Pentachloronitrobenzene Metabolism in Peanut. 3. Metabolism in Peanut Cell Suspension Cultures

Gerald L. Lamoureux,* Jean-Marie Gouot,[1] David G. Davis, and Donald G. Rusness

The metabolism of pentachloronitrobenzene-*UL*-^{14}C (PCNB) was studied in peanut (*Arachis hypogaea* L.) cell suspension cultures over a 14-day period. The primary metabolic pathways involved an initial conjugation with glutathione. Seven major metabolites were detected by high-performance liquid chromatography, and five of these were identified by mass spectrometry of suitable derivatives: *S*-(pentachlorophenyl)glutathione, *S*-(*ar*-tetrachloronitrophenyl)glutathione, *S,S'*-(*ar*-tetrachlorophenylene)dicysteine, *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine, and *S*-(pentachlorophenyl)-*N*-malonylcysteine. Several precursor–product relationships were demonstrated. Nonextractable residue, *S*-(pentachlorophenyl)-*N*-malonylcysteine, *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine, and metabolite III appeared to be terminal metabolic products. PCNB metabolism in peanut cell suspensions cultures was compared to PCNB metabolism in the roots of intact peanut plants. The primary differences between the two systems appeared to be quantitative. Pentachloroaniline and nonextractable residue were produced in larger amounts in intact peanut plants than in the cell suspension cultures. Several advantages and disadvantages of conducting metabolism studies in cell suspension cultures were discussed.

Pentachloronitrobenzene (PCNB) is marketed worldwide as a soil- or seed-treatment fungicide. In the United States, the primary use of PCNB is to control *Rhizoctonia* in cotton (*Gossypium hirsutum* L.) and *Sclerotium* in peanut (*Arachis hypogaea* L.). The metabolism of PCNB has been studied in detail in peanut plants (Lamoureux and Rusness, 1980a; Rusness and Lamoureux, 1980), in onion (*Allium cepa* L.) plants (Begum et al., 1979), and with an in vitro enzyme system from onion roots (Lamoureux and Rusness, 1980b). Two competing initial metabolic reactions, aryl nitro reduction and glutathione (GSH) conjugation, were evident in peanut and onion. Pentachloroaniline, the major product of aryl nitro reduction, was converted only slowly to other products in peanut. Three GSH conjugates were observed in peanut: *S*-(pentachlorophenyl)glutathione, *S*-(*ar*-tetrachloronitrophenyl)glutathione, and *S,S'*-(*ar*-tetrachlorophenylene)-diglutathione. These GSH conjugates were thought to be precursors of other metabolites identified from peanut and onion.

The purpose of this study was (1) to determine the metabolic fate of PCNB in peanut cell suspension cultures, (2) to determine the sequence of metabolic reactions, and (3) to determine which metabolites were stable in peanut cell cultures. A preliminary report of this study was presented as part of a review of the catabolism of glutathione conjugates of pesticides in higher plants (Lamoureux and Rusness, 1981).

MATERIALS AND METHODS

Chemicals. The origin and purity of the following compounds were described previously: Pentachloronitrobenzene-*UL*-^{14}C (specific activity 1.76 mCi/mmol), pentachloronitrobenzene, pentachloroaniline, pentachlorothiophenol, pentachlorothioanisole, pentachlorothioanisole sulfoxide, and *S*-(pentachlorophenyl)-2-thioacetic acid (Lamoureux and Rusness, 1980a; Rusness and Lamoureux, 1980). *S*-(Pentachlorophenyl-*UL*-^{14}C)glutathione, *S*-(*ar*-tetrachloronitrophenyl-*UL*-^{14}C)glutathione, and *S,S'*-(*ar*-tetrachlorophenylene-*UL*-^{14}C)diglutathione were synthesized enzymatically (specific activity 1.76 mCi/mmol, each compound); *S*-(pentachlorophenyl-*UL*-^{14}C)cysteine and *S,S'*-(*ar*-tetrachlorophenylene-*UL*-^{14}C)dicysteine (specific activity 0.0245 mCi/mmol, each compound) were synthesized by a base-catalyzed reaction; *S*-(pentachlorophenyl-*UL*-^{14}C)-*N*-malonylcysteine and *S*-(*ar*-tetrachloronitrophenyl-*UL*-^{14}C)-*N*-malonylcysteine (specific activity 0.477 mCi/mmol, each compound) were synthesized in vivo in peanut plants (Lamoureux and Rusness, 1980a). *S*-(Pentachlorophenyl-*UL*-^{14}C)-γ-glutamylcysteine (specific activity 0.90 mCi/mmol) was synthesized in vivo in onion plants (Lamoureux and Rusness, 1980b).

Chromatographic Methods. Thin-layer chromatography (TLC) was performed as described previously (Lamoureux and Rusness, 1980a). The following solvents were used: (A) hexane–chloroform–glacial acetic acid (15:4:1), (B) chloroform–methanol–water (13:7:1), (C) hexane–chloroform (9:1), (D) 1-butanol–glacial acetic acid–water (12:3:5), (E) chloroform–absolute ethanol (20:1), and (F) benzene–ethyl acetate (9:1).

High-performance liquid chromatography (HPLC) was performed with two Model M-600 pumps, a Model 660 solvent programmer, a U6K injector, and either a 3.9 mm × 30 cm μBondapak C18 column or a 7.8 mm × 30 cm μBondapak C18 column (Waters Associates, Inc.). Nonradioactive peaks were detected by UV absorption at 254 nm with a Model 210 UV detector (Chromatronix). Analyses with the 3.9 mm i.d. column (HPLC system A) were performed at a flow rate of 1.5 mL/min with a 72-min concave gradient (curve F) of acetonitrile–glacial acetic acid–water (18:1:81 to 32:1:67). After completion of the gradient, the column was eluted isocratically with acetonitrile–glacial acetic acid–water (32:1:67) for 40 min before the column was washed with acetonitrile–glacial acetic acid (99:1). From 15000 to 25000 dpm of cell extract was injected for each analysis, and 0.75-mL (0.50-min) fractions were collected. A profile of the ^{14}C eluted from the column was obtained by quantifying each fraction by liquid scintillation counting. Preliminary experiments showed that a counting efficiency of 80% could be assumed. Recovery of ^{14}C from the chromatograms was from 74 to 107%. Preparative HPLC was performed with the 7.8 mm i.d. column (HPLC system B). The column was eluted at 5.3

U.S. Department of Agriculture, Science and Education Administration, Metabolism and Radiation Research Laboratory, Fargo, North Dakota 58105.

[1]Visiting scientist from Rhone-Poulenc, Centre Nicolas Grillet, 13, quai Jules-Guesde, F94400 Vitry-sur Seine, France.

mL/min with acetonitrile–glacial acetic acid–water (20:1:79) for 30 min, with acetonitrile–glacial acetic acid–water (30:1:69) for 60 min, and then with acetonitrile–glacial acetic acid (99:1). Preparative chromatograms were monitored qualitatively for ^{14}C with a Packard Model 317 radioactive flow monitor equipped with a 1-mL anthracene-filled flow cell or with a Berthold Model LB 503 radioactive flow monitor equipped with a 400-μL glass scintillator-filled flow cell. Three-milliliter fractions were collected.

Radioassays. Radioactivity from treating solutions, plant extracts, nonextractable plant residues, thin-layer plates, etc. was measured or monitored by methods previously described (Lamoureux and Rusness, 1980a).

Development of the Peanut Cell Suspension Culture. Peanut (*Arachis hypogaea* L. var. Spanish) seeds were surface sterilizied in 2.1% sodium hypochlorite for ~3 min and germinated aseptically on B5 agar medium (Gamborg and Wetter, 1975). Stem sectons from the resulting seedlings were transferred to a B5 agar medium that contained 0.1 mg/L 2,4-D. Callus cells that developed from the stem sections were transfered to 50 mL of liquid B5 medium that contained 0.1 mg/L 2,4-D. These cells were grown in 250-mL Erlenmeyer flasks in the dark at 25 °C on rotary shakers (100 cycles/min, 2.54 cm/stroke). The cell suspension cultures obtained in this manner were maintained by weekly transfer to fresh media of the same composition. Various studies were then conducted in 250-mL Erlenmeyer flasks that contained 50 mL of fresh medium and 10 mL of a stock suspension of cells (inoculum). The fresh cell suspensions were incubated for 4 days before test materials were added.

Toxicity Studies. Toxicity studies were conducted under the conditions described above. The flasks were inoculated with 21 ± 1 mg of cells (dry weight). Pentachloronitrobenzene in acetone (35 μg/200 μL) was added to the cultures at 2-h intervals to yield 0, 35, 140, and 420 μg of PCNB/flask. When the desired concentration of PCNB was reached, acetone was added at the succeeding time intervals until each flask had been treated with 0.8 mL of acetone. An acetone-free control culture was included. Three replicates were run for each control and each concentration of PCNB. Cell growth was estimated by a dry weight method. The cells were filtered on preweighed, oven-dried miracloth, dried at 60 °C for 3 days, and weighed. Averages and standard deviations were calculated from the three replicates of each concentration.

Assay for Microbiological Contamination. Bacterial and fungal contamination of cell cultures from time study and metabolite preparation experiments was checked by inoculating Petri plates of cooked meat medium (Difco) with the flask contents at harvest time. The Petri plates were examined for bacterial and fungal colonies after a 7-day incubation period at 28 ± 1 °C.

Treatment of Cell Cultures for the Time Study and Metabolite Identification. The time study was conducted in duplicate in 250-mL Erlenmeyer flasks that contained 50 mL of liquid B5 medium (0.1 mg/L 2,4-D). The flasks were inoculated with 32 ± 3 mg of cells (dry weight). After 4 days of incubation, 35 μg of [^{14}C]PCNB in 50 μL of acetone was added to each flask to be harvested 3, 9, and 24 h later. Four additions of 35 μg of [^{14}C]PCNB in 50 μL of acetone were made at 2–3-h intervals to flasks that were to be harvested 3, 7, and 14 days later. Cultures to be harvested after 3, 7, and 14 days were treated with a higher dose of [^{14}C]PCNB to facilitate metabolite detection in the presence of larger quantities of cells. The [^{14}C]PCNB was added at 2–3-h intervals when the higher total dose was used so the solubility of [^{14}C]PCNB would not be exceeded. It was assumed that interval dosing would not effect significantly the results of the 3-, 7-, and 14-day harvests.

For metabolite isolation and identification, six flasks of cells were treated with four 70-μg doses of [^{14}C]PCNB (each dissolved in 50 μL of acetone) at 1–2-h intervals and harvested after 5 days of normal incubation. A second set of four flasks were each treated with one 70-μg dose of [^{14}C]PCNB in 50 μL of acetone. Two of these flasks were incubated as described previously and two were incubated without shaking to reduce the oxygen tension in the media. The cultures were harvested after 20 h. In addition, one peanut cell suspension culture was treated with a single dose of *S*-(pentachlorophenyl-*UL*-^{14}C)cysteine (66 μg), incubated for 24 h, and harvested as described for the time study.

Metabolite Extraction. Cell cultures from the time study and 5-day metabolite identification study were handled in the same manner. The cells were filtered on miracloth under low vacuum, washed with water, and homogenized 3 times for 3 min in cold methanol–water (80:20) with a Sorvall omnimixer cooled in an ice–water bath. The homogenates were centrifuged at 1600*g*, and the clarified extracts were decanted, combined, and assayed for radioactivity. The extracts were concentrated on vacuum evaporators at 30 °C, diluted with water, and partitioned 3 times with equal volumes of methylene chloride. The aqueous fractions were evaporated to dryness, dissolved in acetonitrile–glacial acetic acid–water (18:1:81), and filtered through 5-μm Teflon membranes. The aqueous filtrates from the time study were analyzed with HPLC system A. Aqueous filtrates used for metabolite identification were purified further as described later. The methylene chloride phases from each sample were combined, quantified, concentrated under vacuum at ambient temperature, redissolved in a small volume of methylene chloride, and analyzed by TLC in systems A and C. The cell residues that remained after extraction were dried at ambient temperature and assayed for radioactivity. The culture media and cell washes from each harvest were combined, extracted with methylene chloride, and quantified by liquid scintillation spectrometry. The methylene chloride soluble extracts were concentrated and examined with TLC systems A and C.

Cells harvested after 20 h for metabolite identification were extracted with cold 70% acetone instead of 80% methanol. The acetone extracts were diluted 1:1 with water and partitioned directly with methylene chloride. The methylene chloride phases were concentrated to dryness at 25 °C and analyzed for volatile components in TLC systems A and C. The cotton plugs used to maintain the sterility of the cultures were extracted with methylene chloride after the incubations were completed. The ^{14}C in these extracts was quantified and then analyzed in TLC systems A and C.

The aqueous filtrates from the metabolite identification experiments, dissolved in acetonitrile–glacial acetic acid–water (18:1:81), were chromatographed with HPLC system B. The column eluants were divided into nine radioactive fractions, and the corresponding fractions from each chromatogram were combined and concentrated to dryness. Each combined fraction was dissolved in water, adjusted to pH 7 with 0.1 N NaOH, washed with methylene chloride to remove any anthracene leached from the flow cell, concentrated, dissolved in acetonitrile–glacial acetic acid–water (18:1:81), and chromatographed with HPLC system A. The radioactive fractions were collected,

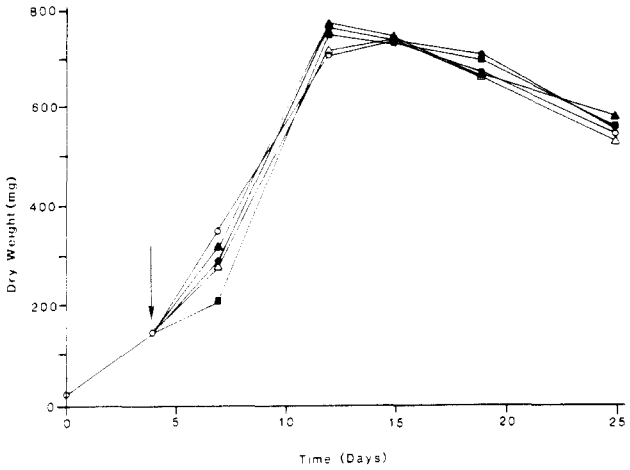

Figure 1. Effect of PCNB and acetone on the growth of peanut cell suspensions. Cells were treated (arrow) with PCNB 4 days after inoculation; (O) acetone-free control; (▲) 200 μL of acetone control; (●) 2.0 μM PCNB in acetone; (▲) 8.1 μM PCNB in acetone; (■) 24.3 μM PCNB in acetone. All controls and treatments were run with three replicates.

concentrated, quantified by liquid scintillation counting, and compared to standards by TLC and HPLC. Metabolites and standards were esterified, acylated, further purified by TLC, and analyzed by mass spectrometry on a Varian MAT CH-5DF as described in Table I.

RESULTS AND DISCUSSION

Toxicity. The toxicity of PCNB to peanut cell suspension cultures was studied by measuring the growth of the cells as a function of PCNB concentration and time (Figure 1). Growth was inhibited by 24.3 μM PCNB during the first 3 days following treatment (statistically significant, $P < 0.01$), but this inhibition was no longer evident by the eighth day. No inhibition of growth was observed with 2.0 or 8.1 μM PCNB, nor was acetone inhibitory to cell growth at the concentration used (statistically significant, $P < 0.05$).

Uptake and Loss of ^{14}C during the Time Study. The metabolism of [^{14}C]PCNB by cell suspension cultures was studied as a function of time by using noninhibitory concentratons of PCNB (2.0–8.1 μM). Each culture was examined at harvest for bacterial and fungal contamination. No contamination was detected in any of the cultures.

The concentrations of ^{14}C in the culture media decreased rapidly during the first 3 h of the time study, suggesting that [^{14}C]PCNB was absorbed by the cells (Figure 2). Absorption of [^{14}C]PCNB was verified by analysis of the cells for ^{14}C. Radioactivity in the cells accounted for 79% of the dose by the third day after treatment and 76% by the fourteenth day. Total recovery of ^{14}C from the system averaged 87%, but decreased from 91% at 3 h to 83% at 14 days (Figure 2). It was shown in a separate study that from 5 to 22% of the applied ^{14}C was volatilized from the system within 20 h following treatment. Most of this was recovered in the cotton plugs used to protect the sterility of the cultures. The ^{14}C in the cottom plugs was shown by the use of TLC systems A and C to be 96–99% PCNB and 1–4% pentachloroaniline. Failure to achieve quantitative recovery of ^{14}C during the first 24 h of the time study was no doubt because of volatilization of [^{14}C]PCNB. The small additional loss of ^{14}C that occurred between the third and fourteenth days could have been caused by the loss of volatile metabolites.

Radioactivity in the Culture Media. The culture media contained 18–21% of the applied ^{14}C 3–9 h following treatment. It was determined from analysis with TLC

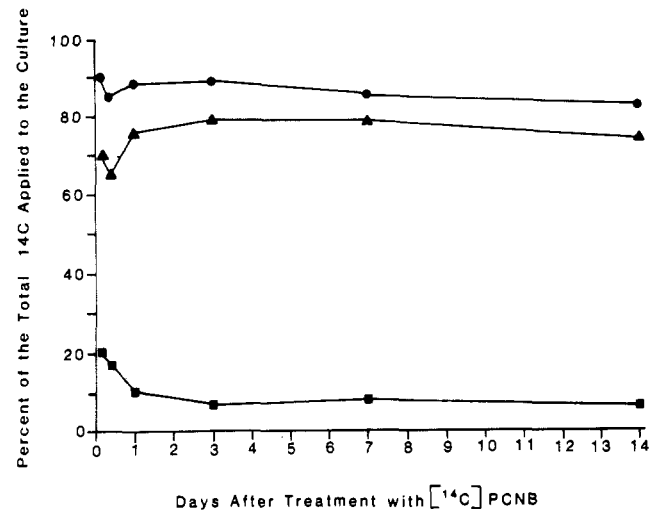

Figure 2. Uptake and recovery of ^{14}C as a function of time by [^{14}C]PCNB-treated peanut cell cultures. Data are presented as averages of two replicates: (●) total recovery of applied ^{14}C; (▲) extractable and nonextractable ^{14}C recovered from cells; (■) ^{14}C recovered from the media.

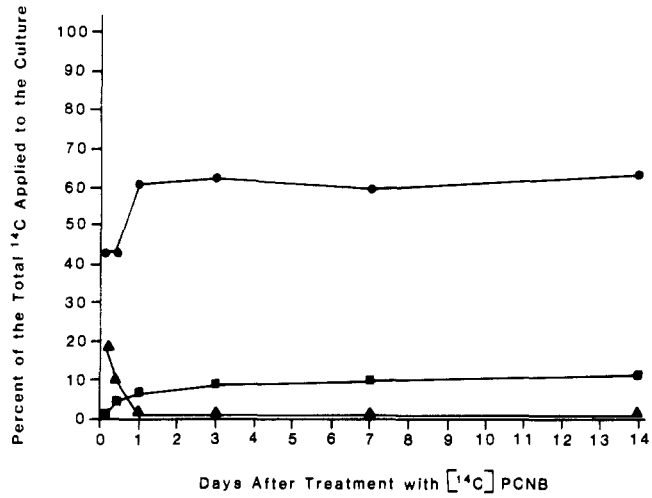

Figure 3. Distribution of ^{14}C in peanut cells as a function of solubility and time. The values presented are an average of two replicates: (●) water-soluble, (▲) methylene chloride soluble, and (■) 80% methanol nonextractable.

systems A and C that this was primarily [^{14}C]PCNB. By 3 days, only 7.5% of the applied ^{14}C remained in the media, mostly in the form of water-soluble products. Identification of these water-soluble products was not attempted.

Methylene Chloride Soluble Metabolites in the Cells. At various times following PCNB treatment, cell cultures were harvested, extracted with methanol, concentrated, and partitioned between water and methylene chloride. The ^{14}C in the nonextractable, methylene chloride soluble and water-soluble fractions was quantified (Figure 3). The cells harvested during the first 9 h following treatment contained appreciable amounts of methylene chloride soluble ^{14}C. A significant loss of ^{14}C occurred when the 80% methanolic extracts from the 3- and 9-h harvests were concentrated before they were partitioned with methylene chloride. For facilitation of the identification of the methylene chloride soluble products, shaken and static cultures were harvested after 20 h and extracted with 70% acetone instead of 80% methanol. The acetone extracts were diluted with water (1:1) and partitioned directly with methylene chloride. The methylene chloride soluble extracts were concentrated under low vacuum. When these procedures were used, the

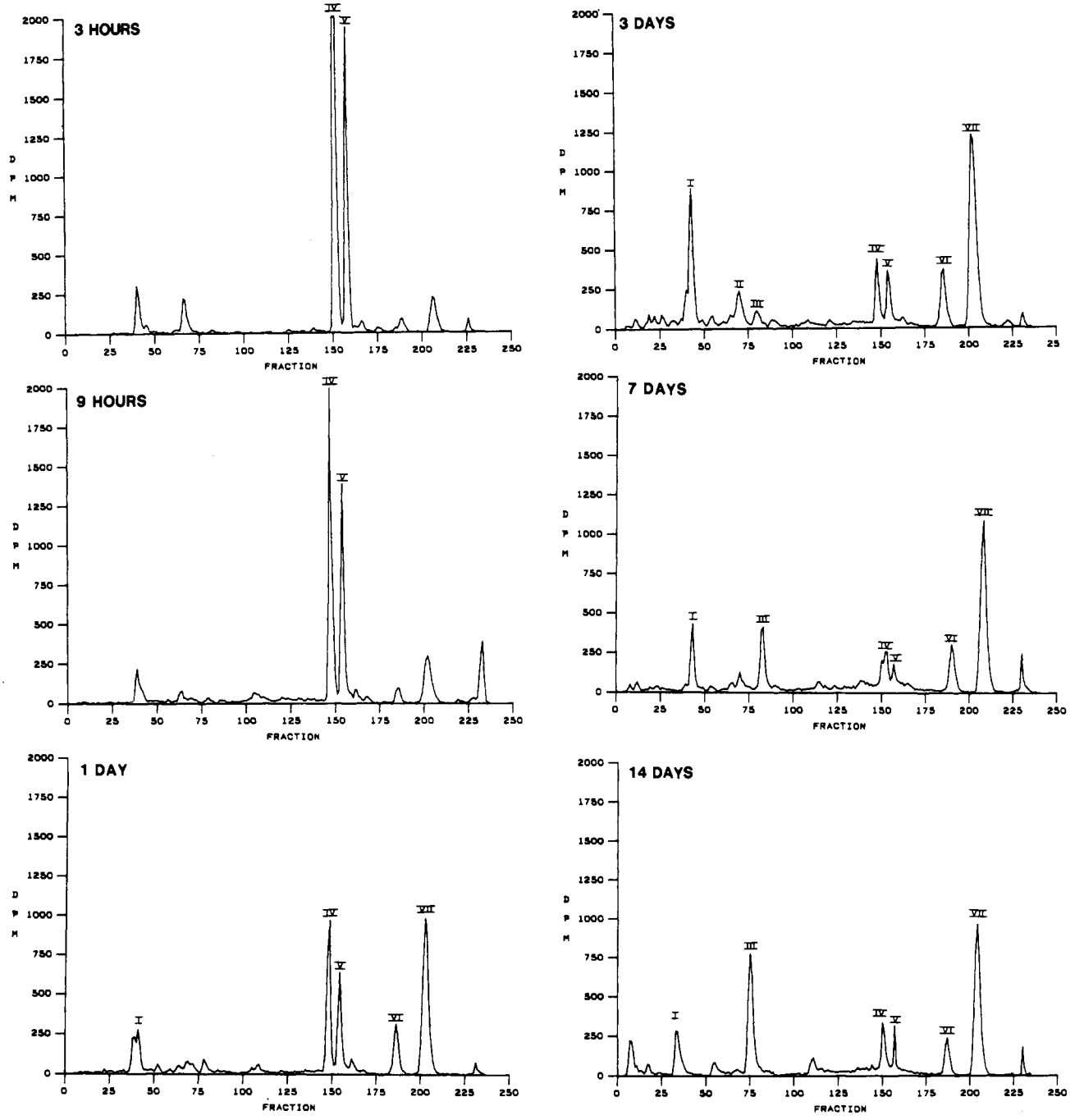

Figure 4. High-performance liquid chromatograms of water-soluble extracts from peanut cells treated with [^{14}C]PCNB.

total recovery of ^{14}C averaged 95%. The methylene chloride soluble cell extracts contained 7.6% of the ^{14}C in the shaken cultures and 22.1% in the static cultures. Radioactivity in the shaken cultures was 1.6% PCNB, 3.9% pentachloroaniline, 1.6% pentachlorothioanisole, and 0.5% unidentified methylene chloride soluble ^{14}C. Radioactivity in the static cultures was 10% PCNB, 10.5% pentachloroaniline, 0.4% pentachlorothioanisole, and 1.2% unidentified methylene chloride soluble ^{14}C. The cotton plugs from the static cultures contained 21.5% of the ^{14}C and those from the shaken cultures contained 4.9%. Pentachloronitrobenzene accounted for over 94% of the ^{14}C in the cotton plugs. Cultures harvested 3–14 days after treatment contained 1–2% of the dose as methylene chloride soluble ^{14}C. No attempt was made to identify this ^{14}C.

Nonextractable Residues from the Cells. Nonextractable ^{14}C increased in the cells with time but accounted for only 13.6% of the applied ^{14}C after 14 days (Figure 3). The nature of the nonextractable residue was not investigated.

Water-Soluble Metabolites in the Cells. Water-soluble metabolites increased rapidly to 60% of the applied ^{14}C during the first 24 h following treatment and then remained constant until the study was terminated after 14 days (Figure 3). Seven major water-soluble metabolites were produced from PCNB during the time study as indicated by separations in HPLC system A (Figure 4). Five of these metabolites were identified by mass spectral comparison to standards, and two were characterized by chromatographic behavior (Table I).

S-(*ar*-Tetrachloronitrophenyl)glutathione (IV) and *S*-(pentachlorophenyl)glutathione (V) were clearly initial products of PCNB metabolism (Figures 4–6). Maximum levels of these metabolites were reached within 3 h following treatment with PCNB. As *S*-(*ar*-tetrachloronitrophenyl)glutathione and *S*-(pentachlorophenyl)glutathione decreased dramatically in concentration, *S*-(*ar*-tetra-

Table I. Isolation, Derivatization, and Mass Spectral Analysis of Water-Soluble Metabolites of PCNB from Peanut Cell Cultures

metabolite[a]	additional purification[b]	derivative for MS[c]	purification of derivative	mass spectra[d]
I, *S,S'*-(*ar*-tetrachlorophenylene)dicysteine	(1) HPLC system A, 31 mL	trifluoroacetyl methyl ester	TLC system A	MI at 672
II, not identified	(1) HPLC system A, 42–56 mL	none prepared	not attempted	not attempted
III, not identified	(1) HPLC system A, 62 mL (2) TLC system C, R_f 0.55 (major), R_f 0.75 (minor) (3) TLC system D, R_f 0.00 (minor), R_f 0.6 (major)	methyl ester		not successful
IV, *S*-(*ar*-tetrachloronitrophenyl)-glutathione	(1) HPLC system A, 110 mL	acetyl ethyl ester	TLC system E (developed 2x)	MI at 662
V, *S*-(pentachlorophenyl)-glutathione	(1) HPLC system A, 115 mL	acetyl ethyl ester	TLC system E, (developed 2x)	MI at 651
VI, *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine	(1) extract into ethyl ether at pH 1 (2) HPLC system A, 137 mL	butyl ester	TLC system A (developed 2x)	MI at 576
VII, *S*-(pentachlorophenyl)-*N*-malonylcysteine	(1) extract into ethyl ether at pH 1 (2) HPLC system A, 150 mL	butyl ester	TLC system A (developed 2x)	MI at 565

[a] Structures are shown in Figure 7. [b] All major isolation steps following purification by HPLC system B and methylene chloride washing at pH 7 are indicated. [c] The derivatization methods have been described previously (Lamoureux and Rusness, 1980a). [d] Derivatives of standards and metabolites yielded identical mass spectra which have been published previously (Lamoureux and Rusness, 1980a); MI = molecular ion.

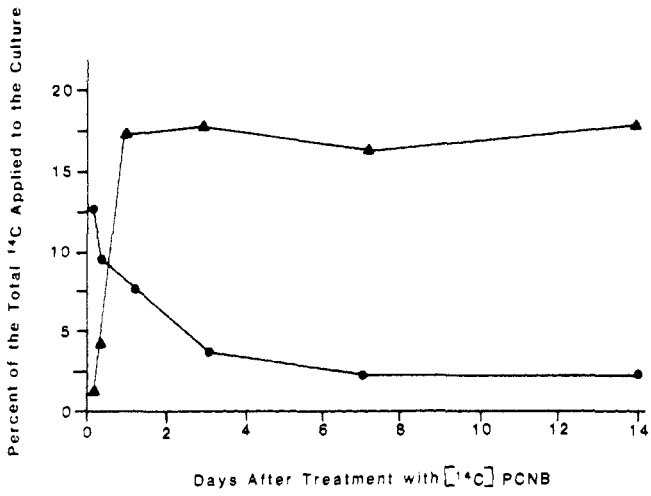

Figure 5. Levels of *S*-(pentachlorophenyl)glutathione and *S*-(pentachlorophenyl)-*N*-malonylcysteine in peanut cell suspension cultures as a function of time following treatment with [^{14}C]PCNB. Data are expressed as an average of two replicate samples: (●) *S*-(pentachlorophenyl)glutathione; (▲) *S*-(pentachlorophenyl)-*N*-malonylcysteine.

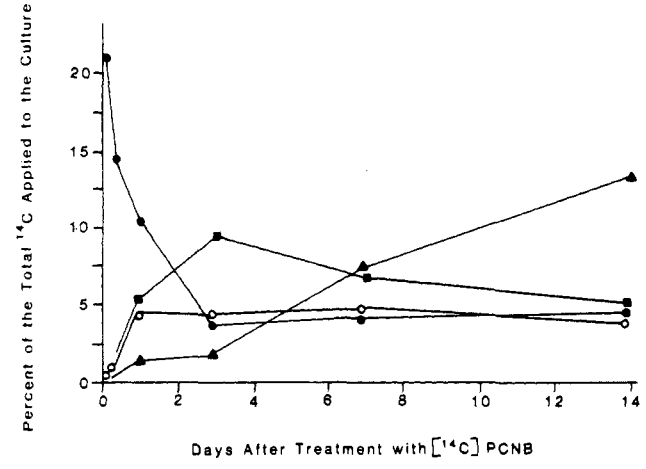

Figure 6. Levels of *S*-(tetrachloronitrophenyl)glutathione, *S*-(tetrachloronitrophenyl)-*N*-malonylcysteine, *S,S'*-(tetrachlorophenylene)dicysteine, and metabolite III in peanut cell suspension cultures as a function of time following treatment with [^{14}C]PCNB. Data are expressed as an average of two replicate samples: (●) *S*-(*ar*-tetrachloronitrophenyl)glutathione; (○) *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine; (■) *S,S'*-(*ar*-tetrachlorophenylene)dicysteine; (▲) metabolite III.

chloronitrophenyl)-*N*-malonylcysteine (VI) and *S*-(pentachlorophenyl)-*N*-malonylcysteine (VII) increased in concentration. Direct evidence that *S*-(pentachlorophenyl)-cysteine was the immediate precursor of *S*-(pentachlorophenyl)-*N*-malonylcysteine was obtained in a study in which a peanut cell culture was treated with *S*-(pentachlorophenyl-*UL*-^{14}C)cysteine. One day after treatment, 90% of the ^{14}C isolated from the cells was *S*-(pentachlorophenyl-*UL*-^{14}C)-*N*-malonylcysteine (identified by mass spectrometry). Only 2.8% of the ^{14}C was nonextractable.

A precursor–product relationship also existed between *S*-(*ar*-tetrachloronitrophenyl)glutathione and *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine, but *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine was apparently not the only product of *S*-(*ar*-tetrachloronitrophenyl)glutathione metabolism (Figure 6). *S,S'*-(*ar*-Tetrachlorophenylene)dicysteine (I) accounted for almost 10% of the applied ^{14}C by the third day following treatment. A probable precursor–product relationship was evident between this metabolite and *S*-(*ar*-tetrachloronitrophenyl)-glutathione. *S,S'*-(*ar*-Tetrachlorophenylene)dicysteine was not stable and declined in concentration after 3 days. This decline corresponded to an increase in metabolite III (Figure 6). Attempts to identify metabolite III were not successful, but because metabolite III was slightly less polar than *S,S'*-(*ar*-tetrachlorophenylene)dicysteine (Figure 4), it was concluded that it might be the mono- or dimalonylcysteine conjugate of *S,S'*-(*ar*-tetrachlorophenylene)dicysteine.

S-(Pentachlorophenyl)-*N*-malonylcysteine and *S*-(*ar*-tetrachloronitrophenyl)-*N*-malonylcysteine were very stable in peanut cells. Little or no decrease in either conjugate was observed between 1 and 14 days (Figures 5 and 6). It seemed unlikely that any further change in the amount of these conjugates would take place unless it occurred during or after the death of the cells.

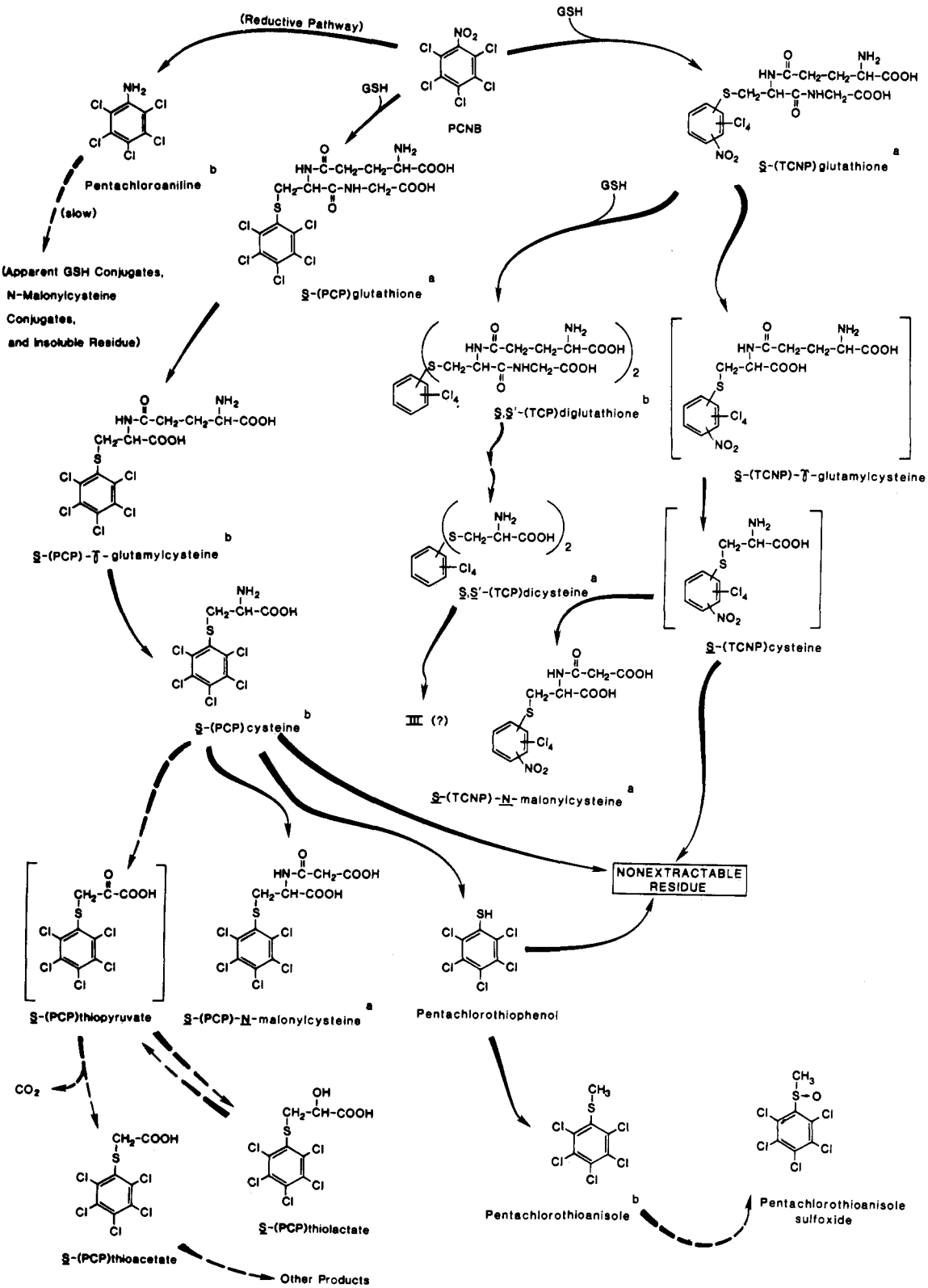

Figure 7. Metabolic pathway of PCNB in peanut cell suspension cultures and in the roots of peanut plants. Pathways indicated by dashed arrows were not observed in cell suspension cultures but were observed in the roots of intact peanut plants. Compounds in brackets were not identified in either peanut roots or peanut cell suspension cultures. Compounds identified in this study by mass spectrometry are followed by a superscript a. Compounds followed by a superscript b are those which have been identified by mass spectrometry in previous studies (Lamoureus and Rusness, 1980a,b; Rusness and Lamoureux, 1980) and characterized by chromatography in this study. Abbreviations used: PCP, pentachlorophenyl; TCP, tetrachlorophenylene; TCNP, tetrachloronitrophenyl.

Intermediates such as the γ-glutamylcysteine and cysteine conjugates were apparently not produced in high concentrations during the metabolism of the glutathione conjugates to their corresponding *N*-malonylcysteine conjugates. *S*-(Pentachlorophenyl)cysteine eluted just before *S*-(*ar*-tetrachloronitrophenyl)glutathione (IV), and *S*-(pentachlorophenyl)-γ-glutamylcysteine eluted just after *S*-(pentachlorophenyl)glutathione (V) in HPLC system A. Small amounts of products with these chromatographic properties were detected (Figure 4). Presumably these intermediates were formed in steps that were rate limiting compared to the formation of the glutathione conjugates

and the conversion of the cysteine conjugates to *N*-malonylcysteine conjugates. An in vitro enzyme system from onion converted PCNB to pentachlorothioansiole through *S*-(pentachlorophenyl)glutathione, *S*-(pentachlorophenyl)-γ-glutamylcysteine, and *S*-(pentachlorophenyl)cysteine intermediates (Lamoureux and Rusness, 1980b). However, in onion the last two intermediates were also not detected in appreciable concentrations.

A proposed pathway of PCNB metabolism in peanut cell suspension cultures is shown in Figure 7. Conjugation with GSH was the first step in the primary routes of PCNB metabolism in peanut cell suspension cultures. All of the metabolites identified from the cell cultures, except pentachloroaniline, were derived from GSH conjugates. Glutathione conjugation was shown previously to be a major pathway in PCNB metabolism in intact peanut plants (Lamoureux and Rusness, 1981). The pathway appeared to be similar in peanut cell suspension cultures and in the roots of intact peanut plants; however, several minor methylene chloride soluble metabolites related to the GSH pathway were detected in peanut roots but not in peanut cell suspension cultures (Figure 7). These metabolites and the levels at which they were found (expressed as a percent of the ^{14}C isolated) are as follows: pentachlorothiophenol (2.6%), pentachlorophenyl methyl sulfoxide (0.5%), *S*-(pentachlorophenyl)-2-thioacetic acid (0.5%), and *S*-(pentachlorophenyl)-2-hydroxy-3-thiopropanoic acid (0.2%). Some of these minor metabolites may have been produced by microfloral action. The absence of these metabolites from the cell suspension cultures does not constitute a major difference in metabolism between the cell suspension cultures and the roots of intact peanut plants.

Incorporation of ^{14}C into nonextractable residue appeared to be less important in peanut cell suspension cultures than in the roots of intact peanut plants. In peanut cell suspension cultures, nonextractable residue accounted for 13.6% of the applied ^{14}C (16.4% of the recovered ^{14}C) 14 days after [^{14}C]PCNB treatment. In comparison, nonextractable residue accounted for 35% of the ^{14}C recovered from peanut roots 21 days after treatment of the intact plants with [^{14}C]PCNB (Lamoureux and Rusness, 1980a). This difference could be partially a result of quantitative differences in competing metabolic reactions that utilize *S*-(pentachlorophenyl)cysteine as a substrate. In intact peanut plants, nonextractable residue accounted for 53.1% of the ^{14}C recovered from peanut roots 20 days following treatment with *S*-(pentachlorophenyl-*UL*-^{14}C)cysteine, and *S*-(pentachlorophenyl-*UL*-^{14}C)-*N*-malonylcysteine accounted for only 28% of the ^{14}C (Lamoureux and Rusness, 1980a). In contrast, when peanut cell suspension cultures were treated for 1 day with *S*-(pentachlorophenyl-*UL*-^{14}C)cysteine, only 2.8% of the ^{14}C recovered from the cells was nonextractable, and ~90% was *S*-(pentachlorophenyl-*UL*-^{14}C)-*N*-malonylcysteine. Since *S*-(pentachlorophenyl)-*N*-malonylcysteine was stable in the peanut cell suspension cultures, large amounts of nonextractable residue would not have been expected with longer incubation times. The possibility that microfloral metabolism may have played a role in the formation of the nonextractable residue in the intact peanut plants should be considered.

A major quantitative difference was observed between peanut cell suspension cultures and the roots of intact peanut plants in the conversion of PCNB to pentachloroaniline. In intact peanut plants, pentachloroaniline accounted for 22.5% of the ^{14}C isolated from the roots 48 h after treatment with [^{14}C]PCNB (Lamoureux and Rusness, 1980a). In contrast, pentachloroaniline accounted for less than 2% of the ^{14}C in the peanut cell suspension cultures at all harvests when the cultures were grown and treated under conditions of normal oxygen tension. Only when the cultures were grown without shaking (to reduce oxygen tension) was pentachloroaniline produced as a major metabolite (up to 10% of the dose).

The methods used to isolate and identify metabolites from peanut cell suspension cultures were similar to those used to isolate and identify metabolites from intact peanut plants. However, the cell cultures were easier to extract, larger aliquots of the extracts could be chromatographed on the HPLC columns, and, in some cases, it was possible to eliminate purification steps that were necessary for the isolation of metabolites from intact plants. The metabolite preparations obtained from the cell cultures were generally of greater purity than those obtained from intact plants. All major metabolites detected in the GSH conjugation pathway in intact peanut plants were also observed in the peanut cell suspension cultures. The peanut cell suspension cultures could probably be used to predict whether a pesticide would be metabolized to a glutathione conjugate in intact peanut plants. The cell cultures could also be used to produce large amounts of certain metabolites to facilitate metabolite isolation and identification.

Some quantitative differences observed between PCNB metabolism in peanut cell suspension cultures and intact peanut plants appeared to be significant. Therefore the peanut cell suspension culture would probably not be useful in evaluating the relative importance of competing metabolic pathways in intact peanut plants. It would be necessary to verify the results of metabolism in cell suspension cultures with the results in intact plants.

The dry weight of the cells in the suspension cultures began to decrease after ~12 days. This may have been caused by exhaustion of nutrients from the media or the release of toxins. If the formation and stability of terminal residues are to be studied in cell suspension cultures, it would be desirable to add fresh nutrient media to the culture before a decline in dry weight of the cells is observed. This would probably make it possible to study the metabolism of the pesticide over a longer time period.

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