated from the pH 2 water-soluble extract of peanut roots as outlined in Figure 1. Chromatography on AG 50W-X2 system A indicated that metabolites I and II accounted for approximately 16 and 31%, respectively, of the ^{14}C in the pH 2 aqueous fraction. Cross-contamination between metabolites I and II obtained by chromatography on AG 50W-X2 was minimized by rechromatography of the overlapping portions of the eluates.

Metabolite I cochromatographed with in vitro product I in high-pressure LC system A (52 mL) and in TLC system A (R_f 0.47). After esterification with ethanol/HCl, acetylation with acetic anhydride, and analysis by TLC system I, both metabolite I and product I yielded a major derivative at R_f 0.75 (68%) and a minor derivative at R_f 0.81. The mass spectra of the major derivative of metabolite I and product I were identical. The mass spectrum of the major derivative of metabolite I and the probable nature of the key ion fragments are shown in Figure 4. The interpretation of this mass spectrum was consistent with the mass spectra obtained from the *N*-acetyl methyl ester, the *N*-acetyl butyl ester, and the *N*-trideuterioacetyl ethyl ester of product I. The mass spectra of these additional derivatives verified the presence of two free carboxyl groups, one free amino group, and a pentachlorothiophenyl nucleus. A parent ion was observed with each derivative. It was concluded that metabolite I and product I were both *S*-(pentachlorophenyl)glutathione. The minor product detected at R_f 0.81 after derivatization of metabolite I and product I may have been an artifact. The mass spectrum of this product was characterized by ion fragments consistent with a pentachlorophenyl nucleus with an alkyl sulfide side chain.

Metabolite II cochromatographed with product II from the in vitro reaction between GSH and PCNB. Both had an elution volume of 47 mL with high-pressure LC system A and an R_f of 0.46 with TLC system A. Both were esterified with ethanol, acetylated with acetic anhydride, and purified by TLC system I. Both yielded a major derivative at R_f 0.62 and a minor derivative at R_f 0.68. The mass spectra of the major derivative of metabolite II and product II were identical. They were characterized by a molecular ion cluster at m/e 662 and ion fragment clusters at m/e 617, 560, 333, and 291 that suggested the presence of four chlorines on an aromatic nucleus. The mass spectrum of the metabolite II derivative and the probable nature of the key ion fragments are shown in Figure 5. The mass spectrum of the metabolite II derivative was very similar to that of the metabolite I derivative. The primary differences between the spectra were the following: (1) aryl fragments of II generally occurred at 11 mass units higher than I, (2) aryl fragments of II indicated the presence of

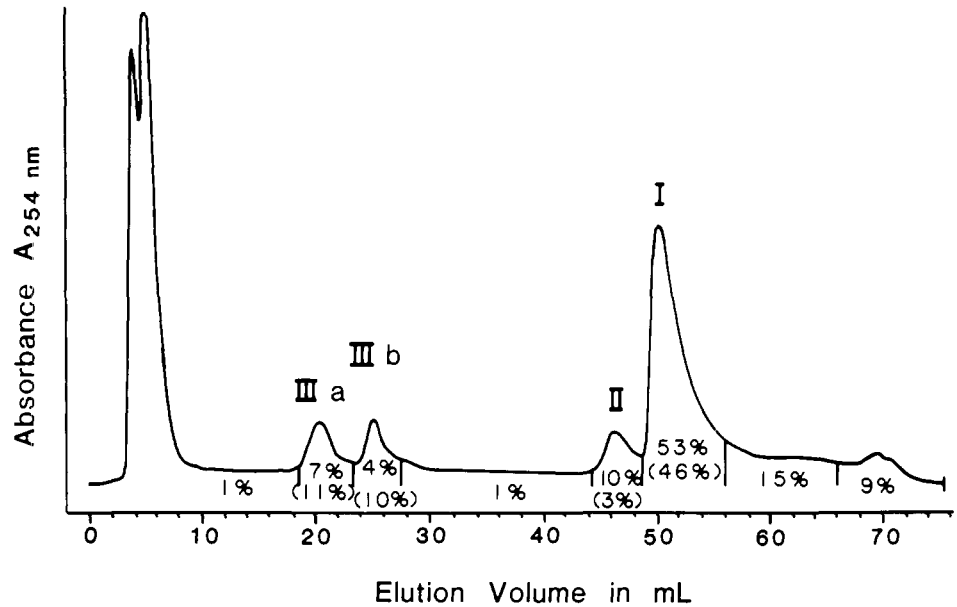

Figure 3. High-pressure LC of the water-soluble products from the in vitro reaction of (^{14}C)PCNB with glutathione. Percent values are based on liquid scintillation counts of radioactive fractions. The upper values were obtained from the enzyme-catalyzed reaction and the lower values were from the corresponding base-catalyzed reaction. The ultraviolet light was monitored from the enzyme-catalyzed reaction.

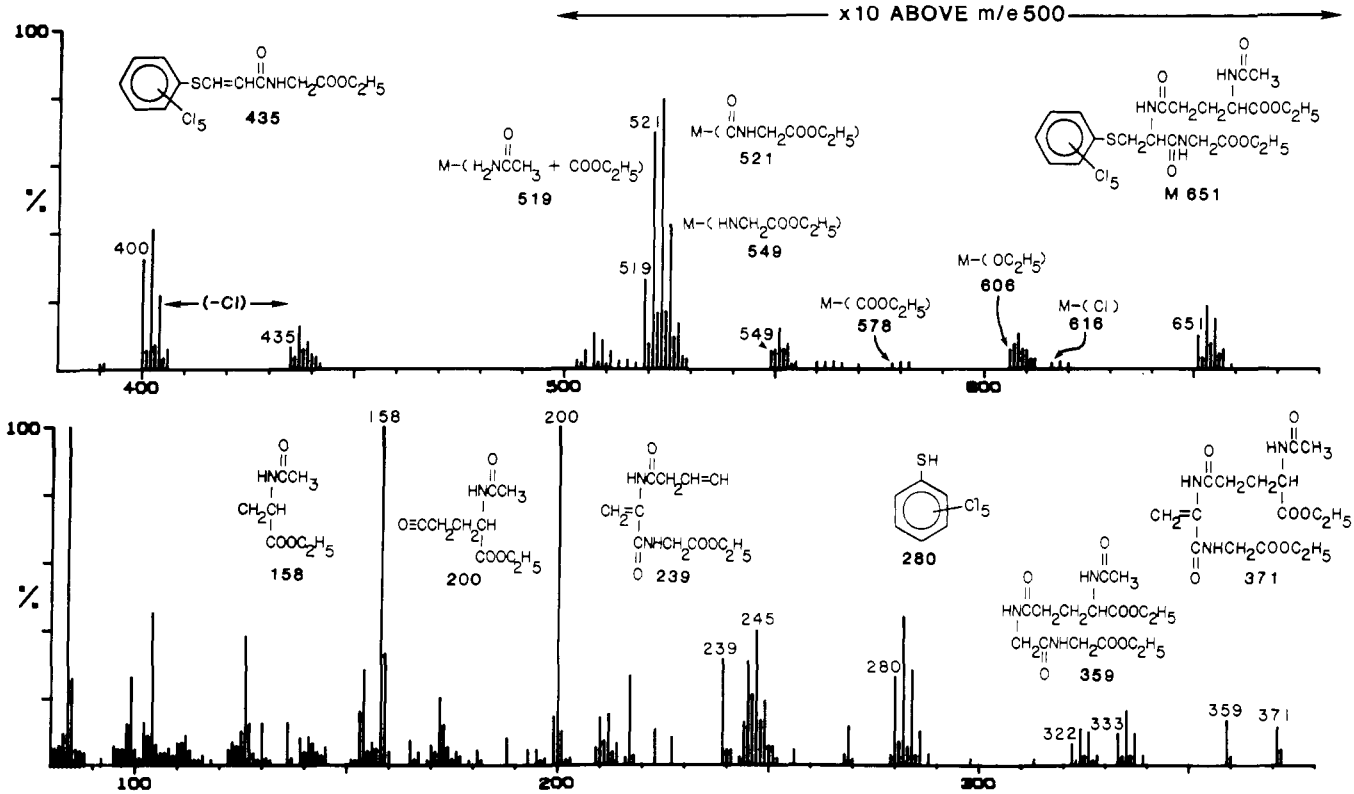

Figure 4. Mass spectrum of metabolite I, *S*-(pentachlorophenyl)glutathione (acetylated ethyl ester derivative). Ion clusters at *m/e* 333 and 322 correspond to $C_6Cl_5SCH{=}CHC{=}O$ and $C_6Cl_5SCH_2CH_2NH$, respectively. The ion cluster at *m/e* 245 corresponds to the loss of one chlorine from the ion cluster at *m/e* 280.

four chlorines while aryl fragments of I suggested the presence of five chlorines, and (3) metabolite II yielded ion fragments at *m/e* 388 and 285 that were not present in the spectrum of I. In the mass spectrum of metabolite II, the ion fragments at *m/e* 388 and 285 were probably formed by a rearrangement in which an oxygen from the nitro group was transferred to the β-carbon of the cysteinyl residue of the side chain. If the thioalkyl side chain was ortho to the nitro group, this rearrangement could have taken place through either a five- or six-membered cyclic intermediate. The elemental composition of the ion at *m/e* 388 was indicated by high-resolution peak matching. The measured value, 388.171 951, agreed to within 0.1 ppm with the theoretical mass for an ion of the composition indicated in Figure 5. Additional proof of structure of metabolite II was obtained by strong acid hydrolysis and amino acid analysis. Metabolite II liberated glycine, glutamic acid, and PCNB equivalents in a ratio of 1.2:1.1:1.0. It was concluded that metabolite II was *S*-(*ar*-tetrachloronitrophenyl)glutathione. The mass spectral data were most

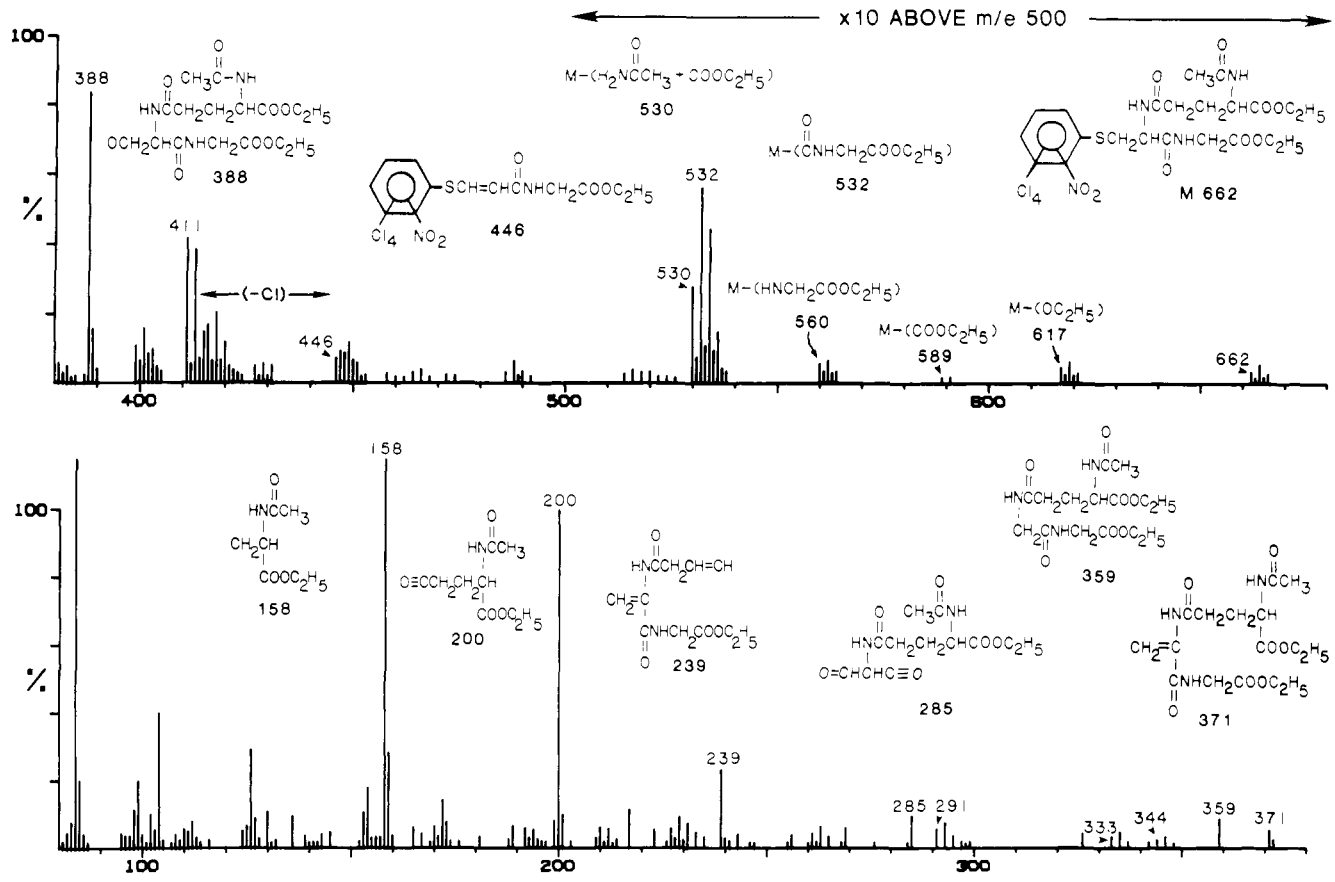

Figure 5. Mass spectrum of metabolite II, *S*-(*ar*-tetrachloronitrophenyl)glutathione (acetylated ethyl ester derivative). Ion clusters at *m/e* 333 and 344 correspond to $C_6Cl_4NO_2SCH_2CH_2NH$ and $C_6Cl_4NO_2SCH{=}CHC{=}O$, respectively. The ion cluster at *m/e* 291 corresponds to $C_6Cl_4NO_2SH$.

consistent with an ortho isomer; however, this was not proven. The minor product detected after derivatization of metabolite II and product II was also examined by mass spectral analysis. This product also appeared to contain a tetrachloronitrophenyl nucleus attached to an alkyl side chain by a sulfide bond. The alkyl side chain appeared to be a dipeptide or an elimination product of a dipeptide. It was assumed that this product was an artifact of derivatization.

Characterization of Water-Soluble Metabolite III, *S,S'*-(*ar*-Tetrachlorophenylene)diglutathione. Fraction III was isolated from the pH 2 water-soluble extract of peanut roots by chromatography on AG 50W-X2 (Figure 1). Fraction III contained 14% of the radioactivity originally present in the pH 2 water-soluble extract and appeared to consist of at least two components; however, attempts to further purify this fraction by high-pressure LC or TLC were not successful. On high-pressure LC, fraction III chromatographed as an unresolved band in the same region as products IIIa and IIIb from the in vitro reaction of PCNB with glutathione (Figure 3). When an XAD-2 methanol eluate of the pH 2 water-soluble fraction was chromatographed on high-pressure LC without prior fractionation on AG 50W-X2, peaks corresponding to in vitro products IIIa and IIIb were detected.

Products IIIa and IIIb, produced in vitro, were esterified with ethanol and acetylated with acetic anhydride. Both yielded derivatives in 70 to 75% yield that chromatographed at R_f 0.23 on TLC system I (developed two times). The mass spectra of these derivatives were similar; however, the spectrum of IIIb was extremely weak above *m/e* 371. The mass spectrum of IIIa is shown in Figure 6. This spectrum contained the key ions associated with the fragmentation of the glutathione side chain, including ions at *m/e* 371 (5.8), 359 (2.0), 239 (19), 200 (32), and 158 (100). A number of aryl fragments suggested that both the nitro group and a chloro group had been displaced by glutathione: *m/e* 677, 461, 426, 306, 278, and 243. The isotope abundance peak ratios of the most intense ion fragments that contained the aromatic moiety were in good agreement for the indicated elemental composition of the ions. The mass spectrum of product IIIa was consistent with a tetrachlorodithiophenyl nucleus that contained two alkyl side chains attached by sulfide bonds. One alkyl side chain appeared to be glutathione and the other appeared to be C_2H_5. The ethyl side chain may have been formed from glutathione as an artifact during derivatization or in the mass spectrometer by pyrolysis or ionization. The molecular weight of the diethyl ester di-*N*-acetyl derivative of *S,S'*-(*ar*-tetrachlorophenylene)diglutathione is 1020. A compound of this molecular weight and polarity would probably not produce a detectable molecular ion.

Products IIIa and IIIb were synthesized from PCNB and GSH both enzymatically and in a mild base-catalyzed reaction. Products IIIa and IIIb appeared to be more polar than *S*-(*ar*-tetrachloronitrophenyl)glutathione by TLC or high-pressure LC, and mass spectral analysis suggested that products IIIa and IIIb contained a tetrachlorophenyl nucleus and two alkyl sulfide side chains. In consideration of these facts, it appears that IIIa and IIIb are most likely two isomeric forms of *S,S*-(*ar*-tetrachlorophenylene)diglutathione.

Isolation and Identification of Water-Soluble Metabolites IV, *S*-(Pentachlorophenyl)cysteine, and V, *S,S'*-(*ar*-Tetrachlorophenylene)dicysteine. Metabolites IV and V were isolated from the pH 2 water-soluble extract of peanut roots as indicated in Figure 1. Metabolites IV and V accounted for 6 to 19% of the ^{14}C in the

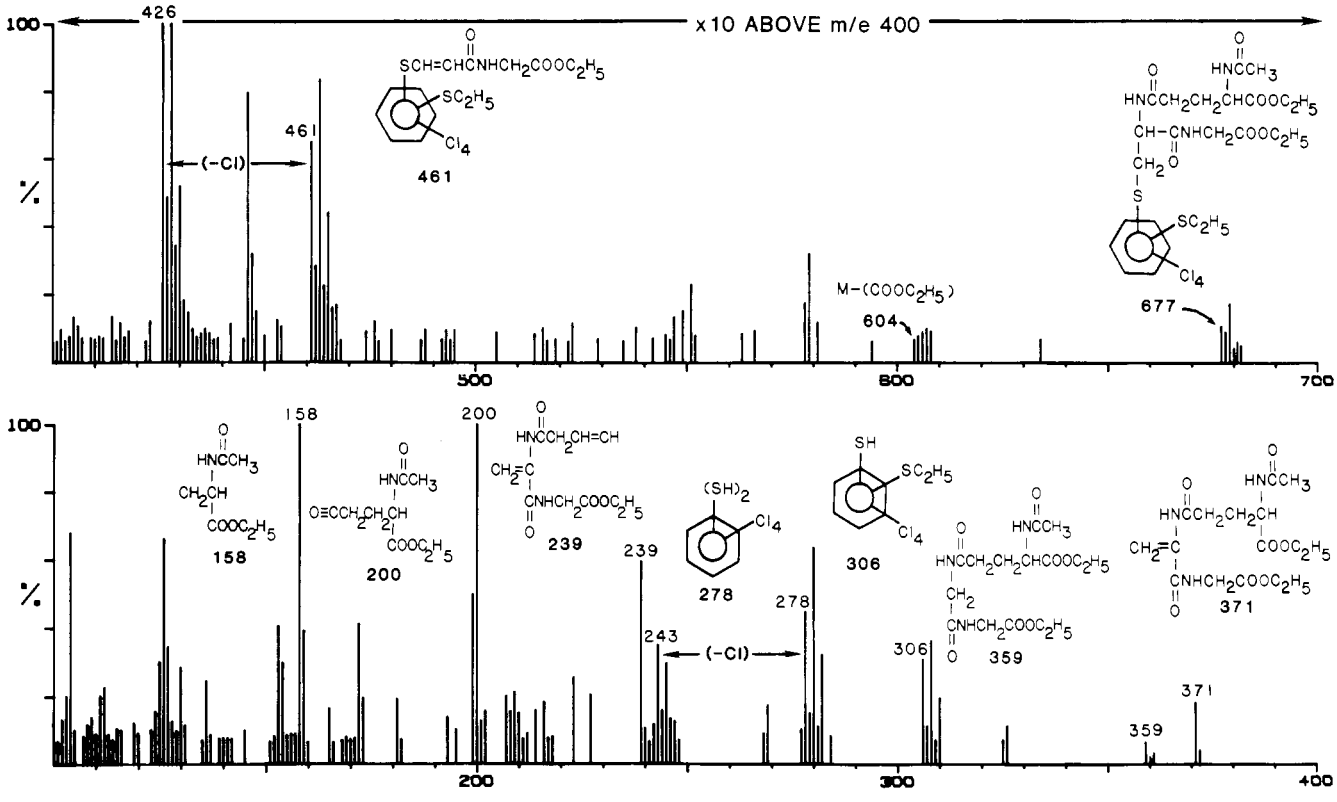

Figure 6. Mass spectrum of in vitro product IIIa, *S,S'*-(*ar*-tetrachlorophenylene)diglutathione (acetylated ethyl ester derivative).

pH 2 water-soluble extract, and they eluted as a single unresolved symmetrical peak at 207 mL when chromatographed on AG 50W-X2. Rechromatography on AG 50W-X2 or TLC with system H showed that the products in this fraction had been changed by chromatography on AG 50W-X2. A mixture of synthetic *S*-(pentachlorophenyl)-cysteine and *S,S'*-(*ar*-tetrachlorophenylene)dicysteine also chromatographed on AG 50W-X2 with an elution volume of 207 mL, and these products were also altered by the chromatographic procedure. For completion of the isolation and identification of metabolites IV and V, a third lot of peanut seedlings was treated with a single dose of (^{14}C)PCNB for 96 h. These seedlings were extracted, partitioned, and treated with XAD-2 as previously outlined in Figure 1. High-pressure liquid chromatography of the resulting XAD-2 methanolic eluate verified the presence of a product, metabolite IV, that cochromatographed with synthetic *S*-(pentachlorophenyl)cysteine on high-pressure LC system A (42–46 mL). Further attempts to purify metabolite IV by TLC resulted in an alteration of the metabolite. It was shown, however, that *S*-(pentachlorophenyl)cysteine was partially soluble in chloroform at pH 5.5 and a metabolite was subsequently found in the chloroform-soluble fraction from peanut roots that cochromatographed with *S*-(pentachlorophenyl)cysteine in TLC system H (R_f 0.50). This metabolite and standard *S*-(pentachlorophenyl)cysteine were esterified, acylated, and subjected to gas chromatographic analysis on a column of SP 2100. The two derivatives cochromatographed. It was concluded that metabolite IV was *S*-(pentachlorophenyl)cysteine.

A product with an elution volume of 12–15 mL, metabolite V, was also detected during high-pressure LC analysis of the XAD-2 eluate (Figure 1). Metabolite V cochromatographed with standard *S,S'*-(*ar*-tetrachlorophenylene)dicysteine in high-pressure LC system A. After esterification with methanol/HCl and acylation with trifluoroacetic anhydride, the resulting derivative of metabolite V cochromatographed with the corresponding derivative of *S,S'*-(*ar*-tetrachlorophenylene)dicysteine. The mass spectra of the two derivatives were identical. The mass spectrum of the derivative of metabolite V is shown in Figure 7.

Isolation and Identification of Ether-Soluble Metabolites VI, (*S*-Pentachlorophenyl)-*N*-malonylcysteine, and VII, *S*-(*ar*-Tetrachloronitrophenyl)-*N*-malonylcysteine. The pH 2 ether-soluble fraction accounted for 13.7% of the radioactivity isolated from peanut roots. Two major metabolites were detected in this fraction by chromatography on AG 50W-X2 (system B). These metabolites, VI and VII, were esterified with methanol or 1-butanol and purified further as described in Figure 1.

The mass spectrum of the methyl ester of metabolite VI and the probable nature of key ion fragments are shown in Figure 8. The spectrum was characterized by a molecular ion cluster at *m/e* 481. The peak ratios of the ion clusters at *m/e* 450, 422, 380, 364, and 322 suggested that these ions contained a pentachlorophenyl moiety. This was consistent with the observation that metabolite VI yielded a negative Bratton–Marshall test, indicating that the nitro group had been displaced or altered. The molecular ion at *m/e* 481, the key aryl ion fragments mentioned previously, and ion fragments derived from the side chain at *m/e* 202, 189, 170, 117, 101, and 88 were all consistent with the methyl ester of *S*-(pentachlorophenyl)-*N*-malonylcysteine. The mass spectrum of the butyl ester of metabolite VI yielded comparable results and verified the interpretation of the spectrum of the methyl ester. Peanut plants treated with *S*-[(^{14}C)pentachlorophenyl]-cysteine formed a product that was indistinguishable from metabolite VI by TLC or mass spectrometry. It was concluded that metabolite VI was *S*-(pentachlorophenyl)-*N*-malonylcysteine.

The mass spectrum of the butyl ester of metabolite VII is shown in Figure 9. A comparison of the key ion clusters

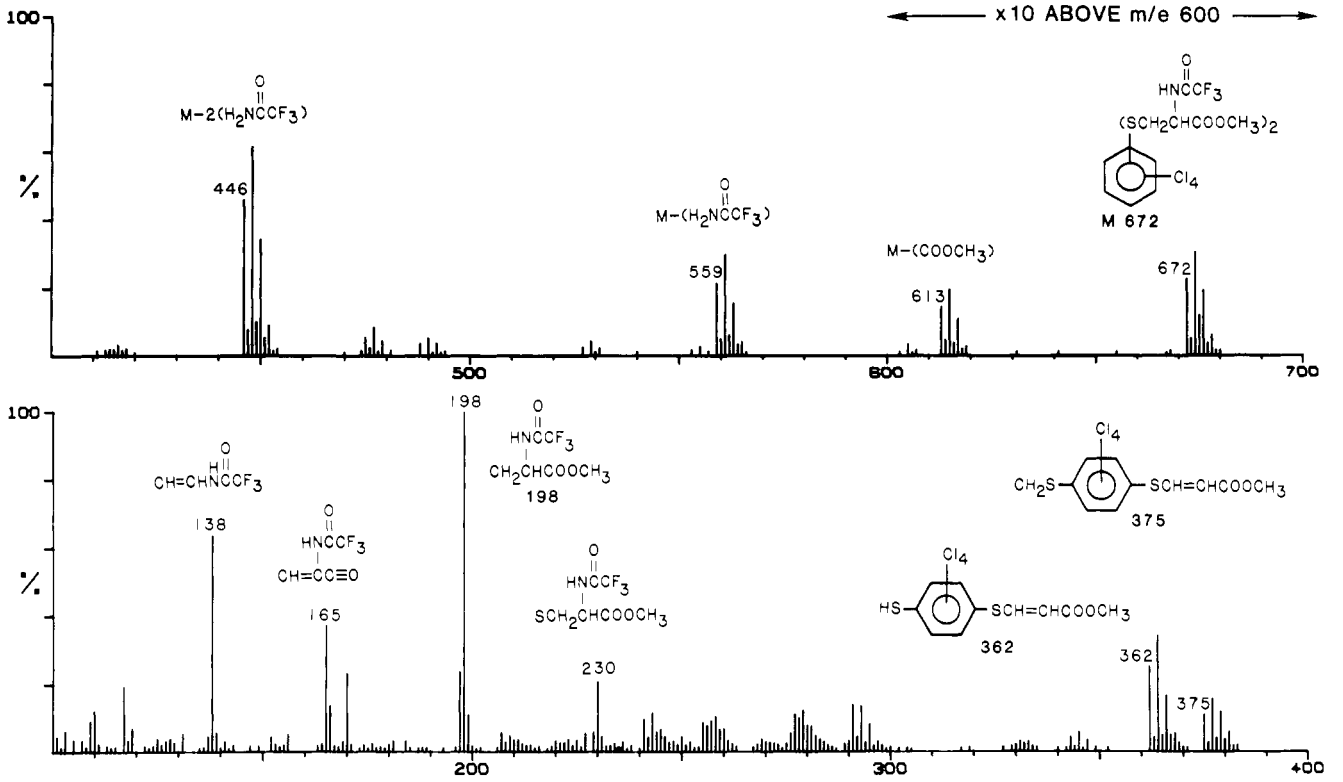

Figure 7. Mass spectrum of metabolite V, *S,S'*-(*ar*-tetrachlorophenylene)dicysteine (trifluoroacetylated methyl ester derivative).

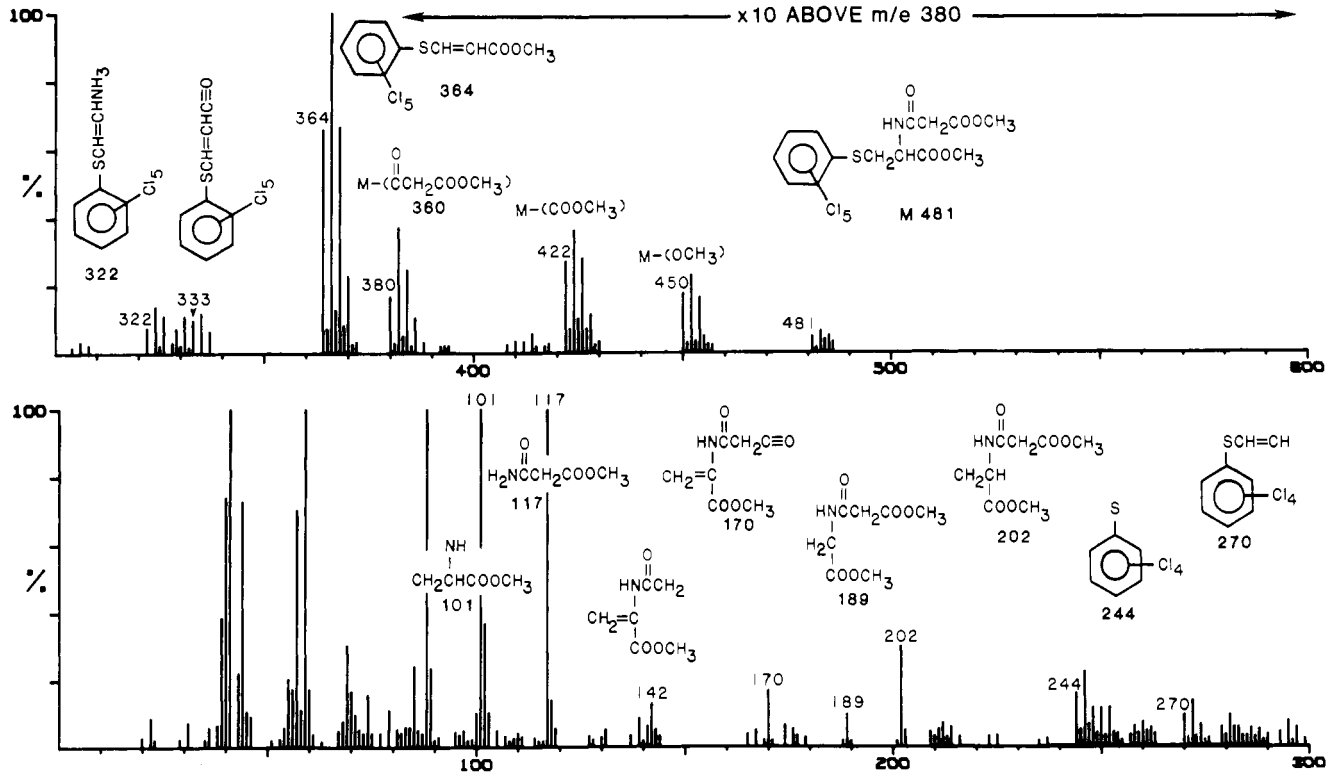

Figure 8. Mass spectrum of metabolite VI, *S*-(pentachlorophenyl)-*N*-malonlycysteine (acetylated methyl ester derivative).

of the butyl esters of metabolites VI and VII from m/e 333 to 576 showed that VII produced comparable ion clusters, but at m/e values 11 units higher (Table II). The isotope abundance clusters of the key aryl fragments between m/e 333 and 503 were consistent with a four chlorine aromatic nucleus for metabolite VII and a five chlorine aromatic nucleus for metabolite VI. Metabolite VII yielded a positive Bratton–Marshall test for a nitro group while VI yielded a negative test. In the spectrum of VII, high-resolution peak matching of the ion at m/e 361 yielded a precise mass of 360.851 699. This agreed to within 0.9 ppm for the theoretical mass of the indicated ion, $C_6Cl_4NO_2S$-CH=CHCOOH. Some important differences were observed between the fragmentation of metabolites VI and VII below m/e 300 (Table II). These differences appeared to be due to the interaction of the aryl nitro group and the

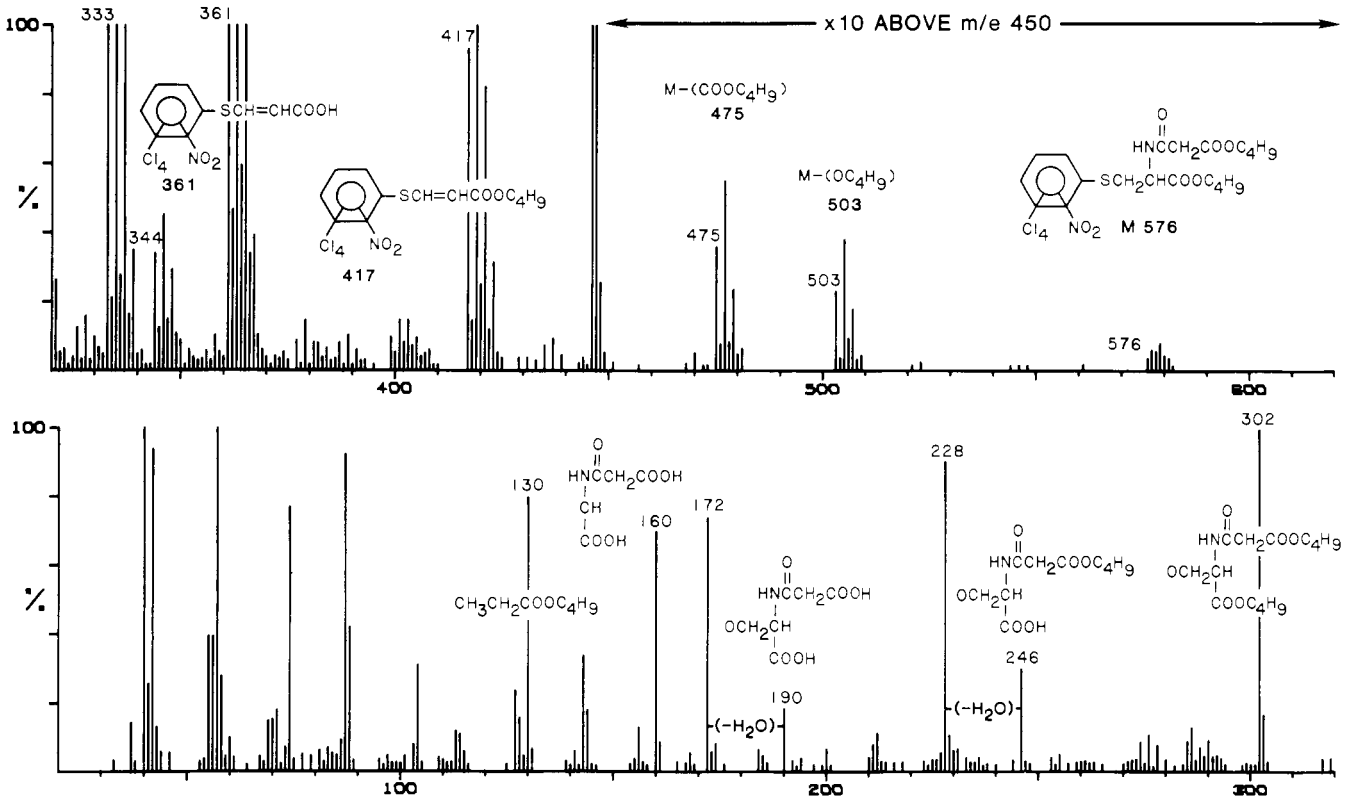

Figure 9. Mass spectrum of metabolite VII, *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine (acetylated butyl ester derivative). Ion clusters at *m/e* 333 and 344 correspond to $C_6Cl_4NO_2SCH_2CH_2NH$ and $C_6Cl_4NO_2SCH{=}CHC{=}O$, respectively.

Table II. Comparison of Key Ion Fragments in the Mass Spectra of the Dibutyl Esters of Metabolites VI and VII

nature of ion	butyl ester of VI		butyl ester of VII	
	m/e	% B	*m/e*	% B
M	565	1.1	576	0.4
M – (OC_4H_9)	492	2.0	503	2.5
M – ($COOC_4H_9$)	464	3.6	475	3.7
M – ($H_2NC(O)CH_2COOC_4H_9$)	406	45.4	417	9.5
M – ($H_2NC(O)CH_2COOC_4H_9$ + C_4H_8)	350[c]	62.9	361	23.8
M – ($HNC(O)CH_2COOC_4H_9$ + HOC_4H_9)	333	6.4	344	3.6
M – ($O{=}C{=}CHCOOC_4H_9$ + $COOC_4H_9$)	322	18.9	333	20.4
$H_9C_4OOC(OCH_2)CHNHC(O)CH_2COOC_4H_9$	302	0.5	302[b]	100
$H_9C_4OOC(CH_2)CHNHC(O)CH_2COOC_4H_9$	286	14.1	286	13.1
$H_9C_4OOC(OCH_2)CHNHC(O)CH_2COOH$	246	0.4[a]	246	30.2
$H_9C_4OOC(CH)CNHC(O)CH_2COOH$	228	1.3	228	90.5
$H_9C_4OOC(CH_2)CNHC(O)CH_2CO$	212	15.5	212	11.5
$HOOC(OCH_2)CHNHC(O)CH_2COOH$	190	0.8	190	18.5
$H_9C_4OOC(CH)CHNHC(O)CH_2$	184	6.4	184	6.9
$HOOC(CH)CNHC(O)CH_2COOH$	172	1.3	172	73.8
$HSCH{=}CHCOOC_4H_9$	160	8.5	160	69.9

[a] Several peaks were observed with a nominal mass of 246, but the mass defect of these peaks was not consistent with the above structure. These peaks had an intensity of 12.7% of B. [b] 302 used as base peak, but *m/e* 57.5 = 212% of base. [c] 352 used as base peak, but *m/e* 57.5 = 144.

side chain. In the spectrum of metabolite VII, the intense ions at *m/e* 302 and 190 were apparently formed by transfer of oxygen from the aryl nitro group to the β-carbon of the cysteine residue on the side chain, with the resulting loss of sulfur. This fragmentation is analogous to that observed with metabolite II. The precise mass of the ion at *m/e* 302 (302.160 637) agreed to within 0.00-ppm error with the theoretical mass for an ion with the structure $H_9C_4OOCCH(CH_2O)HNC(O)CH_2COOC_4H_9$. The intense ion fragments in the spectrum of VII at *m/e* 228 and 172 were very weak in the spectrum of VI, and their formation also may have been assisted by the nitro group; however, the important ion fragments at *m/e* 212 and 184 were present at nearly the same intensity in both VI and VII. Spectra of the butyl esters of VI and VII were nearly identical from *m/e* 40 to 160. It was concluded that metabolite VII was *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine. The mass spectral data were most consistent with the ortho isomer, but additional studies are needed to establish the isomeric form of metabolite VII.

Ion-exchange chromatography of the pH 2 ether extracts indicated that metabolites VI and VII accounted for ca. 90% of the ^{14}C in this fraction (Figure 1) as compared to 40% when chromatographed by high-pressure LC system B. When metabolites VI and VII, isolated by high-pressure LC system B, were rechromatographed by system B, the recovery of ^{14}C was frequently poor. Therefore, the quantitative data obtained by AG 50W-X2 chromatography may more nearly reflect the true composition of this fraction. Derivatization of metabolites VI and VII with

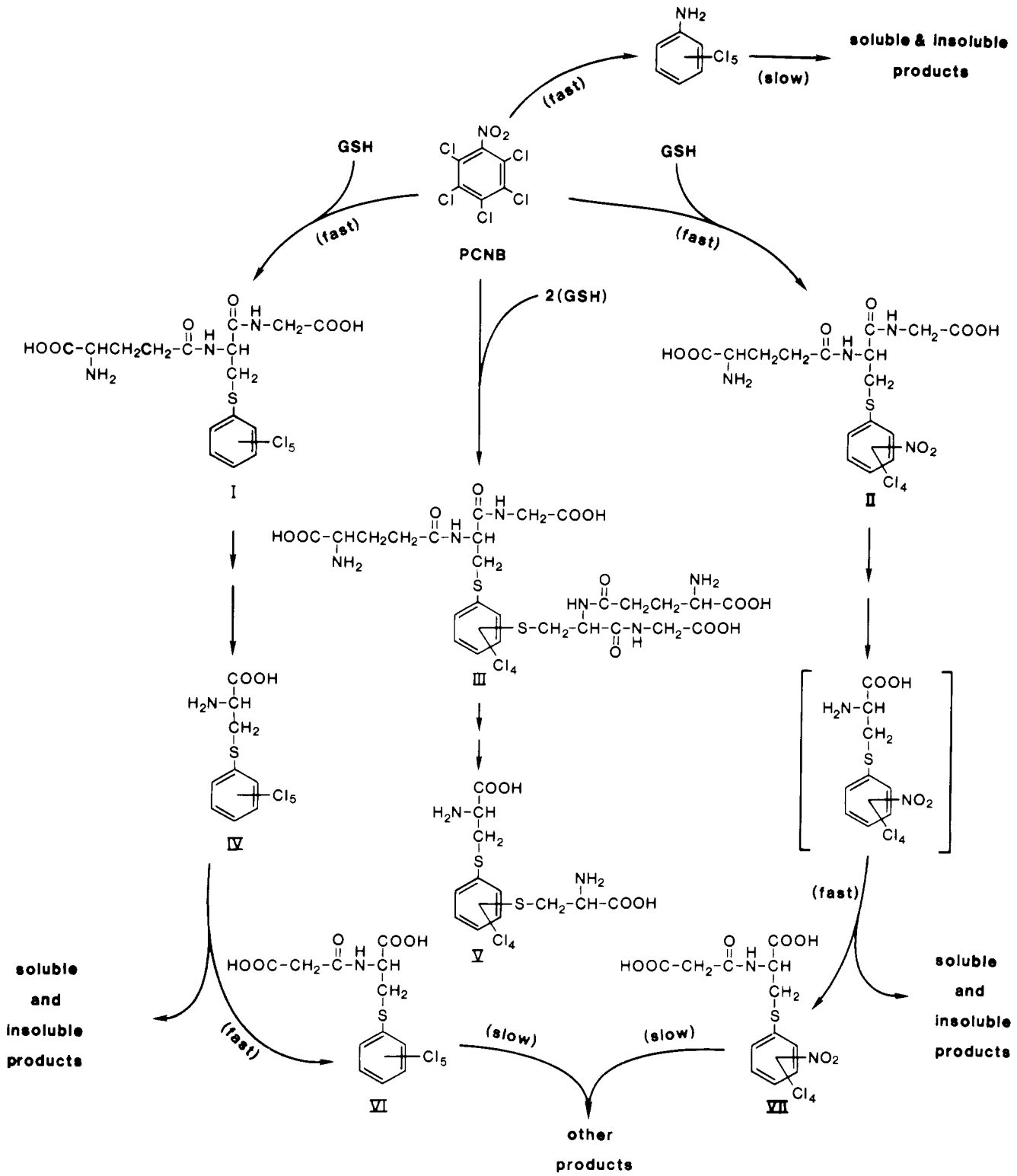

Figure 10. PCNB metabolism in peanut roots. The structure in brackets was not isolated and identified.

absolute alcohol and HCl resulted in only 60 to 70% conversion to esters with the expected TLC properties. This may also have been due to the instability of VI and VII. Rosa and Neish (1968) reported that a similar compound, *N*-malonylphenylalanine, was unstable at pH 2 and underwent spontaneous decarboxylation.

Metabolites VI and VII appeared to be formed by direct malonylation of the corresponding cysteine conjugates. Insoluble residue and *S*-(pentachlorophenyl)-*N*-malonylcysteine were among the primary products formed when intact peanut plants were treated with *S*-[(^{14}C)pentachlorophenyl]cysteine. The formation of *N*-malonyl conjugates of a variety of D-amino acids has been demonstrated in many plant species (Rosa and Neish, 1968; Zenk and Scherf, 1964; Ladesic et al., 1970); however, the identification of metabolites VI and VII appears to be only the second report of a pesticide being converted to an *N*-malonylcysteine conjugate in a plant. Shimabukuro et al. (1976) previously reported *N*-malonyl-*S*-[2-nitro-4-(trifluoromethyl)phenyl]cysteine as a metabolite of fluorodifen in peanut. Malonyl conjugates may be potentially important in pesticide metabolism in plants since malonyl

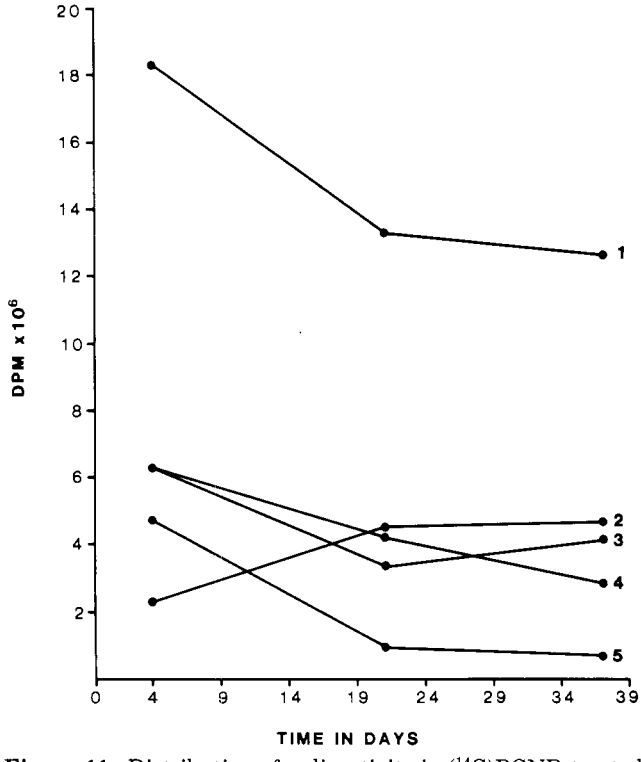

Figure 11. Distribution of radioactivity in (^{14}C)PCNB-treated peanut roots: (1) total recovered from the roots, (2) insoluble in 80% methanol, (3) water soluble, (4) ether soluble, and (5) methylene chloride soluble.

transferase enzymes are widely distributed in the plant kingdom and appear to have broad substrate selectivity.

SUMMARY AND CONCLUSIONS

Structures of metabolites characterized or identified in this study and their probable relationships are shown in Figure 10. Initially the metabolism of PCNB appears to involve three competing reactions: (1) aryl nitro reduction; (2) nucleophilic displacement of a nitro group; and (3) nucleophilic displacement of a chloro group. These three reactions were demonstrated with enzyme preparations isolated from pea or peanut (Lamoureux and Rusness, 1976; Rusness and Lamoureux, 1980). *S*-(*ar*-Tetrachloronitrophenyl)glutathione (metabolite II) was a minor product in vitro with the enzyme from pea. However, in intact peanuts, metabolite II and products derived from it appeared to be nearly as important as products derived from *S*-(pentachlorophenyl)glutathione (metabolite I). Dipeptide conjugates corresponding to metabolite I and II were not isolated and identified in this study, but minor products were detected in fractions containing metabolites I and II. These minor products were present at 6 to 7% of the concentration of I and II and had properties consistent with peptide conjugates. Identification of these minor products was not attempted because of their low concentrations. It was assumed that *S*-(pentachlorophenyl)cysteine (metabolite IV) was formed by the breakdown of the corresponding glutathione conjugate (metabolite I). This is consistent with a previous report in which the glutathione conjugate of atrazine was shown to be systematically metabolized to the corresponding cysteine conjugate (Lamoureux et al., 1973). In addition, metabolite IV was not formed nonenzymatically at an appreciable rate at physiological pH, nor was it formed by direct transfer of cysteine to PCNB under the enzymatic conditions described by Rusness and Still (1977). The formation of *S*-(pentachlorophenyl)-*N*-malonylcysteine (metabolite VI) from *S*-(pentachlorophenyl)cysteine (metabolite IV) was demonstrated by direct in vivo feeding studies. It appears likely that both *N*-malonylcysteine conjugates, metabolites VI and VII, are formed by the transfer of a malonyl moiety to the corresponding cysteine conjugate. Mass spectral and chromatographic evidence indicated that two forms of *S,S'*-(*ar*-tetrachlorophenylene)diglutathione (metabolites IIIa and IIIb) were synthesized both enzymatically and in a base-catalyzed reaction. Products chromatographically identical with IIIa and IIIb were detected in a crude plant extract. Further presumptive evidence for the presence of IIIa and IIIb in vivo was obtained by the isolation of *S,S'*-(*ar*-tetrachlorophenylene)dicysteine (metabolite V) from plant extracts. Metabolite V would be an expected catabolic product of III.

Products insoluble in 80% methanol were clearly among the most important end products of PCNB metabolism in peanut, accounting for 36.8% of the ^{14}C present in roots 33 days after treatment (Figure 11). Because only nucleophilic displacement by glutathione and aryl nitro reduction reactions were detected in the primary phases of metabolism, it was assumed that the insoluble residue must be formed from glutathione-related conjugates or pentachloroaniline. When plants were root treated with (^{14}C)-pentachloroaniline, 32.4% of the ^{14}C isolated from the roots was insoluble in 80% methanol 21 days following treatment. When peanut seedlings were treated in a similar manner with *S*-[(^{14}C)pentachlorophenyl]cysteine, 53.1% of the ^{14}C isolated from the roots was insoluble in 80% methanol. It thus appeared that both pentachloroaniline and products from the glutathione conjugation pathway were involved in the formation of insoluble residue. Several other pesticides metabolized in higher plants by glutathione conjugation are also known to form residues insoluble in 80% methanol: atrazine (Lamoureux et al., 1973), propachlor (Lamoureux et al., 1971), and fluorodifen (Shimabukuro et al., 1973). It appears that insoluble residues are also common end products of pesticides metabolized in plants by an initial reaction involving conjugation with glutathione.

A number of organo-soluble metabolites were isolated and identified from the roots of peanut plants. The nature of these metabolites and their relationship to the metabolism of PCNB via GSH conjugation are described in a separate paper (Rusness and Lamoureux, 1980). Preliminary studies were also conducted on the metabolism of PCNB in excised peanut shoots. Analysis of the products by AG 50W-X2 chromatography system A or by TLC system A suggested that metabolism in the shoots followed the same general pattern as that observed in the roots.

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Pentachloronitrobenzene Metabolism in Peanut. 2. Characterization of Chloroform-Soluble Metabolites Produced in Vivo

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Chloroform-soluble metabolites of pentachloronitrobenzene-*UL*-^{14}C (PCNB) were isolated, identified, and quantitated from roots of peanut plants (*Arachis hypogae* L. cv. Spanish) grown under hydroponic conditions. After 4 days, 59.2% of the radioactivity was chloroform soluble, and the distribution of these metabolites in the peanut root was the following: pentachlorothioanisole, 3.1%; pentachloroaniline, 22.5%; pentachlorothiophenol, 2.6%; pentachlorophenyl methyl sulfoxide, 0.50%; *S*-(pentachlorophenyl)-2-thioacetic acid, 0.48%; *S*-(pentachlorophenyl)-2-hydroxy-3-thiopropanoic acid and *S*-(pentachlorophenyl)-3-thiopropenoic acid, 0.15%; *S*-(pentachlorophenyl)cystiene, 0.04%; unidentified, 1.1%; and parent fungicide (PCNB), 28.7%. All products except *S*-(pentachlorophenyl)cysteine were identified by mass spectral analysis. An aryl nitro reductase enzyme activity that catalyzed the reduction of PCNB to pentachloroaniline was isolated from peanut root. Key chloroform-soluble ^{14}C metabolites of PCNB (pentachloroaniline, pentachlorothiophenol, *S*-(pentachlorophenyl)-2-thioacetic acid, and *S*-(pentachlorophenyl)cysteine) were reintroduced into peanut plants to determine if further metabolism occurred. The proposed pathway for the formation of the chloroform-soluble PCNB metabolites and their significance are discussed.

INTRODUCTION

Preliminary reports on the metabolism of the fungicide pentachloronitrobenzene (PCNB) in peanut (*Arachis hypogaea* L. cv. Spanish) were presented by Lamoureux and Rusness (1976, 1979). A detailed report on the isolation, identification, and significance of water-soluble PCNB metabolites in peanut root also has been presented (Lamoureux and Rusness, 1980). This report is part 2 of a series of reports on PCNB metabolism in which we will describe the isolation, identification, and significance of chloroform-soluble metabolites of PCNB from peanuts.

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MATERIALS AND METHODS

Chromatographic Methods. Thin-layer chromatography (TLC) was performed on silica gel HF_{254} (type 60) (E. Merck, Darmstadt, Germany). The following solvent systems were employed: (A) chloroform–methanol–water (13:7:1), (B) hexane–chloroform–acetic acid (15:4:1), (C) hexane–chloroform (9:1), (D) benzene–acetone (19:1), (E) cyclohexane, (F) hexane–acetic acid (19:1), and (G) benz-