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EPTC Metabolism in Corn, Cotton, and Soybean: Identification of a Novel Metabolite Derived from the Metabolism of a Glutathione Conjugate

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A new class of xenobiotic metabolite derived from the metabolism of a glutathione conjugate was produced by corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), and corn cell suspension cultures treated with [^{14}C]EPTC. This metabolite was characterized as *S*-(*N*,*N*-dipropylcarbamoyl)-*O*-malonyl-3-thiolactic acid by fast atom bombardment and chemical ionization mass spectrometry. It was acid labile and accounted for up to 33% of the [^{14}C] isolated from corn plants 28 days after exposure to [^{14}C]EPTC. It was not detected in soybean (*Glycine max* L.) plants or in peanut (*Arachis hypogaea* L.) cell suspension cultures. A second major metabolite of EPTC, *S*-(*N*,*N*-dipropylcarbamoyl)-*N*-malonylcysteine, was present in all tissues examined. This metabolite was also characterized by mass spectral methods.

EPTC (*S*-ethyl dipropylthiocarbamate) is a selective herbicide used to control weeds in a wide variety of crops including corn, cotton, and beans. It is usually soil incorporated at a rate of 2.2–6.7 kg/ha and has a half-life of about 1 week in moist loam soil (Beste, 1983). The first step in EPTC metabolism in higher plants such as corn is thought to be oxidation of EPTC to the sulfoxide or the sulfone. EPTC sulfoxide can react enzymatically with glutathione (GSH) to form *S*-(dipropylcarbamoyl)GSH (EPTC–GSH) (Lay and Casida, 1976). The sulfone of EPTC can react nonenzymatically with GSH to form the same product (Horvath and Pulay, 1980). In vivo studies showed that EPTC–GSH and EPTC–cysteine are major metabolites of EPTC in corn (Hubbell and Casida, 1977). Other metabolites of EPTC were detected in corn, but they were not identified. In the rat, EPTC is also metabolized to EPTC–GSH, which is then further metabolized to the mercapturic acid and other products. These products appeared to be different from those found in corn (Hubbell and Casida, 1977).

In the course of a general study to determine whether *N*-malonylcysteine conjugates were common end products of GSH conjugate metabolism for a variety of pesticides in higher plants, EPTC metabolism was examined in corn plants, excised corn leaves, corn cell suspension culture, cotton plants, excised cotton leaves, and soybean plants (Lamoureux and Rusness, 1983). During that study, the *N*-malonylcysteine conjugate and an unidentified metabolite were detected as major metabolites of EPTC in several plant systems. This paper reports on the characterization of the unidentified metabolite, which is derived from the GSH conjugation pathway, and also presents details on the identification of the *N*-malonylcysteine conjugate.

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METHODS

Synthesis of [^{14}C]-Labeled Compounds. [*carbamoyl*-^{14}C]-*S*-(*N*,*N*-Dipropylcarbamoyl)GSH, [*carbamoyl*-^{14}C]-*S*-(*N*,*N*-dipropylcarbamoyl)cysteine, and [*carbonyl*-^{14}C]EPTC were prepared by the methods of Hubbell and Casida (1977). The specific activity of [^{14}C]EPTC was 3.45 μCi/μmol. The structure of [^{14}C]EPTC was verified by electron impact mass spectrometry (EI MS). By HPLC, the purity of the [^{14}C]EPTC was estimated to be >97%. The identity and purity of the GSH and cysteine conjugates were verified by chromatographic properties and EI MS of the acetylated methyl esters. For preparation of metabolites, [^{14}C]EPTC was diluted with nonradioactive EPTC.

Treatment of Corn for Isolation of Metabolites. Corn (*Zea mays* L.; Northrup King PX 443) was germinated between layers of moistened paper under the following conditions: 14-h day at 27 °C, 21 °C night, relative humidity 50%. After 5 days, the seedlings were transferred to paper wetted with an aqueous 30 ppm solution of dichlormid (*N*,*N*-diallyl-2,2-dichloroacetamide) and placed in a greenhouse. Dichlormid is a herbicide antidote that

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increases the rate of metabolism of EPTC by GSH conjugation (Lay and Casida, 1976). After a 24-h exposure to dichlormid, the seedlings were placed in nutrient culture (six groups of 22 seedlings/100-mL beaker). Each group of seedlings was treated with 41.7 mL of water that contained 29.3 nmol/mL [^{14}C]EPTC (specific activity 0.366 nCi/nmol). Additional water was added after 28 h. The seedlings were removed from the solution after 48 h, and the roots were rinsed. The treating solution and rinses accounted for 10.7% of the original ^{14}C. On the basis of thin-layer chromatographic analysis by the methods of Horvath and Pulay (1980), most of the ^{14}C in rinses and treating solution was shown to be EPTC. The seedlings were then placed in six 350-mL beakers that contained 250 mL of one-fourth-strength Hoagland's solution. The solutions were aerated, and water was added as needed. After 6 days, the seedlings appeared healthy and were harvested. The Hoagland's solution contained 1.92% of the original dose of radioactivity but was not further analyzed. The tissue (356 g) was extracted four times for 5-min intervals with 1.5-L portions of cold 70% acetone. A polytron homogenizer cooled in an ice bath was used for extraction. The extracts were filtered, combined, and concentrated under vacuum on a rotary evaporator at 40 °C, and the ^{14}C was quantitated (0.761 μCi). The extract was diluted with water and washed four times with equal volumes of methylene chloride. The aqueous phase was adjusted to pH 1.5, and the resulting 200-mL solution was partitioned six times against 250-mL volumes of ethyl ether. The aqueous phase contained 37.3% and the ether phase 48.3% of the soluble ^{14}C. Metabolites in the ether fraction were purified further by HPLC. The experiment was repeated three times.

Treatment of Corn, Cotton, and Soybean for Metabolite Distribution. Corn, cotton (*Gossypium hirsutum* L.; Stoneville 519), and soybean (*Glycine max* L.; Wilkin) were seeded in vermiculite and grown in a greenhouse. After 2 weeks, individual plants were transferred to 500-mL jars of aerated one-third-strength Hoagland's solution and grown in a growth chamber for an additional 1 week prior to treatment. Growth chamber conditions: 30 °C during the 12-h photoperiod (approximately 1600 lx), 27 °C during the 12-h dark period, constant relative humidity 50%. Each plant was pulse treated with 200 mL of 1.3 μM [^{14}C]EPTC in one-third-strength Hoagland's solution. After 2 days, the plants were again treated with an equal amount of [^{14}C]EPTC added in 45 μL of acetone. After an additional 2 days, the plants were transferred to 1-L jars containing fresh Hoagland's solution and aeration was resumed. One week after the first treatment, aeration was discontinued and the plants were treated with 500 mL of 1.0 μM [^{14}C]EPTC for 48 h. The plants were then placed in fresh one-third-strength Hoagland's solution and grown under aeration until 28 days after the first treatment. The roots and shoots of the plants were then extracted separately as described above; however, the ^{14}C present in each 70% acetone extract was measured before the extracts were combined and concentrated. No loss of ^{14}C was observed on concentration of the extracts. Two plants were used for each species, and the experiment was repeated twice.

Treatment of Excised Cotton and Corn Leaves. Leaves from cotton plants 17 weeks old (young bolls forming on plants) were excised under water at the axil and placed in 20-mL vials that contained 64 nmol of [^{14}C]EPTC dissolved in 13 mL of aqueous 0.15% acetone. Two leaves (3.0 g of tissue) were placed in each vial. The vials of cotton leaves were placed in a growth chamber under the previously described conditions and incubated for 8 days. After 48 h, when nearly all of the treating solution had been taken up, water was added as needed to make up for transpiration and evaporative losses. After 8 days, the cotton leaves appeared healthy and were then extracted with aqueous 70% acetone as previously described. The aqueous extracts were analyzed by HPLC systems I–III. The experiment was repeated twice with different lots of cotton plants.

The foliar portions of six 14-day-old corn seedlings were excised (1.4 g/excised seedling, 27-cm length) and individually treated for 48 h in conical tubes that contained 3 mL of 9.2 μM [^{14}C]EPTC and 0.13% acetone. Water was added as needed during the 48-h period in which the leaves were under treatment in the growth chamber. The leaves were divided into two lots, extracted with 70% acetone, and analyzed for metabolites as described previously. The experiment was repeated twice with different lots of plants.

Treatment of Corn and Peanut Cell Suspension Cultures. The corn cell suspension culture, originally obtained from C. E. Green, University of Minnesota, was maintained in the dark in BM7 medium (0.5 mg of 2,4-D/L) (Green, 1977). The peanut (*Arachis hypogaea* L.) cell suspension culture was maintained in the light in B5 medium (0.1 mg of 2,4-D/L) and has been described previously (Lamoureux et al., 1981). The general methods of treatment of the cell cultures were as previously described (Lamoureux et al., 1981); however, the corn cell cultures (50 mL of suspension in 250-mL flasks) were treated with three 175-nmol doses of [^{14}C]EPTC at 2-day intervals and harvested 14 days after the first treatment. The first treatment was made 5 days after the cultures had been transfered to fresh media. The peanut cell suspension cultures (50 mL of suspension in 250-mL flasks) were treated with single 130-nmol doses of [^{14}C]EPTC 4 days after the cultures has been transferred to fresh media. These cells were harvested and extracted after 7 days. Studies with both cultures were conducted with two replicates.

Chromatography. High-performance liquid chromatography (HPLC) was accomplished with a Beckman Model 334 HPLC system equipped with a Berthold Model LB 503 radioactive monitor. A 500-μL flow cell, filled with a solid scintillator, was used in the radioactive monitor. Except where otherwise noted, all HPLC separations were performed on a 3.9 mm × 30 cm μBondapak C_{18} column (Millipore Corp., Milford, MA) eluted at 1.5 mL/min with solvents that contained 1% glacial acetic acid. The following elution profiles were employed: HPLC system I, aqueous 22% acetonitrile for 10 min followed by a linear 20-min gradient to aqueous 32% acetonitrile and then held isocratic for an additional 5 min; HPLC system II, aqueous 33% methanol for 5 min followed by a linear 25-min gradient to 50% aqueous methanol and then held isocratic for 5 min; HPLC system III, aqueous 15% tetrahydrofuran for 10 min followed by a linear 30-min gradient to aqueous 35% tetrahydrofuran and then held isocratic for an additional 5 min; HPLC system IV, aqueous 22% acetonitrile for 10 min followed by a linear 40-min gradient to 52% aqueous acetonitrile; HPLC system V, stepwise with 40% acetonitrile for 16 min, with 45% acetonitrile for 10 min, and with 75% acetonitrile for 15 min; HPLC system VI, 7.8 mm × 30 cm Aminex ion-exclusion column for organic acids (Bio-Rad Laboratories, Richmond, CA). This column was eluted at 0.50 mL/min with 0.01 N sulfuric acid in aqueous 5% acetonitrile.

Gas chromatography was performed with previously described instrumentation (Lamoureux and Rusness,

1980). A 2 mm × 1 m column of 3% SP-2100 on 80/100-mesh Supelcoport was used with helium carrier gas (21 mL/min). After on-column injection of the sample, the initial oven temperature of 150 °C was held for 2 min and then programmed to increase at 10 °C/min to 250 °C. The injector and detector temperatures were 175 and 390 °C, respectively. A split ratio of 8:1 was used between the flame ionization and gas proportional detectors.

Mass Spectrometry. Electron impact and chemical ionization mass spectrometry was performed on a Varian MAT 112S mass spectrometer equipped with a dual electron impact (EI) and chemical ionization (CI) source. EI and CI spectra were obtained by introducing the sample with a heated solid sample probe. Fast atom bombardment (FAB) mass spectrometry was performed on a Varian MAT CH5-DF equipped with an IonTek Saddlefield gun used to produce a xenon atom beam. Samples for FAB mass spectrometry (2–5 μg) were dissolved in a matrix of 0.2 μL of glycerol, 0.1 μL of methanol, and 10 μg of oxalic acid and applied to a copper probe tip. Gas chromatography/mass spectrometry was performed with a Hewlett-Packard 5992A gas chromatograph/mass spectrometer equipped with a 12-m OV-101 capillary column.

Radioactive Analyses. Radioactivity in various solutions, extracts, and plant residues and on thin-layer plates was measured by methods previously described (Lamoureux and Rusness, 1980).

Derivatization Procedures. Esterification reactions were run in sealed vials at 90 °C for 30 min in 300 μL of absolute methanol that was 3 N with respect to hydrogen chloride. Acetylation reactions were run in sealed vials for 30 min at 90 °C with 200 μL of methylene chloride and 100 μL of acetic anhydride. Excess reagents from both reactions were removed under gentle vacuum.

RESULTS AND DISCUSSION

Detection of EPTC Metabolites in Corn, Cotton, and Soybean Grown and Treated in Hydroponic Culture. EPTC is metabolized in plants by oxidation followed by conjugation with GSH (Lay and Casida, 1976). GSH conjugates are usually unstable in plants; however, the only plant metabolites of EPTC derived from the GSH pathway that have been characterized are the GSH, cysteine, and malonylcysteine conjugates (Hubbell and Casida, 1977; Lamoureux and Rusness, 1983). When hydroponically grown corn, cotton, and soybean plants were treated with [^{14}C]EPTC and analyzed 28 days later, the root and foilar tissues from the three species did not contain either the GSH or the cysteine conjugate as a major metabolite, but all tissues did contain a product that cochromatographed with the previously characterized malonylcysteine conjugate, metabolite I (Figure 1). In addition, the root tissue of corn and the foilar tissues of corn and cotton also contained a major unidentified metabolite, metabolite II. Metabolite II appeared to be the most abundant metabolite in the root and foilar tissues of corn and in the foliar tissue of cotton. This metabolite was less polar than the cysteine, GSH, or malonylcysteine conjugates of EPTC.

Isolation and Identification of Metabolite I [*S*-(*N*,*N*-Dipropylcarbamoyl)-*N*-malonylcysteine] and Metabolite II [*S*-(*N*,*N*-Dipropylcarbamoyl)-*O*-malonyl-3-thiolactic Acid]. Metabolite II was isolated from whole corn plants that had been pretreated with dichlormid and then treated with [^{14}C]EPTC for 2 days and grown for an additional 6 days. The sequence of procedures for isolation of metabolite II are outlined in Figure 2. Recovery of ^{14}C from these and related experiments was comparatively low due to the volatility of EPTC. Low recoveries of ^{14}C were also observed in EPTC metabolism studies conducted by Hubbell and Casida (1977). On the basis of HPLC system I, metabolite II accounted for 15.5% of the extracted ^{14}C in the corn plants. When metabolite II was further purified by chromatography on an anion-exclusion column (HPLC system VI), a single radioactive peak was eluted, but when this radioactive peak was concentrated and examined by HPLC system II, two products were observed (II, IIB). The ratio of II to IIB was 0.33. Metabolite IIB had a retention time of 19 min with HPLC system I and was assumed to be an artifact. Metabolite II had a retention time of 24 min and was assumed to be the unaltered metabolite. The original retention time of metabolite II on HPLC system I was 24 min (Figure 2). When metabolite II was purified by HPLC systems I and II, without the use of HPLC system VI, both II and IIB were detected, but the ratio of II to IIB was 4.0.

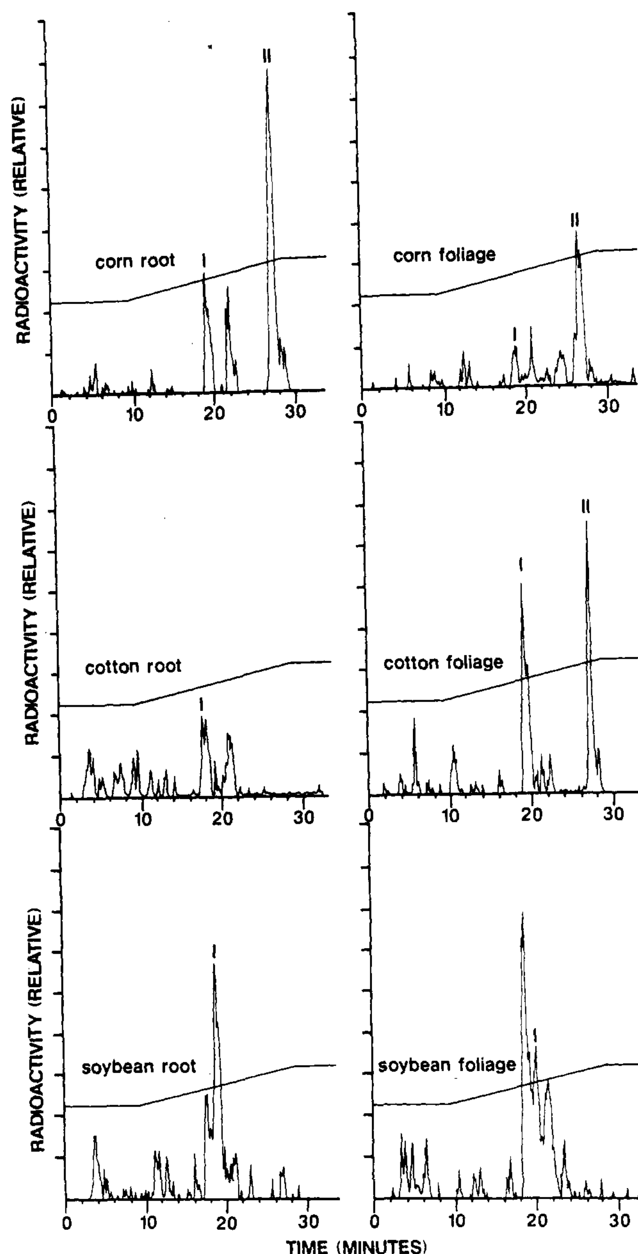

Figure 1. HPLC of 70% acetone extracts of root and foliar tissues of corn, cotton, and soybean plants 28 days after initial exposure in hydroponic culture to [^{14}C]EPTC. Extracts were washed with methylene chloride and purified by Sep-pak C_{18} prior to analysis by HPLC system I. EPTC–GSH and EPTC–cysteine were unresolved and had a retention time of 8.8 min in this system. Metabolite I was determined by internal standarization.

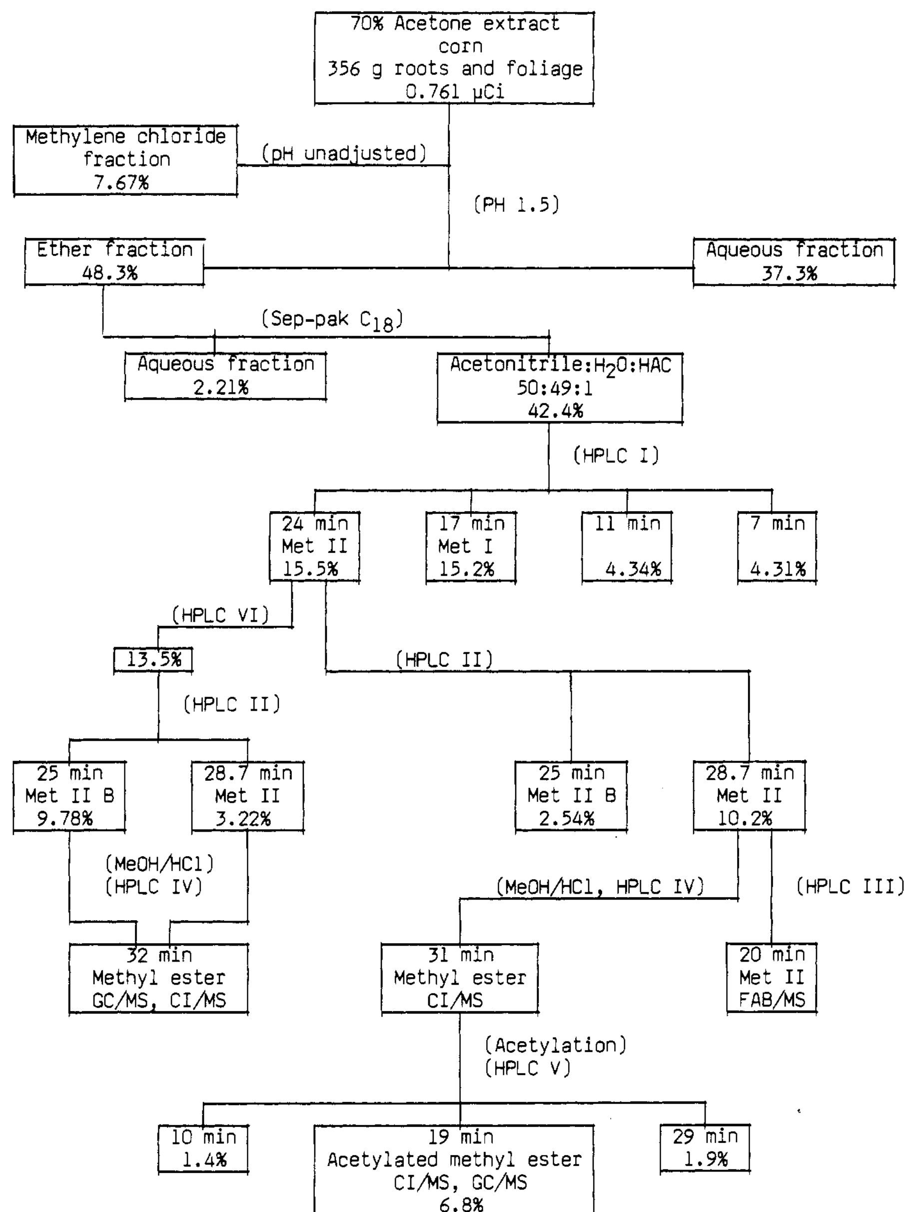

Figure 2. Summary of isolation methods employed in the purification of metabolites I and II from corn tissues.

Metabolites II and IIB appeared to yield the same product upon esterification with methanol/HCl (retention time 31–32 min on HPLC system IV). The esterification product appeared to be volatile, and in some cases it was lost during concentration of samples. The esterification product had a GC retention time and temperature of about 4 min and 166 °C. Upon GC/MS, the product produced a base peak at m/z 128 and ion fragments at m/z 160 (7.0%), 161 (7.3%), and 204 (12.6%). The isobutane CI MS of the methyl ester of metabolites II and IIB were identical. The spectrum of the methyl ester of metabolite II isolated without HPLC system VI is shown in Figure 3A. The spectrum was consistent with that of *S*-(*N*,*N*-dipropylcarbamoyl)-3-thiolactic acid methyl ester. When this esterified metabolite was acetylated and purified, the major product yielded a CI MS that was consistent with the corresponding acetylated derivative (Figure 3B). This product had a GC retention time of 5.8 min. On the basis of studies with the methyl ester and the acetylated methyl ester of phenyllactic acid, the differences in the GC retention time between the methyl ester and the acetylated methyl ester of metabolite II were as expected for the indicated structure.

Since metabolite IIB appeared to be an artifact of metabolite II, and since both metabolites II and IIB yielded the same product upon derivatization, it was desirable to isolate unaltered metabolite II and to obtain mass spectral data without derivatization. Subsequently, metabolite II was isolated from the leaves of whole corn plants grown and treated hydroponically as described previously. Metabolite II was purified from aqueous 70% acetone extracts by successive chromatography with HPLC systems I–III as indicated in Figure 2. Metabolite II obtained in this manner had a retention time of 24 min when rechromatographed with HPLC system I. It was analyzed by FAB MS. The FAB MS of metabolite II was characterized by an intense M + 1 ion and M + Na and M + K ions that verified the molecular weight of the metabolite to be 335 (Figure 3C). Metabolite II was concluded to be *S*-(*N*,*N*-dipropylcarbamoyl)-*O*-malonyl-3-thiolactic acid (Figure 3C). Metabolite II from cotton cochromatographed with metabolite II from corn on HPLC systems I–III but was not identified by mass spectrometry.

Metabolite I [*S*-(*N*,*N*-dipropylcarbamoyl)-*N*-malonylcysteine] was previously characterized as a metabolite of EPTC from soybean (Lamoureux and Rusness, 1983). However, characterization was based on EI and CI MS of various esters, and no spectra were published. Metabolite

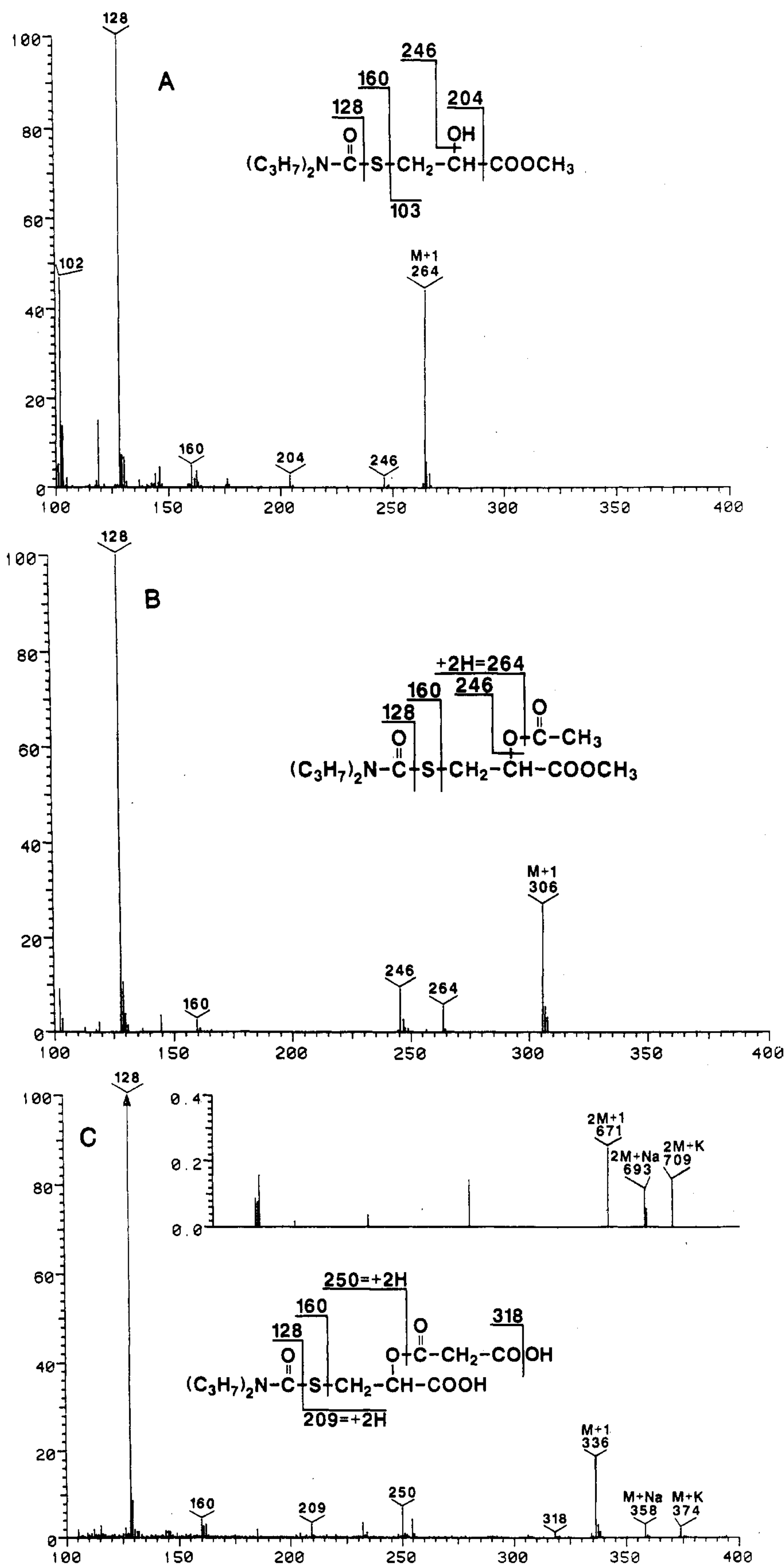

Figure 3. Mass spectra of metabolite II and derivatives of metabolite II: (A) CI (isobutane) mass spectrum of metabolite II after esterification; (B) CI (isobutane) mass spectrum of metabolite II after esterification and acetylation; (C) FAB mass spectrum of parent metabolite II.

I was isolated from corn by successive chromatography in HPLC systems I–III as previously described with metabolite II. The CI MS of the methyl ester and the FAB MS of the parent metabolite confirm that the characterization of metabolite I was correct (Figure 4).

Metabolite II [*S*-(*N*,*N*-dipropylcarbamoyl)-*O*-malonyl-3-thiolactic acid] was hydrolyzed to metabolite IIB [*S*-(*N*,*N*-dipropylcarbamoyl)-3-thiolactic acid] under the

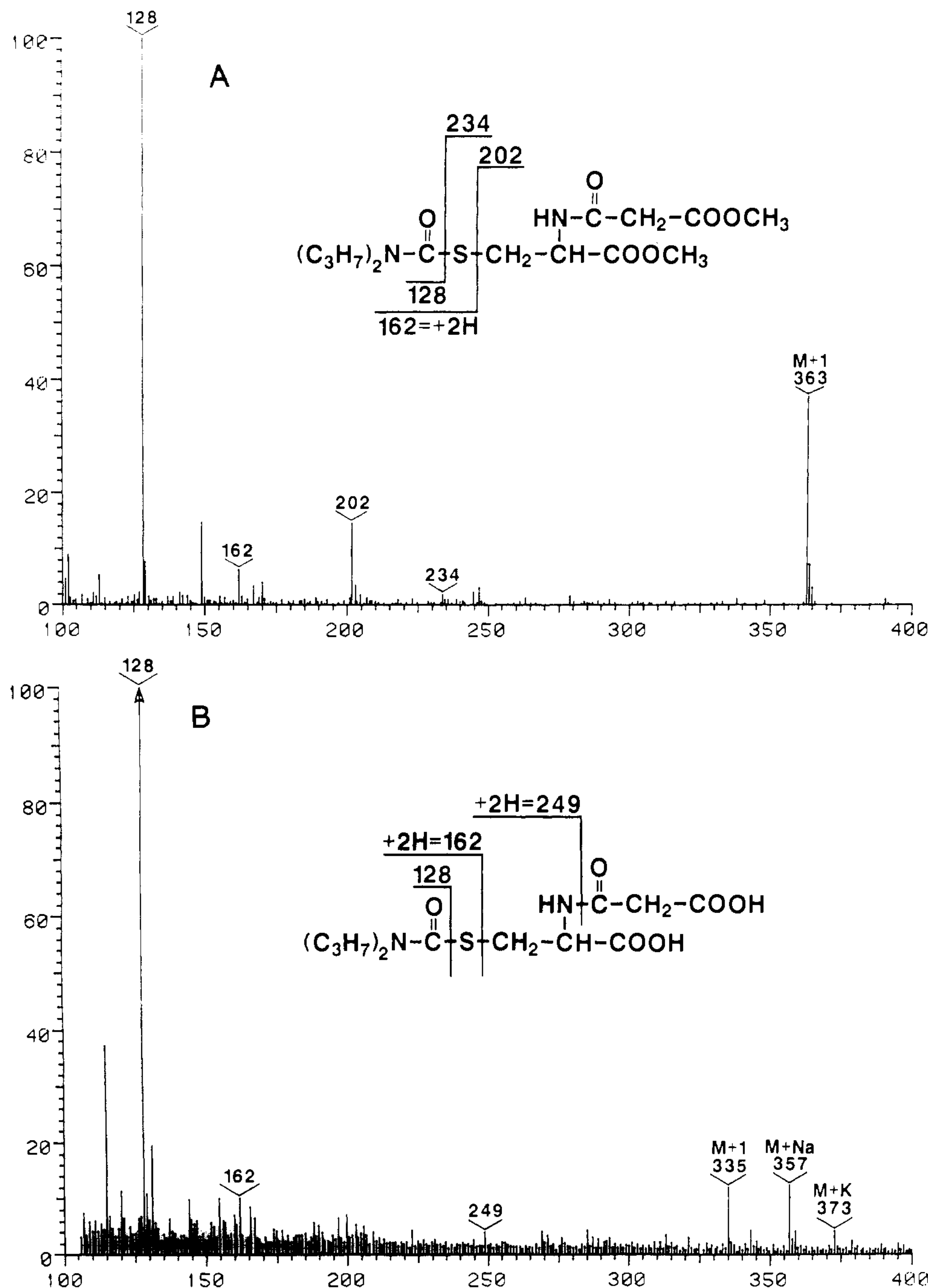

Figure 4. Mass spectra: (A) CI (isobutane) mass spectrum of metabolite I methyl ester; (B) FAB mass spectrum of parent metabolite I.

mildly acidic conditions encountered during chromatography and/or evaporation of HPLC solvents. The hydrolysis of 6-*O*-malonylglucosides has been previously reported to occur under mildly acidic conditions such as those used in this study (Lamoureux and Rusness, 1986). During the purification of a 6-*O*-malonylglucoside of the herbicide propachlor (2-chloro-*N*-isopropylacetanilide) by HPLC system I, some hydrolysis of the malonyl group occurred during chromatography or concentration of solvents. The retention time of the malonylglucoside in HPLC system I was 13.5 min, and the retention time of the free glucoside was 8.9 min (unpublished research). The conditions of hydrolysis and the differences in retention time between the malonyl derivative and the free hydroxy form appear to be very similar for metabolite II of EPTC and the 6-*O*-malonylglucoside of propachlor. *N*-Malonylcysteine conjugates such as metabolite I are more resistant to mild acid hydrolysis than *O*-malonyl conjugates.

The quantitative distribution of metabolites I and II in corn, cotton, and soybean plants grown under hydroponic culture and treated by incorporation of EPTC into the nutrient solution is shown in Table I. Metabolite I was detected in the root and foliar tissues of all three species. Metabolite II was a major metabolite in the foliar tissues of corn and cotton and in corn root, but it was not detected in soybean tissues. Since these analyses were performed 28 days after treatment, it can be concluded that both metabolites I and II are relatively stable. It was previously reported that malonylcysteine conjugates were stable in plant tissues (Lamoureux and Rusness, 1983). It is assumed that EPTC metabolism in soybean proceeds through a homoglutathione conjugate rather than through a GSH conjugate (Frear et al., 1985). The absence of metabolite II in soybean is probably unrelated to the initial difference between conjugation with GSH vs. conjugation with homoglutathione since metabolite I was detected in soybean. The probable pathways of EPTC metabolism

Table I. Percent Distribution of [^{14}C]EPTC Metabolites[a]

species	root tissue				foliar tissue			
	extractable				extractable			
	Met II	Met I	all others	bound	Met II	Met I	all others	bound
corn	20	9.2	20	6.6	13	7.4	16	7.4
cotton	0	1.2	9.0	2.0	17	21	35	15
soybean	0	8.2	21	3.4	0	10	51	6.9

[a] Plants in hydroponic culture were analyzed for metabolites by HPLC system I 28 days after initial exposure to [^{14}C]EPTC. Values are averages of two completely replicated experiments. Met II = *S*-(*N*,*N*-dipropylcarbamoyl)-*O*-malonyl-3-thiolactic acid and Met I = *S*-(*N*,*N*-dipropylcarbamoyl)-*N*-malonylcysteine.

Table II. Distribution of [^{14}C]EPTC Metabolites in Plant Tissue[a]

tissue	incub time, days	% ^{14}C in tissue: Met II	Met I	all other soluble	bound
corn leaves, excised	2	14	11	70	5
corn cell s. culture	14	16	21	61	2
cotton leaves, excised	8	22	22	51	5
peanut cell s. culture	7	0	78	18	4

[a] Excised leaves or cell suspension cultures were treated with [^{14}C]EPTC by previously described methods. Tissues or cells were analyzed by HPLC systems I and II. Values are averages of two completely replicated studies. Abbreviations are the same as in Table I.

and the biosynthesis of metabolites I and II are shown in Figure 5.

When excised leaves of corn and cotton were treated with EPTC, both species appeared to produce metabolites I and II in relatively high yield (Table II). Since both excised leaves and whole plants were treated with EPTC under nonsterile conditions, it was not certain whether the observed metabolism was due to plant tissue, microbial activity, or a combination of both. Therefore, peanut and corn cell suspension cultures were treated with EPTC, and the extracts produced from these cell cultures were examined by HPLC. The peanut cell suspension culture produced metabolite I almost to the complete exclusion of all other metabolites. Like soybean plants, The peanut cell suspension culture did not produce metabolite II. Unlike soybean, peanut utilizes GSH instead of homoglutathione in the formation of xenobiotic conjugates (Lamoureux and Rusness, 1986).

The corn cell suspension culture produced metabolites I and II in about the same yield as excised leaves or intact plants (Table II). It was concluded that metabolite II was produced by the plant tissues.

3-Thiolactic acid and 3-thiopyruvic acid conjugates have occasionally been reported as xenobiotic metabolites in plants (Rusness and Lamoureux, 1980) and animals (Lamoureux and Bakke, 1984). These metabolites are usually thought to occur with the xenobiotic conjugated via a sulfide bond to the 3-position of lactic acid or pyruvic acid. These metabolites are actually formed by catabolism of the corresponding GSH conjugates. *S*-(*N*,*N*-Dipropylcarbamoyl)-*O*-malonyl-3-thiolactic acid appears to be the first 3-thiolactic acid conjugate reported to be formed in a living organism in which the hydroxyl group is acylated with malonic acid.

The presence of metabolite II as a major terminal residue of EPTC in two diverse plant species, corn and cotton, suggests that yet another class of terminal residue should be considered in studies that deal with the metabolism of pesticides that are converted to GSH conjugates in plants. It is also interesting to note that malonyl conjugates have now been identified as primary conjugates of anilines, secondary conjugates of glucosides, and tertiary conjugates of cysteine and thiolactic acid conjugates derived from the GSH pathway (Lamoureux and Rusness, 1986). It seems likely that malonylation may be a reaction utilized by plants to compartmentalize and/or terminate metabolism.

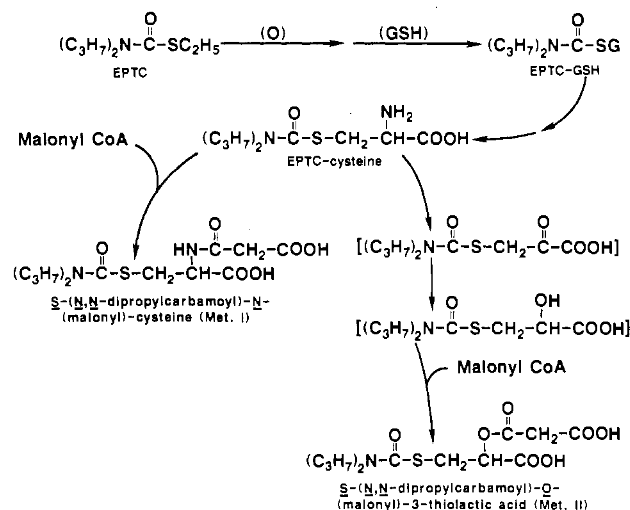

Figure 5. Likely route of formation of metabolites I and II in plants.

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