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

Donald G. Rusness and Gerald L. Lamoureux*

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-

ene–95% ethanol (3:1). No paper lining was used in the chromatography tanks containing solvent systems B through G. For isolation of compounds separated by TLC, the silica gel zones were removed from the plates, transferred to a glass wool-plugged Pasteur pipet, and eluted with chloroform or methanol. High-pressure LC was performed with a 0.45 × 183 cm column of C/18 bondapak on 37–50 mesh Corasil (Waters Associates Inc., Milford, MA). The column was eluted at 1.0 mL/min with 60% methanol in water (v/v) (high pressure LC system A). Gas chromatography (GC) was performed on a Varian Model 3700 gas chromatograph equipped with a flame ionization detector (FID) and a 4 mm × 182 cm column of 3% SP 2100 on 80–100 mesh Supelcoport. The GC conditions employed were as follows: injector temperature 220 °C, FID temperature 350 °C, helium carrier gas flow rate 20 mL/min, and a linear temperature program of 130 to 250 °C at 5 °C/min (GC system A) or 170 to 310 °C at 5 °C/min (GC system B). During some GC analyses a Packard Model 894 gas proportional counter (radioactive monitor) or a capillary dry ice trap was used in conjunction with a stream splitter and the FID detector.

Chemicals. Pentachloronitrobenzene-*UL*-^{14}C (PCNB) (1.76 mCi/mmol) was synthesized and purified (99.5%) by Kadunce and Lamoureux (1976), and nonradioactive PCNB (98%) was obtained from K & K Laboratories. Pentachlorothiophenol (PCPSH, tech) and pentachloroaniline (PCA, 98% pure) were products of Aldrich Chemical Co., and pentachlorothiophenol-*UL*-^{14}C (1.78 mCi/mmol) was a gift from Vernon J. Feil. Pentachlorothioanisole was synthesized by methylation (Schlenk and Gellerman, 1960) of pentachlorothiophenol. The methylated product was separated from pentachlorothiophenol and other minor UV-absorbing material by TLC system C. Pentachlorophenyl methyl sulfoxide (PCP methyl sulfoxide) was synthesized from pentachlorothioanisole (0.20 mmol) and sodium metaperiodate (0.21 mmol) by a modification of the method of Johnson and Keiser (1973). The pentachlorothioanisole in 1 mL of chloroform–methanol (1:1) was slowly added to an aqueous metaperiodate solution (5 mL) with vigorous stirring. After 6 h, the PCP methyl sulfoxide was extracted from the reaction mixture with chloroform and purified by TLC systems C and D. Phenyl-^{14}C or nonradioactive *S*-(pentachlorophenyl)cysteine (PCP-*S*-cysteine) was synthesized from phenyl-^{14}C or nonradioactive PCNB and L-cysteine as described by Lamoureux and Rusness (1980). Nonradioactive or phenyl-^{14}C *S*-(pentachlorophenyl)-2-thioacetic acid (PCP-*S*-acetate) was synthesized by reaction of nonradioactive or phenyl-^{14}C pentachlorothiophenol with iodoacetic acid. Ten milliliters of 0.2 N sodium hydroxide was added to a mixture of 1 mmol of pentachlorothiophenol and 1 mmol of iodoacetic acid in 10 mL of methanol. After 15 min at 40 °C, the reaction system was acidified to pH 2 with HCl and partitioned with chloroform. The chloroform-soluble product was purified by TLC system B, and 84% conversion to (phenyl-^{14}C)-PCP *S*-acetate was observed.

Plant Materials, Treatment, and ^{14}C Metabolite Extraction. Peanut plants (*Arachis hypogaea* L. cv. Spanish) were treated with (^{14}C)PCNB (four doses per day for 2 days) as described by Lamoureux and Rusness (1980). Four days after the initial dose the ^{14}C metabolites were extracted from root tissues with 80% methanol as described previously. Duplicate experiments with multiple-dosed (^{14}C)PCNB-treated plants were conducted.

Additional in vivo plant treatments with (^{14}C)PCNB, (^{14}C)PCA, (^{14}C)PCPSH, and (^{14}C)PCP-*S*-acetate were administered as a single dose dissolved in acetone to give a final concentration of 12–35 μM (^{14}C)pentachlorophenyl equivalents and 1% acetone (v/v) in the 50-mL hydroponic treatment solution. The potassium salt of (^{14}C)PCP-*S*-cysteine was administered as a single dose in aqueous solution. The peanut plants were allowed to absorb the labeled compound from 1 to 4 days in a nonaerated system, and uptake of ^{14}C radiolabel was monitored by measuring radioactivity present in the residual treatment solution. For treatment beyond 4 days, the peanut plants were transferred to fresh aerated one-third strength Hoagland's solution (Blankendaal et al., 1972) for an additional 16 or 17 days. The ^{14}C metabolites were extracted from the roots with 80% methanol as above.

Isolation and Analysis of ^{14}C Metabolites. The 80% methanolic extracts from the replicated (^{14}C)PCNB incubation systems were analyzed directly by chromatography in TLC systems C and/or E to estimate quantitatively the volatile ^{14}C metabolites and unchanged (^{14}C)PCNB. Aliquots of the 80% methanolic extracts were streaked onto TLC plates overspotted with authentic nonradioactive standards; no forced air or heat was used during sample application in order to ensure quantitative recovery of applied radiolabel. Upon TLC development, the radioactivity was localized by a radiochromatogram scanner and the appropriate R_f zones were quantitated by liquid scintillation counting. The remainder of the 80% methanolic extracts were concentrated in vacuo to an aqueous phase. The concentrated aqueous samples (pH 5.5) were partitioned with chloroform, acidified with HCl (pH 2), and repartitioned with diethyl ether. Analysis of the water-soluble and acidic-ether-soluble ^{14}C metabolites is described elsewhere (Lamoureux and Rusness, 1980).

The chloroform-soluble ^{14}C metabolites were separated and purified by TLC, high-pressure LC, and GC as summarized in Figure 1. Esterifications, acylations, radioassays, and mass spectrometry (MS) were performed as described previously. The chloroform-soluble extracts from plants receiving multiple doses of (^{14}C)PCNB were concentrated to about 3 mL, streaked onto 5 cm × 20 cm × 500 μm TLC plates at a rate of 400 nmol of PCNB-equivalents per plate, and developed in TLC system A. Three radioactive fractions were separated: A, 85%, B, 5%, and C, 8%. Fraction A was eluted with chloroform and purified further by TLC systems C and D or TLC systems B, E, and F. Metabolites were localized by radiochromatogram scans or autoradiography and analyzed directly by GC/MS. Fractions B and C were eluted with methanol. Fraction B remained as one radioactive component in TLC system G (R_f 0.55), and attempted derivatizations did not change its R_f. Fraction B also could not be analyzed by GC (system B), and so remains unidentified. Fraction C was rechromatographed in TLC system B and, after the solvent had air-dried, the chromatogram was redeveloped in the same direction in TLC system A. Metabolites VI and VII, obtained by these procedures, were esterified (methylation and *n*-butylation) and purified in TLC system B. The esterified derivatives of VII were analyzed in the MS via a solid probe, and the esterified derivatives of VI were analyzed by GC/MS. Metabolite IX was methylated, trifluoroacetylated, and then chromatographed in TLC system B and in GC system B.

The procedures outlined in Figure 1 also were utilized for the analysis of metabolites from plants receiving single-dose treatments of (^{14}C)PCNB, (^{14}C)PCA, (^{14}C)PCP-*S*-cysteine, (^{14}C)PCPSH, and (^{14}C)PCP-*S*-acetate.

Aryl Nitro Reductase Enzyme Preparation, Incubation, and Assay. Roots (5 g) from peanut plants were diced and homogenized (mortar and pestle) at 4 °C in 2

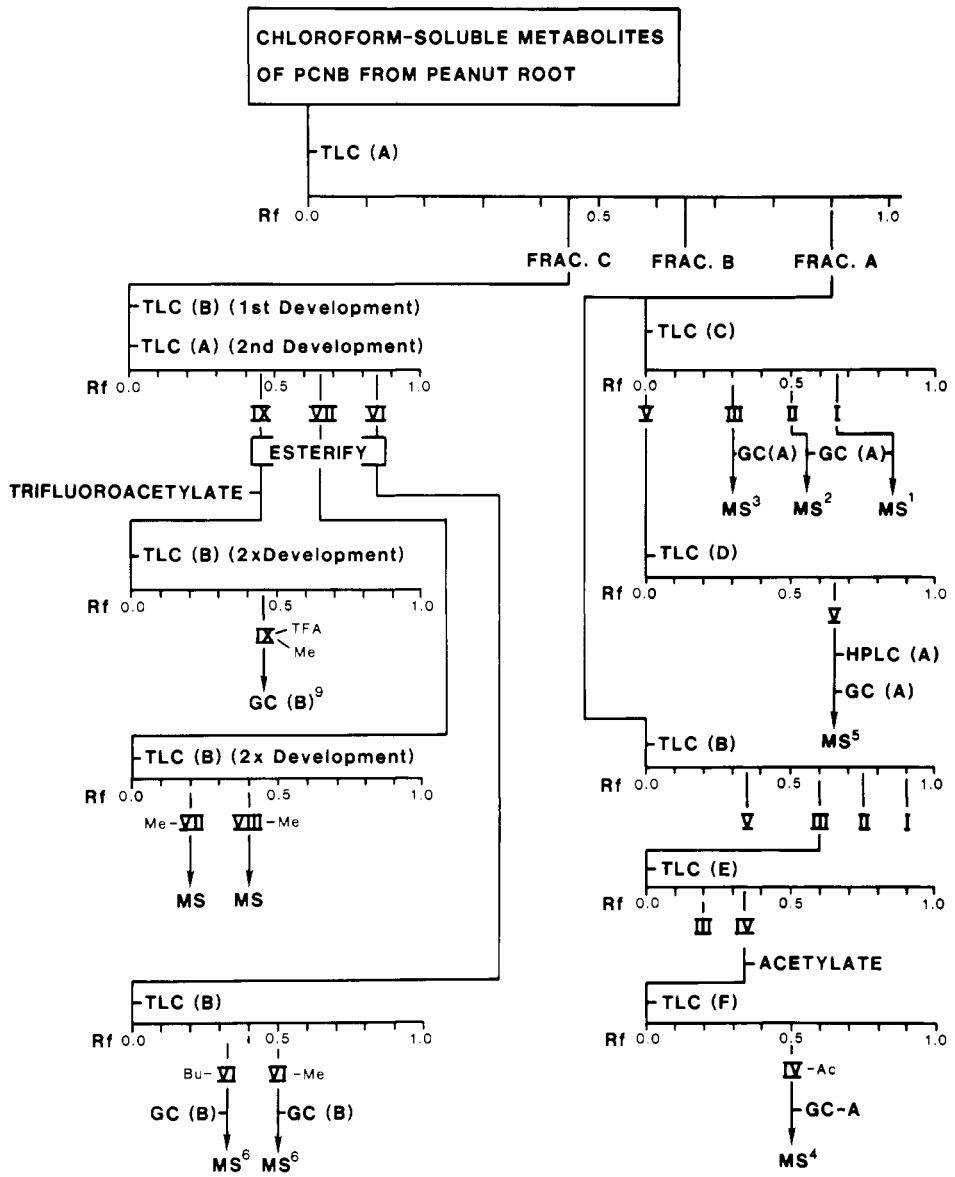

Figure 1. Isolation of chloroform-soluble PCNB metabolites from peanut root. Chromatographic systems are defined in the methods. ^{14}C metabolites I through VI or their derivatives exhibited chromatographic properties and mass spectra identical with those of the comparable standards or their derivatives; (1) standard pentachlorothioanisole, (2) PCNB, (3) PCA, (4) PCPSH or its *S*-acetyl derivative, (5) PCP methyl sulfoxide, and (6) PCP-*S*-acetate or its methyl or *n*-butyl ester. ^{14}C metabolite IX or its derivative exhibited chromatogrpahic properties identical with those of standard PCP-*S*-cysteine (9) or its *N*-acetyl-*O*-methyl ester. Authentic standards were not available for comparisons with metabolites VII–VIII.

volumes (w/v) of 0.3 M sucrose in 0.2 M Tris-HCl (pH 7.5). The brei was squeezed through eight layers of cheesecloth and centrifuged at 10 000*g* for 20 min at 4 °C to remove cell debris and mitochondria. Magnesium chloride (1 M, aqueous) was added to the supernatant at a rate of 50 μmol/mL (Diesperger et al., 1974), and the system was centrifuged at 25 000*g* for 20 min at 4 °C to precipitate microsomal material. Solid ammonium sulfate was added to the supernatant to 80% saturation. After centrifugation at 10 000*g* for 15 min (4 °C) the precipitated protein was redissolved in 0.1 M Tris-HCl (pH 7.5) at a rate of 1 mL/g of fresh weight root tissue. The protein concentration was measured by 280- and 260-nm absorbance as described by Layne (1957). The enzyme (1.3 mg of protein) was incubated at 30 °C for 100 min in a 0.5-mL reaction mixture which contained 35 μmol of Tris-HCl (pH 7.5), 31.5 nmol of (^{14}C)PCNB, 50 nmol of FAD, 200 nmol of NADPH and 15% *tert*-butyl alcohol (v/v). *tert*-Butyl alcohol has been reported to stimulate several enzyme activities (Tan and Lovrien, 1972; Lamoureux and Rusness, 1980). Immediately before initiation of the in vitro enzymatic reaction by addition of the enzyme, the reaction components and the atmosphere above the system were purged with nitrogen. The sealed anaerobic system containing the indicated components was defined as the complete reaction system. The enzymatic reactions were terminated by addition of 1 vol of methylene chloride. The (^{14}C)PCNB substrate and the (^{14}C)PCA product were partitioned into the organic phase which was chromatographed in TLC system C. Standard nonradioactive PCNB and PCA were overspotted and cochromatographed with ^{14}C samples. The separated ^{14}C substrate and ^{14}C product were quantitated by liquid scintillation spectroscopy.

RESULTS AND DISCUSSION

Identification of Compounds I–V. ^{14}C Metabolites I–V (fraction A) were cochromatographed with the respective standards, pentachlorothioanisole, PCNB, PCA, PCPSH, and PCP methyl sulfoxide in TLC systems A thru F and GC system A as described in Figure 1. Metabolite

Table I. Characterization of Chloroform-Soluble Metabolites I, III, and V by GC/MS Analysis[a]

metabolite	authentic standard	GC (A) retention, °C (min)	mass, m/e	relative intensity[b] m/e	m/e + 2	m/e + 4	proposed fragment
I	pentachlorothioanisole	194 (12.8)	294	67	100	72	M^+
			279	14	23	8	$M^+ - CH_3$
			261	20	25	15	$M^+ - SH$
			244	34	45	27	$M^+ - CH_3 - Cl$
			212	3	4	2	$M^+ - SCH_3 - Cl$
			209	5	6	5	$M^+ - CH_3 - 2Cl$
III	pentachloroaniline	184 (10.8)	263	69	100	66	M^+
			235	3	4	2	$M^+ - CNH_2$
			228	10	12	5	$M^+ - Cl$
			201	11	15	7	$M^+ - Cl - CNH$
			193	13	12	3	$M^+ - 2Cl$
			192	22	20	7	$M^+ - 2Cl - H$
			165	10	11	5	$M^+ - 2Cl - CNH_2$
			158	14	5	1	$M^+ - 3Cl$
			130	34	30	3	$M^+ - 3Cl - CNH_2$
			123	19	12	7	$M^+ - 4Cl$
			95	32	16	6	$M^+ - 4Cl - CNH_2$
V	pentachlorophenyl methyl sulfoxide	218 (17.7)	310	20	32	23	M^+
			295	61	100	65	$M^+ - CH_3$
			275	9	11	8	$M^+ - Cl$
			263	13	16	13	$M^+ - SCH_3$
			247	7	11	8	$M^+ - SOCH_3$
			244	8	13	8	$M^+ - Cl - OCH_3$
			232	14	22	10	$M^+ - CCl - OCH_3$
			225	4	4	3	$M^+ - 2Cl - CH_3$
			212	14	22	10	$M^+ - Cl - SOCH_3$
			177	13	13	5	$M^+ - 2Cl - SOCH_3$
			142	22	19	2	$M^+ - 3Cl - SOCH_3$

[a] GC system A is defined in the text. Metabolite II and PCNB cochromatographed at 177 °C (9.4 min) in this system. [b] The number of chlorines was determined from isotope ratios (McLafferty, 1967).

I (pentachlorothioanisole), metabolite III (PCA), and metabolite V (PCP methyl sulfoxide) were identified by direct comparison of gas chromatographic and mass spectral properties (Table I). Compound II was identified as PCNB by gas chromatography and mass spectral analysis. The mass spectrum of II was identical with that reported by Begum et al. (1979). Acetylated derivatives of metabolite IV and standard pentachlorothiophenol cochromatographed in TLC system F and GC system A (190 °C, 12.0 min). The mass spectrum of the *S*-acetylpentachlorothiophenol derivative was characterized by a five-chlorine-containing parent ion cluster (M^+) at *m/e* 322 (7.8%) and a four-chlorine-containing cluster at *m/e* 287 (65.3%). Intense chlorine ion clusters at *m/e* 279 (24.4%) and 244 (100%) apparently were formed by the loss of the acetyl moiety from the ions at *m/e* 322 and 287. The ion clusters with three chlorines at *m/e* 209 (33.1) and the ion clusters with two chlorines at *m/e* 174 (37.6) were apparently formed by the successive loss of chlorine from the ion at *m/e* 244.

Identification of Metabolites VI–IX. ^{14}C metabolites VI–IX (fraction C) were separated by TLC, derivatized, and identified by MS as summarized in Figure 1. After the first chromatographic development (TLC-B), radiolabel was detected at or near the origin and at R_f 0.30. Standard PCP-*S*-cysteine and PCP-*S*-acetate migrated to R_f 0.0 and 0.30, respectively, in TLC system B. The metabolites were separated further after redevelopment in TLC system A (Figure 1).

Metabolite VI and standard PCP-*S*-acetate and their respective methyl or butyl esters cochromatographed throughout the purification procedures. Derivatization of metabolite VI or standard PCP-*S*-acetate resulted in 81–84% conversion to the esters, and the remaining 16–19% of the radiolabel remained unchanged. The methyl and butyl ester of both metabolite VI and the standard exhibited retention at 224 (10.7 min) and 248 °C (15.5 min), respectively, in GC system B. Mass spectra of both the methyl and butyl esters of metabolite VI and standard PCP-*S*-acetate were identical. The complete mass spectrum of the metabolite VI methyl ester derivative is shown in Figure 2. A molecular ion cluster at *m/e* 352 (19%) and the ion cluster at *m/e* 293 (40%) due to the loss of $COOCH_3$ were both consistent with the presence of five chlorines. Ion clusters at 317 (65%) and 258 (25%) were formed by the loss of one chlorine from the ions at 352 and 293. Mass spectra of the butyl ester of VI and the standard were consistent with this interpretation. Therefore, metabolite VI was *S*-(pentachlorophenyl)-2-thioacetic acid (PCP-*S*-acetate).

A negative quinoxaline test (Neish, 1957) indicated that metabolite VII was not *S*-(pentachlorophenyl)-2-oxo-3-thiopropanoic acid (PCP-*S*-pyruvate). Two major derivatives were formed in nearly equal quantities in an overall yield of 60% when metabolite VII was esterified. These products, designated VII-Me and VIII-Me (Figure 1), were subjected to mass spectral analysis. The mass spectrum of VII-Me (Figure 3) was consistent with that of the methyl ester of *S*-(pentachlorophenyl)-2-hydroxy-3-thiopropanoic acid (PCP-*S*-lactate). Key ion fragments at *m/e* 382 (M^+), 323, and 288 were indicative of a substituted lactic acid molecule. The strong five chlorine ion clusters at 364 showed a loss of water from the parent ion at 382. Other four or five chlorine ion clusters at *m/e* 333, 329, 293, 279, 258, and 244 were characteristic of the fragmentation pattern exhibited by various PCP-*S*-substituted metabolites (Lamoureux and Rusness, 1980). Since no authentic standard was available for comparison with VII, 3-phenyl-2-hydroxypropanoic acid (phenyllactic acid) was utilized for comparative studies. The fragmentation pattern of the methyl ester of phenyllactic acid was consistent with the spectrum of the methyl ester of VII (Table II). Therefore, metabolite VII was *S*-(pentachlorophenyl)-2-hydroxy-3-thiopropanoic acid (PCP-*S*-lactate).

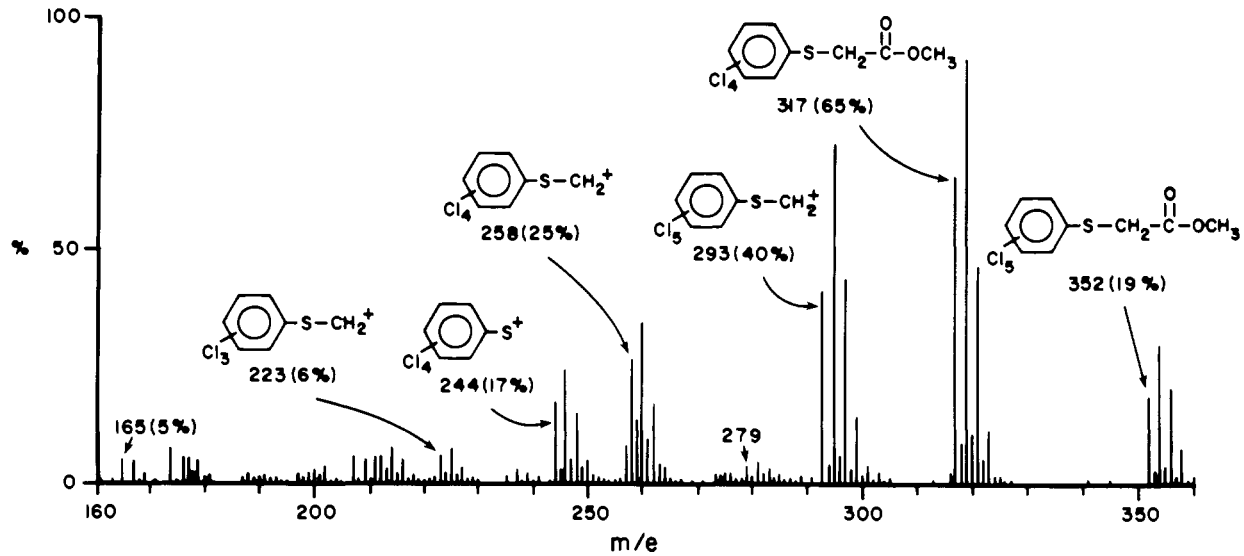

Figure 2. GC/mass spectrum of the methyl ester of metabolite VI.

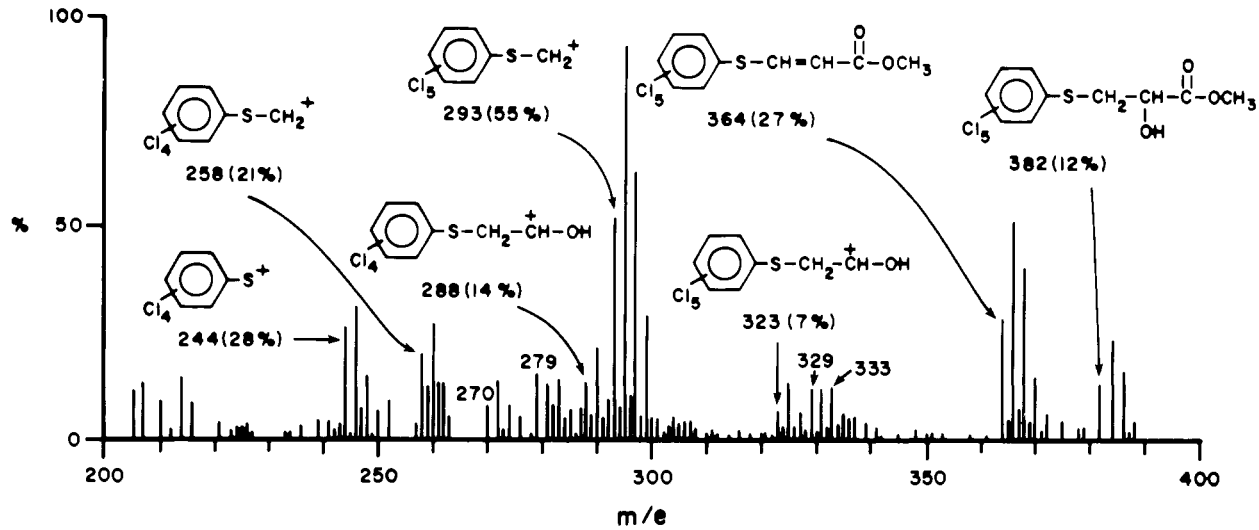

Figure 3. Mass spectrum of the methyl ester of metabolite VII. The spectrum was corrected for background.

The mass spectrum of VIII-Me gave a fragmentation pattern similar to that of VII-Me (Figure 3), except no chlorine ion clusters were observed at m/e 382 and 288. A five chlorine ion cluster at m/e 364 (M^+, 100%) plus other chlorine ion clusters consistent with the fragmentation of VII-Me were observed. The mass spectrum suggested that metabolite VIII was *S*-(pentachlorophenyl)-3-thiopropenoic acid (PCP-*S*-propenoate). It appears that either metabolite VIII resulted from dehydration of metabolite VII during derivatization or metabolite VIII was present in an unresolved mixture with metabolite VII prior to derivatization.

Metabolite IX and PCP-*S*-cysteine or the respective *N*-(trifluoroacetyl) *O*-methyl esters cochromatographed in TLC systems A and B and GC system B as described in Figure 1. However, GC/MS was obtained only for the standard (Lamoureux and Rusness, 1980). Metabolite IX appeared to be *S*-(pentachlorophenyl)cysteine (PCP-*S*-cysteine). A small amount of PCP-*S*-cysteine was observed both in the water-soluble and chloroform-soluble root extracts.

Quantitation of Metabolites I–IX. Significant loss of radioactivity (up to 60%) was observed upon in vacuo concentration of the 80% methanolic extracts and the subsequent chloroform-soluble phase from PCNB-treated peanut roots. Therefore, direct TLC analyses of the 80% methanolic extracts was necessary. This consistently resulted in a 95–100% recovery of applied ^{14}C radiolabel. Quantitative comparisons between the direct TLC assay of the 80% methanolic extract and the isolation method outlined in Figure 1 indicated that pentachlorothioanisole (I), PCNB (II), and PCA (III) were volatile ^{14}C components. Autoradiographic analyses of thin-layer chromatograms before and after in vacuo concentration of the 80% methanolic extract suggested that no other volatile components were present. Therefore, chloroform-soluble metabolites I–IX were quantitated by direct TLC analysis, and the distribution of these metabolites is reported in Table III.

Table II. Comparison of Key Ions Produced in the Mass Spectra of the Methyl Ester of Phenyllactic Acid and the Methyl Ester of Metabolite VII

	m/e and intensity values (%) of ion fragments	
nature of ion fragment	phenyl lactate methyl ester	metabolite VII methyl ester
M^+	180 (2)	382 (12)
$M^+ - H_2O$	162 (77)	364 (27)
$M^+ - (H_2O + OCH_3)$	131 (18)	333 (10)
$M^+ - (COOCH_3)$	121 (28)	323 (7)
$M^+ - (CHOHCOOCH_3)$	91 (100)	293 (55)

Table III. Quantitation of Chloroform-Soluble Metabolites of PCNB from Peanut Roots

product	identification	% distribution in peanut root[a]
I	pentachlorothioanisole	3.1
II	pentachloronitrobenzene	28.7
III	pentachloroaniline	22.5
IV	pentachlorothiophenol	2.6
V	pentachlorophenyl methyl sulfoxide	0.50
VI	*S*-(pentachlorophenyl)-2-thioacetic acid	0.48
VII	*S*-(pentachlorophenyl)-2-hydroxy-3-thiopropanoic acid	0.15[b]
VIII	*S*-(pentachlorophenyl)-3-thiopropenoic acid	
IX	*S*-(pentachlorophenyl)cysteine	0.04[c]
	fraction B	0.43[d]
	other compounds	0.70
	total chloroform soluble	59.20

[a] The data are averages of two separate experiments. [b] Since compound VIII may be an artifact from derivatization, the quantities of VII and VIII were combined. [c] Radioactivity from the pH 2 water-soluble phase cochromatographed with *S*-(pentachlorophenyl)cysteine. [d] Fraction B (Figure 1) remained as one ^{14}C component in two TLC systems (A and G) but was not identified.

Table IV. Aryl Nitro Reductase Activity from Peanut Root: Reduction of (^{14}C)PCNB to (^{14}C)PCA

reaction system[a]	nmol of (^{14}C)PCA/100 min	% conversion/100 min
complete (PCNB, NADPH, FAD, enzyme, N_2)	11.43	36.2
complete (at 0 min or aerobic)	0.12	0.4
minus enzyme	0.23	0.7
minus FAD	0.34	1.1
minus FAD and NADPH	0.12	0.4

[a] The concentrations used in the complete reaction system are defined in the methods.

In Vitro Enzyme Studies: Aryl Nitro Reductase. An aryl nitro reductase enzyme system capable of converting (^{14}C)PCNB to (^{14}C)PCA was isolated from peanut roots. The reaction was dependent upon the presence of the enzyme, FAD, NADPH, and anaerobic conditions (Table IV). Under the conditions described, the reaction approached equilibrium (36% conversion to PCA) after 100-min incubation. Only 42% conversion to PCA was observed under similar conditions when the reaction incubation was extended to 16 h. (^{14}C)PCNB and (^{14}C)PCA were the only radioactive components detected in the enzyme reaction by TLC system C. Enzyme activity was stable at −20 °C for at least 5 months. The addition of 1 mM dithiothreitol to the reaction system neither stimulated nor inhibited the formation of PCA, and dithiothreitol could not be used to replace NADPH and FAD. The data from Table IV show that peanut roots possess the enzymatic capability to synthesize PCA from PCNB. It is noteworthy that PCA accounted for 22% of the ^{14}C in intact peanut roots treated with (^{14}C)PCNB for 4 days.

Additional in Vivo Studies. Peanut plants were treated with (^{14}C)PCNB and key ^{14}C-labeled chloroform-soluble metabolites. The 80% methanol extracts and nonextractable residues from these plants were analyzed for metabolites (Table V). Significant amounts of nonextractable radioactive products were isolated from the plants treated with PCNB, PCA, PCPSH, or PCP-*S*-cysteine. The amount of chloroform-soluble metabolites isolated from the 3-week PCNB treatment (5.4%, Table V) was significantly less than from the 4-day PCNB treatment (59.2%, Table III). PCP methyl sulfoxide was the only chloroform-soluble metabolite that did not appear to decrease between 4 and 21 days. Treatment with (^{14}C)pentachloroaniline (Table V) did not result in the formation of any PCNB or other new chloroform-soluble metabolites; however, significant amounts of polar conjugates (water-soluble and pH 2 ether-soluble ^{14}C metabolites) were observed. At least four polar metabolites of (^{14}C)PCA exhibited TLC and high-pressure LC patterns that were similar to those exhibited by the glutathione- and *N*-malonylcysteine conjugates of PCNB (Lamoureux and Rusness, 1980). The esterified, acetylated derivatives of the (^{14}C)PCA metabolites also displayed TLC properties similar to those of the glutathione- and *N*-malonylcysteine conjugates. Treatment of plants with (^{14}C)PCP-*S*-cysteine resulted in no detectable pentachlorothioanisole or PCP methyl sulfoxide in the roots; however, PCP-*S*-acetate and pH 2 ether-soluble metabolites with high-pressure LC properties similar to those of *N*-malonylcysteine-related conjugates were characterized. Product/precursor studies were also undertaken using PCP-*S*-acetate and PCPSH as substrates (Table V). Preliminary studies showed that both compunds were rapidly metabolized; thus shorter treatment periods were used with these compounds. After incubation for 48 h, PCP-*S*-acetate was converted to several polar metabolites which did not behave like glutathione-related conjugates. One of these polar metabolites was subjected to an esterification reaction with methanol/hydrogen chloride. The product yielded a mass spectrum identical with that of the methyl ester of PCP-*S*-acetate. This polar metabolite was apparently a labile conjugate of PCP-*S*-acetate similar to those reported for

Table V. Results of Treatment of Intact Peanut Plants with Pentachlorophenyl-Related ^{14}C Metabolites of PCNB

substrate (treatment concentration, time)	percent distribution of metabolites isolated from root systems: penta-chloro-aniline	penta-chloro-thioanisole	PCP methyl sulfoxide	PCP-S-acetate	polar conjugates	non-extractable
PCNB (35 μM, 21 d)	2.5	0.6	0.9	0.1	60.6	34.0
pentachloroaniline (35 μM, 20 d)	41.9	ND[a]	ND	ND	27.7	30.4
PCP-*S*-cysteine[b] (12 μM, 20 d)	ND	ND	ND	7.3	42.8	31.8
PCP-*S*-acetate (23 μM, 2 d)	ND	ND	ND	51.8	43.5	4.0
pentachlorothiophenol[c] (22 μM, 1 d)	ND	3.9	5.2	ND	22.6	47.3

[a] ND = not detected. [b] The remaining 18.1% cochromatographed (R_f 0.45, TLC systems B plus A) with PCP-*S*-cysteine. [c] The remaining 21.0% cochromatographed (R_f 0.75, TLC system C) with the oxidized form of pentachlorothiophenol.

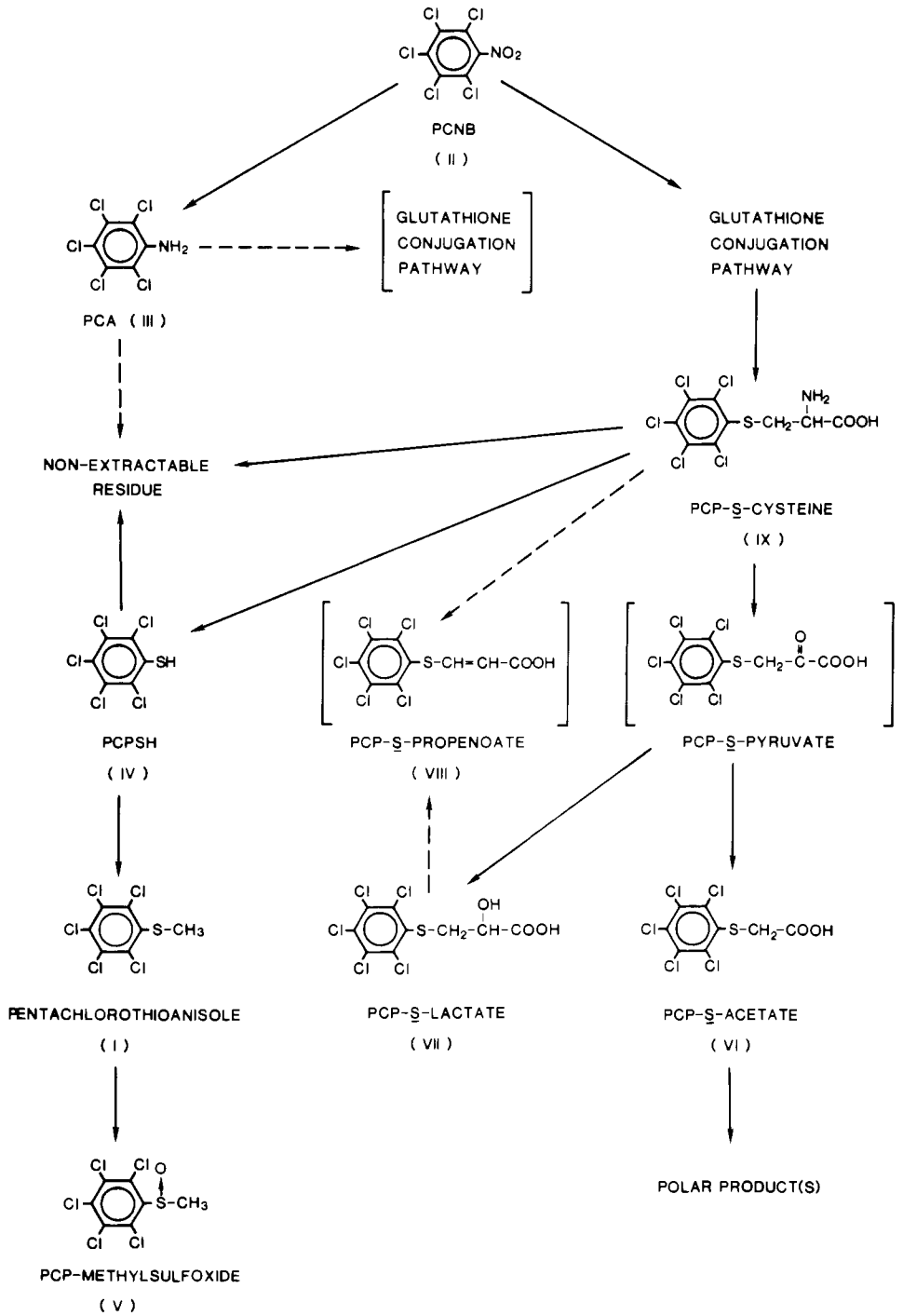

Figure 4. Proposed pathway for the biosynthesis of chloroform-soluble PCNB metabolites in peanut root. Water-soluble metabolites of the glutathione conjugation pathway are discussed in detail by Lamoureux and Rusness (1980). Dashed lines represent speculative pathways.

2,4-dichlorophenoxyacetic acid (Feung et al., 1978) and 3-amino-2,5-dichlorobenzoic acid (Frear et al., 1978). No (^{14}C)pentachlorothioanisole or (^{14}C)PCP methyl sulfoxide was detected in the roots of plants treated with PCP-*S*-acetate. On the other hand, (^{14}C)pentachlorothioanisole and PCP methyl sulfoxide were identified by TLC, GC, and MS and were quantitated from roots exposed to (^{14}C)PCPSH.

CONCLUSIONS

The chloroform-soluble metabolites of PCNB identified in this study and the proposed metabolic pathways are summarized in Figure 4. Pentachloroaniline (III) was a major chloroform-soluble metabolite in vivo, and an enzyme system that formed PCA from PCNB in the presence of NADPH and FAD was isolated from peanut root. Data summarized in Figure 4 suggested that PCP-*S*-cysteine (IX) is a key intermediate for additional catabolic and anabolic reactions. The isolation and identification of pentachlorothiophenol (IV), pentachlorothioanisole (I), and PCP *S*-methyl sulfoxide (V) suggested that these products may be formed by catabolism of the PCP-*S*-cysteine intermediate. This hypothesis was substantiated by parallel in vitro enzyme studies described by Lamoureux and Rusness (1979). The isolation and identification of PCP-*S*-lactate (VII) and PCP-*S*-acetate (VI) from peanut root suggested that yet another pathway was present for the catabolism of PCP-*S*-cysteine. The presence of PCP-S-

lactate (VII) suggested that the oxidized form of this metabolite (PCP-*S*-pyruvate) may have existed as a transient intermediate formed from PCP-*S*-cysteine via a transaminase-related reaction. A further reaction of PCP-*S*-pyruvate with a decarboxylase-related enzymatic activity would explain the formation of PCP-*S*-acetate (VI) as a metabolite of PCNB.

An S-substituted 2-thioacetic acid intermediate in the metabolism of *S*-ethyl *N*,*N*-dipropylthiocarbamate (EPTC) in rat has been identified by Hubbell and Casida (1977). The isolation and identification of PCP-*S*-acetate and PCP-*S*-lactate from peanut root extracts reported herein confirmed and extended this observation. An additional chloroform-soluble compound (VIII) was identified as the methyl ester after derivatization of VII. This ^{14}C compound may have been formed as an artifact during the derivatization of the PCP-*S*-lactate (VII); however, its existence as a metabolite of PCNB cannot be excluded. It is noteworthy that the reactions suggested for the conversion of metabolite IX to VI and VII (Figure 4) are characteristic of metabolic pathways utilized for the catabolism of phenylalanine (Mahler and Cordes, 1966; Neish, 1965). Since phenylalanine also undergoes deamination to cinnamic acid in a reaction catalyzed by a phenylalanine ammonia-lyase enzyme activity in potato tuber (Havir and Hanson, 1968a,b) and maize (Marsh et al., 1968), it is possible that PCP-*S*-cysteine also might undergo a similar reaction to form PCP-*S*-propenoate (VIII) in peanut root.

Significant nonextractable residues were also observed in peanut root after (^{14}C)PCNB, (^{14}C)PCP-*S*-cysteine, and (^{14}C)pentachlorothiophenol were administered to peanut plants. This suggested that products from glutathione conjugation may be converted to insoluble residue. (^{14}C)PCA metabolism also gave rise to nonextractable residue. This insoluble residue may have been formed by direct incorporation of PCA into the lignin fraction in a manner similar to incorporation of the chloroaniline-related compounds reported by Balba et al. (1979). Alternatively, it may have been incorporated into insoluble residue through the glutathione conjugation pathway since the water- and ether-soluble metabolites of PCA had properties suggestive of glutathione and *N*-malonylcysteine conjugates.

All of the chloroform-soluble ^{14}C metabolites isolated and identified in the roots from (^{14}C)PCNB-treated peanuts contained the pentachlorophenyl moiety. Although tetrachloronitrophenyl moieties and *S*,*S*′-tetrachlorophenylene moieties were observed in the water and ether extracts from PCNB-treated peanut roots (Lamoureux and Rusness, 1980), related ^{14}C metabolites were not isolated from the chloroform-soluble phase. Various tetrachlorophenyl-containing metabolites of PCNB have been isolated from anion (Begum et al., 1979) and from Rhesus monkey (Kogel et al., 1979).

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