Comparative Degradation of [^{14}C]-2,4-Dichlorophenoxyacetic Acid in Wheat and Potato after Foliar Application and in Wheat, Radish, Lettuce, and Apple after Soil Application

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The fate of 2,4-dichlorophenoxyacetic acid (2,4-D) applied foliarly as the 2-ethylhexyl ester (EHE) to wheat and potatoes, to the soil as the dimethylamine (DMA) salt under apple tree canopies, and preplant as the free acid for wheat, lettuce, and radish was studied to evaluate metabolic pathways. Crop fractions analyzed for ^{14}C residues included wheat forage, straw, and grain; potato vine and tubers; and apple fruit. The primary metabolic pathway for foliar application in wheat is ester hydrolysis followed by the formation of base-labile 2,4-D conjugates. A less significant pathway for 2,4-D in wheat was ring hydroxylation to give NIH-shift products 2,5-dichloro-4-hydroxyphenoxyacetic acid (4-OH-2,5-D), 4-OH-2,3-D, and 5-OH-2,4-D both free and as acid-labile conjugates. The primary metabolic pathway in potato was again ester hydrolysis. 2,4-D acid was further transformed to 4-chlorophenoxyacetic acid and 4-OH-2,5-D. For the soil applications, ^{14}C residues in the crops were low, and characterization of the ^{14}C residues indicated association with or incorporation into the biochemical matrix of the tissue. The degradative pathways observed in wheat are similar to those characterized in other intact plant studies but differ from those in studies in wheat cell suspension culture in that no amino acid conjugates were observed.

Keywords: *2,4-Dichlorophenoxyacetic acid; 2,4-D; herbicide; metabolism; wheat; potato; apple; lettuce; radish; rotational crop*

INTRODUCTION

2,4-Dichlorophenoxyacetic acid (2,4-D) is the active ingredient (ai) in a variety of herbicidal formulations that are primarily used in pre- and post-emergence applications for broadleaf weed control in turf and field crops. Due to its potent auxin activity, 2,4-D is also used as a plant growth regulator. The metabolic fate of 2,4-D in various plants, in callus tissue, and in cell suspension cultures has been studied extensively (*1*). Reviewed collectively, several pathways for the degradation of 2,4-D in various plant systems are apparent (Figure 1). The major pathways of 2,4-D metabolism include side-chain degradation (*1*−*3*), side-chain elongation, ring hydroxylation (*3*−*8*), conjugation reactions (*2*−*4*), and incorporation into biochemicals or the biochemical matrix of the plant (*9*, *10*).

The metabolism of 2,4-D by wheat was studied using cell suspension culture (*4*). The pathways identified were ring hydroxylation, sugar conjugation, and amino acid conjugation. An intact plant metabolism study (*3*) compared the metabolic pathways operating in a variety of plant tissues: wheat, soybean, saska bean, sunflower, strawberry, and timothy grass. The residues identified in wheat were unaltered 2,4-D (30%), free 2,5-dichloro-4-hydroxyphenoxyacetic acid (4-OH-2,5-D) and 4-OH-2,3-D (0.8%), glucose ester of 2,4-D (24%), phenolic glycosides of 4-OH-2,5-D and 4-OH-2,3-D (10%), and a 2,4-DCP glycoside (2.2%). Amino acid conjugates were not detected. Side-chain elongation products have not been detected in either intact plant systems or tissue culture with wheat exposed to 2,4-D.

As a plant growth regulator, 2,4-D formulations are used at a low concentration in potato [0.07 lb of acid equivalent (ae)/acre]. To obtain sufficient residues in the plant tissue for characterization, a significantly higher application rate was needed but not so high that 2,4-D would exhibit its broad-leaf herbicidal activity. A review of the literature for 2,4-D suggested that a metabolism study at exaggerated application rates could be performed in potato if application was made to more mature plants (*2*, *11*). Potato plants are less susceptible to the herbicidal effects of 2,4-D during flowering and flower-drop growth stages. Also, the use of an early- to mid-season variety of potato will shorten the time between the last application and the harvest of mature tubers to minimize growth dilution of residues for analysis.

This paper presents data on the fate of 2,4-D in spring wheat, potato, and apple after 2,4-D 2-ethylhexyl ester (EHE) was applied to wheat and potato plants and 2,4-D dimethylamine (DMA) salt was applied to the turf beneath an apple tree. Also, degradation of 2,4-D was studied after soil application with 30- and 139-day aging

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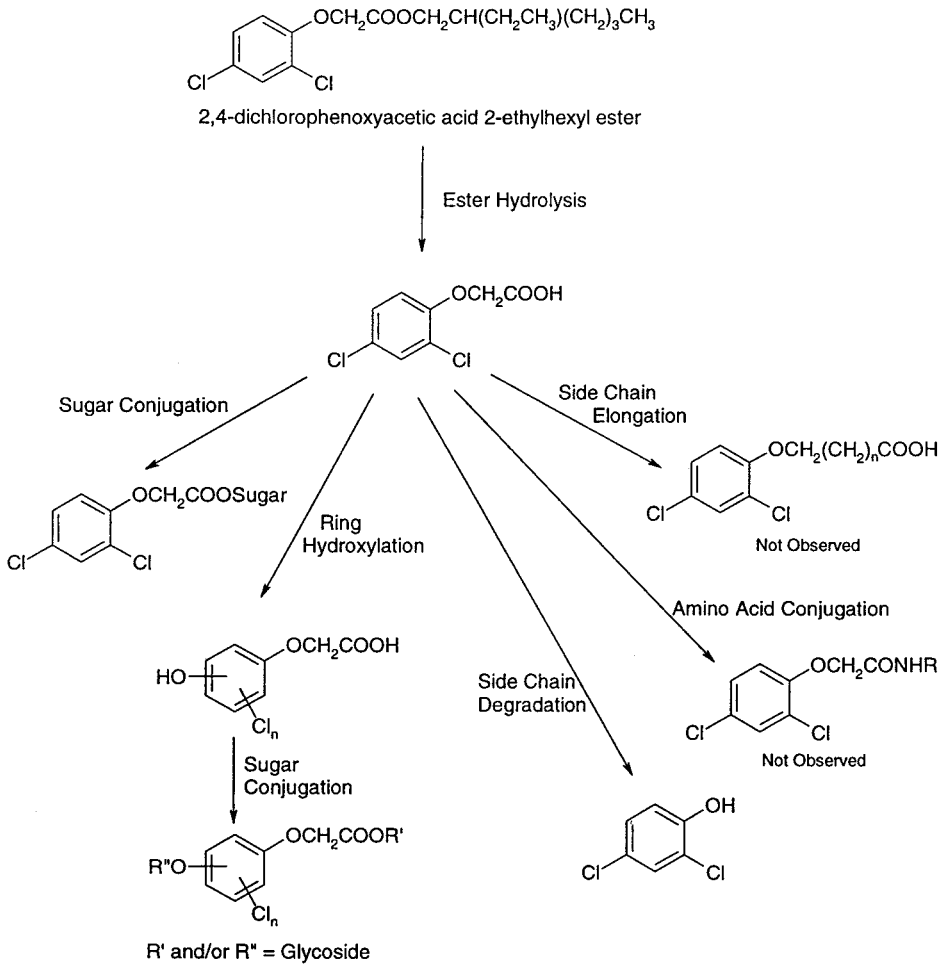

Figure 1. Degradative pathways for 2,4-D in plants.

Table 1. Test and Reference Substances

compound	CAS Registry No.[a]	FW	chem/ radiochem purity,[b] %	sp act. × 10^{-11},[c] Bq/mol	acronym
test substances					
2,4-dichlorophenoxy acetic acid	94-75-7	221.0	>99/>98	8.21−8.99	2,4-D
2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester	1928-43-4	333.3	>99/≥98	6.77−7.51	2,4-D EHE
reference substances					
2,4-dichlorophenol	120-83-2	163.0	≥99/>99	4.70	2,4-DCP
2,3-dichloro-4-hydroxyphenoxyacetic acid	3004-84-0	237.0	95.0/—[d]		4-OH-2,3-D
2,5-dichloro-4-hydroxyphenoxyacetic acid	2639-78-3	237.0	≥99/—		4-OH-2,5-D
2,5-dichloro-4-hydroxyphenoxyacetic acid, potassium salt		275.1	95.5/—		4-OH-2,5-D K
2,5-dichloro-4-hydroxyphenoxyacetic acid, methyl ester	66789-84-2	251.1	97.7/—		4-OH-2,5-D ME
2,4-dichloro-5-hydroxyphenoxyacetic acid	2639-79-4	237.0	≥99/—		5-OH-2,4-D
2,4-dichlorophenoxyacetic acid methyl ester	1928-38-7	235.1	>99/-		2,4-D ME
2,4-dichloroanisole	553-82-2	177.0	99/—		2,4-DCA
4-chlorophenoxyacetic acid	122-88-3	186.6	≥99/—		4-CPAA
4-chlorophenol	106-48-9	128.6	>99/—		4-CP

[a] CAS Registry No. have been provided by the author. [b] For nonradiolabeled test substance. [c] For *phenyl*(U)-^{14}C-labeled chemical. [d] Not available or applicable.

intervals before planting spring wheat, radish, and lettuce crops.

MATERIALS AND METHODS

Test and Reference Substances. The identity, purity, and, when appropriate, specific activity for the test and reference substances are given in Table 1. The radiolabeled test substances were uniformly ^{14}C-labeled in the phenyl ring.

Crops, Application Parameters, and Tissue and Soil Sampling. Table 2 summarizes the pertinent data for the biological phase of the studies. After treatment, wheat and potato plants were irrigated manually at the soil surface only to ensure the foliarly applied [^{14}C]-2,4-D EHE would not be washed from the leaf surface and would remain available for uptake by the plants. All crops were maintained according to good agricultural practices throughout the studies. Plant tissues were frozen after collection. For the soil-applied studies, multiple, 45-cm soil cores (2-cm diameter) were taken prior to application, on the day of application, at planting (if appropriate), and at each tissue sampling. The soil cores were frozen after collection and segmented into either 7.5- or 15-cm sections. Like soil sections were composited from the multiple cores taken at each sampling point.

Processing of the Plant Tissues and Soil Samples. The immature potato vines [1 and 7 days after treatment (DAT)]

Table 2. Summary of the Conditions during the Biological Phase for the Foliar Applications

biological phase	crops	
test system		
identity	spring wheat	potato
variety	Marshal	Dark Red Norland
test plots		
size, m^2	0.89	2.0
location[a]	greenhouse	greenhouse
soil type	sandy loam	sandy loam
test materials		
test substances	2,4-D EHE and [*phenyl*-(U)-^{14}C]-2,4-D EHE	
formulation/diluent	emulsifiable concentrate/water	
final sp act., Bq/mol	7.36×10^{10}	5.71×10^{11}
appln parameters		
equipment	glass container/flat-fan nozzle/CO_2	
method/site	broadcast/foliage	
no. of appln	1	2
crop stage at appln	tillering	tuber formation
appln rate, lb of ae/acre/appln	1.5	0.31
max labeled use rate, lb of ae/acre/appln	1.5	0.07
sampling		
crop fraction	forage (10)[b]	vines/tubers (1)
	grain (49)	vines/tubers (7)
	straw (49)	tubers (20)

[a] ABC Laboratories, Inc., Columbia, MO. [b] DAT given in parentheses.

were rinsed with acetonitrile prior to further processing. The 1-DAT vines were rinsed in a beaker containing ~0.7 L of acetonitrile; the 7-DAT vines were rinsed with ~4 L of acetonitrile. Wheat forage, straw, and grain, potato tubers, acetonitrile-rinsed potato vines, lettuce, radish roots and tops, and apple fruit were ground mechanically to a fine powder in the presence of dry ice or liquid nitrogen. The freezing agent was allowed to sublime or evaporate, and then the processed plant tissues were weighed and stored frozen. Composited soil sections were processed mechanically in the presence of dry ice similar to the procedure for the plant tissue.

Radioassay. Radioactivity in plant tissues, soil samples, and the tissue pellet remaining after solvent extraction [post-extracted sample (PES)] was quantified by combustion of an aliquot of the sample in commercial biological oxidizers followed by liquid scintillation counting (LSC) of the base-trapped CO_2. Moisture analyses were conducted on the soil samples, and the total radioactive residue (TRR) in the soil was reported on a dry-weight basis. Radioactivity in the plant tissues and PESs was reported at the moisture content of the harvested tissues. Total radioactivity in the various liquid extracts was quantified by LSC with commercial instrumentation.

Isolation of Organosoluble and/or Aqueous Soluble ^{14}C Residues from Plant Tissues. For wheat forage, grain, and straw, lettuce, and radish root and top, aliquots of the frozen, processed tissue samples were weighed and then extracted with multiple aliquots of the appropriate solvent(s) as summarized in Figure 2. Mechanical tissue homogenizers were used as needed to ensure satisfactory contact between the matrix and the solvent. Upon addition of diethyl ether to the acidified aqueous slurry of wheat grain, the grain matrix swelled, requiring the separation of the aqueous phase from the matrix prior to the extraction of the matrix with aqueous ethanol. In a separate procedure, wheat grain from the foliar application was extracted with 4:1 acetonitrile/water followed by a water extraction.

For potato tubers, the ^{14}C residues in 50 g of tissue were solubilized with acetonitrile ($5 \times 20-25$ mL); an acidified (pH 2) acetonitrile extraction was conducted initially ($4 \times 25-50$ mL). Solubilization of the ^{14}C residues in apple fruit was pursued by Soxhlet extraction of 700 g of processed fruit with 500 mL of 95:5 methanol/acetic acid for 72 h. The organic phase was removed from the extracted tissue; the solvent was removed from the organic phase, and the remaining residue

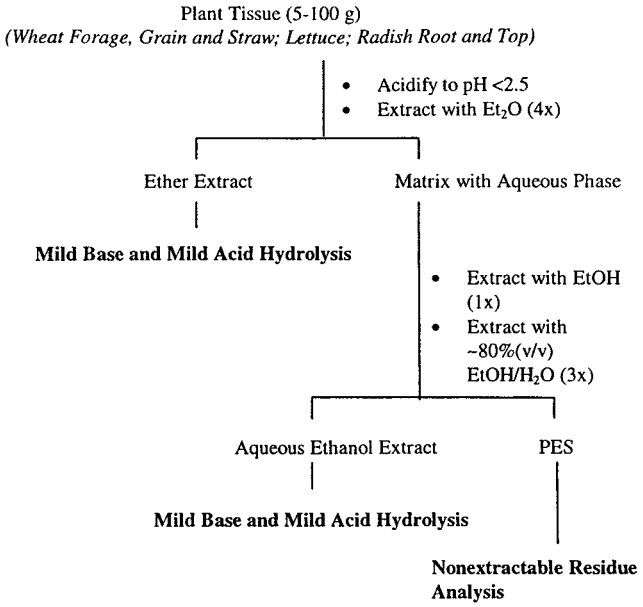

Figure 2. General extraction scheme for plant tissues.

was reconstituted in 200 mL of water. This aqueous solution was partitioned against ethyl acetate (4×100 mL).

Isolation of Aqueous Soluble and Organosoluble ^{14}C Residues from Soil. Aliquots of the soil samples were extracted according to the method of McCall et al. (*12*). Briefly, the aliquot was dispersed in 1.5 M H_3PO_4 and extracted with diethyl ether. The ether and aqueous acid phases were separated from the soil, and the extracted soil was further extracted with 1.0 N NaOH. This procedure yielded four fractions: ether, aqueous acid, NaOH, and extracted soil.

Characterization of the Soluble ^{14}C Residues. Prior to chromatographic analysis of the soluble ^{14}C activity, typically the extract or an aliquot of the extract was reduced under vacuum and the residue was reconstituted in a small volume of a solvent compatible with the chromatographic technique to be used. As needed, the extracts were subjected to the appropriate hydrolytic procedure (Figure 2). Individual ^{14}C components were tentatively identified on the basis of their chromatographic mobility compared to that of reference standards. Tentative chromatographic identifications were confirmed by gas chromatography−mass spectroscopy (GC-MS). Acidic ^{14}C isolates were methylated with diazomethane prior to GC-MS. When reference standards for the methylated ^{14}C isolates were not available, the appropriate nonmethylated reference standard was carried through the methylation procedure along with the ^{14}C isolate.

Mild Hydrolytic Techniques. The PES from the final aqueous extraction or organoextraction procedure was then subjected to mild base or mild acid hydrolytic procedures.

Mild Base Hydrolysis (MBH). The organic solvent, if present, in the extract to be hydrolyzed was removed under vacuum before the solution was made 1 M in base with sodium hydroxide. After 1 h at room temperature, the basic hydrolysate was acidified to pH 1 with hydrochloric acid and partitioned against diethyl ether to give ether-MBH and aqueous-MBH fractions.

Mild Acid Hydrolysis (MAH). The organic solvent, if present, in the extract to be hydrolyzed was removed under vacuum before it was acidified to 1 M acid with hydrochloric acid. The acidic solution was heated at or near reflux for 2 h. The hydrolysate was partitioned against diethyl ether to give ether-MAH and aqueous-MAH fractions.

The acetonitrile extract from potato tubers was acidified to pH 2 with 6 M hydrochloric acid. The acidified extract was heated at 45−50 °C under nitrogen gas for 16 h; HPLC analysis indicated incomplete hydrolysis by the presence of peaks for both 2,4-D and the putative conjugate, so the hydrolytic reaction was continued for another 20 h under

nitrogen gas at 90 °C. HPLC analysis still indicated incomplete hydrolysis. The pH value was dropped to 1, and hydrolysis was continued for another 24 h under nitrogen gas at 90 °C.

Characterization of Nonextractable ^{14}C Residues. The PES from the mild hydrolytic procedures was then subjected to strong base or enzymic hydrolytic procedures. In addition, procedures to isolate natural products were employed when bound residues remained in the PES after the strong base or enzymic hydrolytic procedures.

Strong Base Hydrolysis (SBH). PESs were extracted at room temperature with 0.5 M potassium hydroxide in 50% aqueous methanol. The strong base extract was then reduced in volume by 50% under vacuum, acidified to pH 1 with hydrochloric acid, and partitioned against diethyl ether to give the ether-SBH and the aqueous-SBH fractions.

Enzymic Digestions. Cellulase digestions were carried out at pH 5 in 0.02 M sodium acetate buffer at 37 °C for 16−48 h. Amylase and protease digestions were conducted similarly except at pH values of 6.8 and 7, respectively.

Cellulose and Lignin Characterization. Crude cellulose and lignin were isolated from bound residues by a modification of a procedure reported by Honeycutt and Adler (*1*). Instead of a 3-h, high-pressure, basic hydrolysis used in the referenced procedure, the PES was heated at reflux for 22 h in 5 N sodium hydroxide; also, the crude cellulose was not washed with a sodium hydroxide solution but dried as the filtrate cake originally collected. The acidic filtrate from the crude lignin precipitate was partitioned against diethyl ether to give alkaline hydrolysate, ether and alkaline hydrolysate, aqueous fractions.

Starch Characterization. The starch fraction was isolated from wheat grain from the soil application according to the procedure of Wargo et al. (*14*).

Glucosazone Isolation. Anhydrous ethanol was used to precipitate the starch from solution in dimethyl sulfoxide. The purified starch was then hydrolyzed to glucose and derivatized with phenylhydrazine to form the glucosazone. The crude glucosazone was isolated and purified to a constant specific activity by recrystallization from acetonitrile. The ^{14}C percentage incorporated into starch was then calculated.

Protein, Starch, and Cellulose Characterization for Bound Residue in Wheat Grain (Foliar). The procedure for isolation of these biochemical fractions is presented in Rouchaud et al. (*15*). Selvendran and O'Neill (*16*), and Wargo et al. (*14*).

HPLC. Commercial high-performance liquid chromatographic (HPLC) equipment was used with Nova-Pak C-8 or C-18 columns (8 × 100 mm) (Waters, St. Louis, MO) and acetonitrile/water mobile phases acidified with 0.5−1% (v/v) acetic acid. Chromatographic conditions for method 1 consisted of two sequential linear gradients, the first, 30 min from 10% acetonitrile to 50% acetonitrile, and the second, 15 min to 100% acetonitrile and then isocratic for 15 min at 100% acidified acetonitrile at a flow rate of 1.5 mL/min. Retention times for 4-OH-2,5-D; 4-OH-2,3-D; 5-OH-2,4-D; 2,4-D; 2,4-DCP; and 2,4-D EHE were ∼13, ∼14, ∼19, ∼25, ∼27, and ∼46 min, respectively. Method 2 consisted of two sequential linear gradients, the first, 20 min from 20% acetonitrile to 50% acetonitrile, and the second, 5 min to 100% acetonitrile and then isocratic for 10 min at 100% acetonitrile at a flow rate of 1.5 mL/min. Retention times for 4-OH-2,5-D; 4-OH-2,3-D; 4-CPAA; 4-CP; 2,4-D; 2,4-DCP; 2,4-D ME; 2,4-DCA; and 2,4-D EHE were ∼7.5, ∼8.5, ∼12.5, ∼14, ∼17, ∼19, ∼24.5, ∼26, and ∼31 min, respectively. Elution of radiolabeled components was monitored by LSC of eluant fractions. Nonradioactive reference standards were detected by ultraviolet (UV) absorbance at 280 nm.

TLC. TLC was performed on silica gel 60 F-254 plates (E. Merck, Darmstadt, Germany) either 250 μm or 1 mm thick. Typical solvent systems were (A) hexane/2-propanol (1:1) containing 5% (v/v) acetic acid, (B) heptane/2-propanol (9:1), and (C) toluene/ethyl acetate/acetic acid (10:10:1). Radiolabeled zones were located by radioscanning with commercial equipment. Nonradioactive species were observed by fluorescence quenching detected under UV light.

GC-MS. GC-MS was performed with a Finnigan Mat 5100 quadrupole mass spectrometer interfaced with a Finnigan model 9611 gas chromatograph. The mass spectrometer was operated in the electron ionization mode with the ionizing voltage set at 70 eV. The separations were performed on a 30-m J&W DB-5 phenylmethylsilicone-coated, fused-silica capillary column.

For the potato study, GC-MS was performed using a Hewlett-Packard 5970B quadrupole mass spectrometer interfaced with a Hewlett-Packard 5890 series II gas chromatograph. The mass spectrometer was operated in the EI mode with the ionizing voltage set at 70 eV. The GC separations were performed on a 30-m J&W DB-1701 cyanopropylphenylmethylsilicone-fused silica capillary column.

Limits of Detection. Limits of detection for LSC methods were calculated according to the method of Currie (*17*).

RESULTS AND DISCUSSION

Total Radioactive Residues (TRR). TRR values in the crops are summarized in Table 4. TRR values ranged from 5 to >600 times higher in the wheat samples from the foliar application versus soil application. The ^{14}C residues in apple fruit were also extremely low after two applications to the turf under the apple tree during the fruit-formation stage of growth. As reviewed by Loos (*1*), plant roots readily adsorbed phenoxyalkanoic acids, such as 2,4-D, with limited translocation to above-ground parts of the plant. Application to the turf beneath the apple tree to control broad-leaf weeds would not be expected to yield significant residue in the fruit because of the restricted translocation of phenoxyalkanoic acids out of the roots. In the case of the crops planted at two aging intervals after soil application, the rapid degradation of 2,4-D by soil microbes seen in this study and others (*1*, *12*, *18*) limits the amount of 2,4-D available for potential root uptake.

Immature vine and tuber samples were taken at 1 and 7 days after the second application to evaluate the uptake of [^{14}C]-2,4-D EHE into the vines. At 1 DAT (after second application), the ^{14}C residues in the rinsate, rinsed vines, and tubers was 5, 10, and 0.3 ppm, respectively; at 7 DAT, these values were 0.3, 10, and 0.6 ppm, respectively; at maturity (20 DAT), these values were 6 and 0.58 ppm for unrinsed vines and tubers, respectively. The majority of the available ^{14}C activity is not releasable by an acetonitrile surface rinse of the vines after 7 days from application. From HPLC of the rinsate, 96 and 74% of the ^{14}C residue in the rinsate was 2,4-D EHE at 1 and 7 DAT, respectively. At 7 DAT ∼17% of the ^{14}C residue in the rinsate was strongly retained under strong elution conditions (i.e., 80−100% acetonitrile in a two-step, linear gradient over 25 min). No further characterization of the strongly retained material was conducted. Also, the amount of activity in the tuber doubled over a 7-day period despite the growth of the tuber during this period. This suggests active translocation of the ^{14}C activity from the leaves to the tubers during this growth period.

In the foliar application study for wheat, low levels (0.1−0.5 ppm) of ^{14}C residues were detected in the untreated (Table 4) and pretreatment samples. Except for wheat grain, the background ^{14}C residue values were insignificant compared to the ^{14}C levels attained by the foliar application of [^{14}C]-2,4-D EHE. The presence of ^{14}C residues in these samples was attributed to the incorporation of $^{14}CO_2$ generated by the microbial degradation of [^{14}C]-2,4-D EHE applied to soil for a

Table 3. Summary of the Conditions during the Biological Phase for the Soil Applications

biological phase	crops			
test system				
identity	apple	lettuce	radish	spring wheat
variety	Regent/M26 dwarf	Waldmans	White Icicle	Proband 775
test plots				
size, m^2	2 m tall, >2 m canopy	0.93	0.93	1.9
location	field[a]		field[b]	
soil type	Londo sandy loam		sandy loam	
test materials				
test substances	2,4-D and [*phenyl*(U)-^{14}C]-2,4-D			
formulation/diluent	DMA salt/water	none/50 (v/v)% acetone/water		
final sp act., Bq/mol	7.40×10^{10}	9.58×10^{10}		
appln parameters				
equipment	glass container/flat-fan nozzle/CO_2	backpack sprayer/8003E nozzle/CO_2		
method/site	broadcast/soil			
no. of appln	2	1	1	1
crop stage at appln	petal fall and fruiting	30 or 139 days preplant		
appln rate, lb of ae/acre/appln	1.9	2.1	2.1	2.1
maximum labeled use rate, lb of ae/acre/appln	2	2	2	2
sampling				
crop fraction	fruit (56)[c]	leaves[d]	top[d] root[d]	forage[d] grain[d] straw[d]

[a] The Dow Chemical Co., Field Research Station, Midland, MI. [b] Pan-Agricultural Labs, Inc., Madera, CA. [c] DAT given in parentheses. [d] At maturity.

Table 4. Total Radioactive Residues in the Crops

	TRR, ppm of 2,4-D ae		
		soil application	
crop	foliar application[a]	30 days preplant	139 days preplant
wheat, forage	34 (0.1)	ND[b]	0.030
wheat, straw	56 (0.5)	0.060	0.084
wheat, grain	0.30 (0.17)	0.049	0.060
potato, tubers	0.58	–[c]	–
apple, fruit	–	0.009[d]	–
radish, top	–	0.043	0.010
radish, root	–	0.010	0.011
lettuce	–	0.019	0.013

[a] The TRR values in parentheses are for untreated wheat forage, straw, and grain. See Results and Discussion. [b] ND, not detected. The limit of detection was 0.001 ppm of 2,4-D ae. [c] No application was made for this use pattern. [d] Applications were made to the ground beneath the apple tree at petal fall and then again 42 days later.

different study in the same greenhouse and allowed to age. The degradation of 2,4-D by soil microbes is known to produce CO_2, with 25−50% of the applied material converted to CO_2 within 20−25 days (*12*, *18*). The greenhouse was ventilated; however, the likely presence of $^{14}CO_2$ in the atmosphere from the soil degradation is the probable source for the ^{14}C activity in the untreated crops as well as a major contributor to the TRR for treated wheat grain.

Radioassay of the composited 0−15-cm section of the 0- and 30-day soil cores showed TRR values of 0.6 and 0.2 ppm, respectively. Less than 0.006 ppm of 2,4-D ae was found in the soil cores at a depth >15 cm at the 30-day planting.

Distribution of Extractable and Nonextractable ^{14}C Residues. Greater than 77% of the TRR was extractable from the crop tissues with organic or aqueous organic solvents for the foliar applications with the exception of wheat grain (Tables 5−7). Only ∼11% of the grain TRR was soluble in acetonitrile and water. The grain head was not yet formed when the foliar application was made. For all aging intervals for the soil applications (Table 8), 55−82% of the crop fraction TRR was not extractable with organic or aqueous solvents. For the ^{14}C residue that was solubilized, 77−100% of the extractable residue was found in the aqueous ethanol extract. Control experiments with [^{14}C]-2,4-D-fortified radish tops and roots, lettuce, and wheat straw and forage from the untreated plots showed that if 2,4-D were present in these crop samples, [^{14}C]-2,4-D would be found in the ether extracts. The control tissues were fortified with [^{14}C]-2,4-D at approximately the TRR value. For these fortified extractions, from 79 to 101% of the fortified ^{14}C activity was recovered in the ether extract.

Chromatographic analysis of the soluble activity in the 0−15-cm segments of the soil cores from the 0- and 30-day plantings indicated that the 2,4-D concentrations were 0.6 and 0.01 ppm, respectively. Thus, even though significant ^{14}C activity was present at the 30-day planting (0.2 ppm), very little 2,4-D remained for uptake by the crops grown in this soil. The non-2,4-D ^{14}C residue in the soil at the 30-day planting was characterized as predominantly nonextractable (70% of TRR) with diethyl ether from an acidic aqueous soil slurry. This nonextractable residue was composed of ^{14}C-labeled fulvic acid, humic acid, and humin at 16, 12, and 14% of the TRR, respectively. Thus, the microbial degradation of [^{14}C]-2,4-D in the soil radiolabeled many soil components that could then be taken up by the roots and transformed into the biochemical structure of the growing plant.

For apple fruit, 56% (0.005 ppm) of the TRR was solubilized, but none of the radioactivity could be quantifiably extracted into ethyl acetate. The remaining residue (0.004 ppm) was nonextractable by the conditions employed. No further characterization was conducted on these very low residue fractions.

Characterization and Identification of the Extractable ^{14}C Residues. *Wheat Straw (Foliar).* The predominant ^{14}C components in the ether extract prior to hydrolysis (Figure 3a) were 2,4-D (6% of TRR) and 2,4-D EHE (2% of TRR) as judged by chromatographic

Table 5. Distribution and Identification of Extractable and Nonextractable ^{14}C Residues in Wheat after Foliar Application of [*pheny*(U)-^{14}C]-2,4-D EHE[a]

fraction	% TRR		structural assignment techniques[b]
	wheat, straw	wheat, forage	
unextracted crop	**100**	**100**	
ether extract	*14*[c]	*20*	
ether-MBH			
4-OH-2,5-D	1.8	2.8	GC-MS
4-OH-2,3,D	0.2	0.4	GC-MS (straw only)
5-OH-2,4-D	0.6	1.0	GC-MS (straw only)
2,4-D	8.1	13	Co-TLC, GC-MS
2,4-DCP	0.4	0.2	GC-MS (straw only)
unidentified (6−10 components)	each ≤0.9	each ≤1.2	
aqueous-MBH	0.3	0.6	
ether-MAH	0.3	0.4	
4-OH-2,5-D	<0.1	<0.1	
2,4-D	<0.1	<0.1	
unidentified (3 components)	each ≤0.2	each ≤0.1	
aqueous-MAH	<0.1	0.1	
aqueous ethanol extract	*63*[d]	*66*	
ether-MBH	49	57	
4-OH-2,5-D	0.5	0.4	GC-MS (straw only)
4-OH-2,3,D	0.1		
5-OH-2,4-D	0.2	0.2	
2,4-D	48	55	co-TLC, GC-MS
2,4-DCP	ND	0.1	
unidentified (1−2 components)	each ≤0.5	each ≤1.1	
aqueous phase-MBH	13	8.8	
ether-MAH	12	7.3	
4-OH-2,5-D	6.2	4.8	GC-MS
4-OH-2,3,D	2.2	1.5	GC-MS (forage only)
5-OH-2,4-D	0.8	0.2	GC-MS (forage only)
2,4-D	0.2	0.4	co-TLC (straw only)
2,4-DCP	0.5	0.2	GC-MS (forage only)
unidentified (2 components)	each ≤1.8	each ≤0.1	
aqueous phase-MAH	1.4	1.5	
strong base hydrolysate	*19*	*12*	
ether-SBH	18	8.9	
2,4-D	16	8.8	
unidentified (1−2 components)	each ≤1.5	each ≤0.1	
aqueous-SBH	0.9	1.6	
cellulase digestible	*1.3*	*0.3*	
amylase digestible	*0.4*	*0.1*	
tissue unextractable	*0.4*	*0.4*	
total recovered	**98**	**100**	

[a] TRR values for wheat straw and wheat forage were 56 and 34 ppm of 2,4-D ae, respectively. [b] Initial structural assignment was made with HPLC by comparison of chromatographic mobility with that of the reference standards. Co-TLC, cochromatography. [c] Figure 3b. [d] Figure 3c.

mobility with comparison to the reference standards. Because xenobiotic esters are rapidly de-esterified by plant esterases, the detection of 2,4-D EHE required confirmation. The identity of 2,4-D EHE was confirmed by TLC cochromatography (solvent system B) and GC-MS analysis of ∼0.5 μg of the ^{14}C component eluting between 46 and 50 min. The presence of 2,4-D EHE in the initial tissue extract can be explained by considering the growing conditions for wheat in the foliar application study. Manual irrigation of the growing plants was directed to the soil surface to prevent removal of the surface-applied [^{14}C]-2,4-D EHE; thus, any ^{14}C activity remaining on the plant surface would not be available to plant esterases and could survive unchanged. In the potato study discussed above, the vines were rinsed with acetonitrile prior to tissue processing and extraction to demonstrate that the ester could be found on the leaf surface at least 7 days after application. Confirmation of the identity of the ^{14}C component eluting with a retention time similar to that of 2,4-D (24.0−26.5 min, HPLC method 1) was conducted on ∼1 μg of the ^{14}C component eluting at 24−26.5 min. The component was isolated, purified, derivatized, and analyzed by GC-MS. The mass spectral data and the GC retention time of the derivatized product matched those of the methylated reference standard for 2,4-D. A minor ^{14}C component (0.5% of TRR) with HPLC elution behavior (27−28 min) similar to that of 2,4-DCP was also observed. A 0.1-μg portion of this component was isolated, purified, derivatized, and confirmed to be 2,4-DCP by GC-MS.

At least five ^{14}C components were observed in the 9−20 min region (Figure 3a) in the ether extract prior to hydrolysis. The ring-hydroxylated reference standards eluted in this region under these HPLC conditions, but more ^{14}C components were observed than could be accounted for by an NIH-shift hydroxylation (*19*). Hydrolytic techniques were used to determine if any of the ^{14}C components possibly were conjugates of 2,4-D moieties. Preliminary mild basic and acidic hydrolytic experiments with the ether- and ethanol-extractable ^{14}C residues from straw indicated that mild base hydrolysis was the preferred method for the conversion of the majority of the ^{14}C residues into 2,4-D. The results of the HPLC analyses of the hydrolysates and the ether extract from the diethyl ether partitioning steps are presented in Table 5 together with a summary of the structural confirmation techniques used in addition to HPLC mobility. In Table 5, no 2,4-D EHE is

Table 6. Distribution and Identification of Extractable and Nonextractable ^{14}C Residues in Wheat Grain after a Foliar Application of [*phenyl*(U)-^{14}C]-2,4-D EHE[a]

wheat grain fraction	%TRR treated	%TRR untreated
unextracted crop	**100**	**100**
acetonitrile/water extract	*3.7*	*2.4*
2,4-D	1.3	–[b]
unidentified (3 components)	≤1.7	–
water extract	*7.7*	*8.3*
ether-MAH	1.3	–
aqueous phase	3.7	–
unaccounted for after MAH	2.7	–
strong base hydrolysate	*28 (41)*[c]	*19 (29)*
ether-SBH	5.4 (7)	3.0 (4)
2,4-D	4.7 (~4)	(~2)
unaccounted for after HPLC	0.7 (~3)	(~2)
aqueous phase-SBH	5.7	5.9
precipitate[d]	17	9.5
amylase digest	*2.3*	*2.4*
cellulase digest	*4.7*	*4.7*
protease digest	*3.0*	*2.4*
enzymic PES	*35*	*40*
ether-MAH	1.3	1.2
aqueous phase-MAH	21	26
unknown (2.0–5.0 min)[e]	16	–
unaccounted for after MAH	3.7	–
tissue bound	*8.9*	*11*
total recovered	**84**	**79**

[a] TRR values for treated and untreated were 0.30 and 0.17 ppm of 2,4-D ae, respectively. [b] Not observed. [c] Results for a strong base hydrolysis only of the unextracted crop. [d] The precipitate was combined with the enzymic PES prior to mild acid hydrolysis. [e] HPLC method 1.

Table 7. Distribution and Identification of Extractable and Nonextractable ^{14}C Residues in Potato Tubers after a Foliar Application of [*phenyl*(U)-^{14}C]-2,4-D EHE[a]

fraction	% TRR
unextracted crop	**100**
acetonitrile extract	*87*
mild acid hydrolysis	80
4-OH-2,5-D (metabolite A)	15[b]
4-CPAA (metabolite B)	24[b]
2,4-D (metabolite C)	40[b]
unidentified (5 components)	≤2.8
bound residue	*14*
mild acid hydrolysis	8.7
4-OH-2,5-D	0.9
4-CPAA	1.6
2,4-D	2.3
unidentified (5 components)	≤1.9
unextractable solid	*6.4*
total recovered	**105**

[a] TRR value for potato tuber was 0.58 ppm of 2,4-D ae. [b] Initial structural assignment was made with HPLC (method 2) by comparison of chromatographic mobility with that of the reference standards; structure was confirmed by GC-MS.

reported because mild base hydrolysis converted the ester to 2,4-D. The concentration of the major ^{14}C components observed in ether-MBH by HPLC analysis was essentially unchanged before and after mild base hydrolysis except for the increase in the 2,4-D concentration by an amount equal to the concentration of 2,4-D EHE in the ether extract. The peaks in the radiochromatogram of ether-MBH were sharper after mild base hydrolysis, allowing the quantification of seven minor ^{14}C components in the 9–20-min region versus two prior to hydrolysis.

Analysis of the aqueous ethanol extract by HPLC method 1 yielded a radiochromatogram with very poor resolution with 50% of the ^{14}C activity eluting over a 30-min region (Figure 3b). Reanalysis of the aqueous ethanol extract after storage at 4 °C for ~4 months showed free 2,4-D. This increase in free 2,4-D concentration with time at freezer temperatures supports the suggestion that the ^{14}C components shown in Figure 3b were somewhat labile conjugates of 2,4-D. The aqueous ethanol extract was subjected to mild base hydrolysis followed by mild acid hydrolysis of the aqueous-MBH fraction to convert the aqueous ethanol-extractable residues to ether-soluble aglycons. In work on the metabolism of 2,4-D by wheat cell suspension Bristol et al. (*4*) noted that the water-soluble metabolites (in this paper represented by the aqueous-ethanol extractable fraction) were incompletely converted to ether-soluble aglycons by either base or acid hydrolysis alone. Both hydrolytic procedures were required. The results of the HPLC analyses of the hydrolysates and the ether extract from the diethyl ether partitioning steps are shown in Table 5 with a summary of the confirmation techniques used to identify the ^{14}C components. 2,4-D, released by mild base hydrolysis, composed 77% of the total radioactivity in the aqueous ethanol extract. Minor amounts of the ring-hydroxylated 2,4-D moieties were also observed. Acid hydrolysis released the majority of the ^{14}C residues remaining in the mild base hydrolysate; however, only a minor amount of 2,4-D was released. The majority of the residue in the mild base hydrolysate (71% of the TRR in the aqueous phases-MBH) was from the ring-hydroxylated 2,4-D moieties.

Wheat Forage (Foliar). The results of the characterization of the extractable ^{14}C residues from forage are also summarized in Table 5. Qualitatively, the same types of HPLC and hydrolytic behavior and degradates were observed for the wheat forage extracts as for the wheat straw extracts described in detail above. Assuming the ethanol-extractable forage residues were predominantly glycosyl conjugates because of their base lability, the distribution of aglycons between ether-MBH and ether-MAH centers on the structure of the aglycons. The base hydrolytic procedure was somewhat milder than the acid hydrolysis step. 2,4-D accounted for 96% of the ether-soluble residues from the aqueous ethanol extract after mild base hydrolysis, whereas the ring-hydroxylated 2,4-D derivatives accounted for 89% of the ether-soluble residues after acid hydrolysis of the aqueous phase-MBH from the aqueous ethanol extract. This suggests the formation of phenolic glycosides with the hydroxylated 2,4-D derivatives. Such glycosides are typically base stable but can be cleaved under acidic conditions.

Wheat Grain (Foliar). The treated and untreated grain samples were analyzed side by side. The results from the characterization of the extractable ^{14}C fractions for both treated and untreated tissues are presented in Table 6. The percentage distribution of ^{14}C activity between the acetonitrile and water extracts is comparable.

Potato Tuber. Concentration of the initial acidic acetonitrile extract of potato tuber indicated that the nature and relative amounts of the ^{14}C residue was affected by the extent of concentration of the original extract. This result was interpreted as a sensitivity of the ^{14}C residues to the mildly acidic conditions of the initial extraction solvent. Subsequently, the ^{14}C residues in potato tuber were characterized from a neutral acetonitrile extract. Figure 3c shows the distribution of ^{14}C residues in the neutral acetonitrile extract. The ^{14}C

Table 8. Extractable/Nonextractable Distribution of ^{14}C Residues in Crops Planted 30 and 139 Days after the Soil Application of [*phenyl*(U)-^{14}C]-2,4-D

	% TRR					
fraction	radish, top (0.043/0.010)[a]	radish, root (0.010/0.011)	lettuce (0.019/0.013)	wheat, straw (0.060/0.084)	wheat, forage (0.030)[b]	wheat, grain (0.049)[c]
unextracted crop	100	100	100	100		100
ether extract	ND[d]/ND	ND/ND	ND/ND	5/5	ND	<2
(aqueous phase						10)[e]
aqueous ethanol extract	26/40	50/27	37/46	28/17	27	8
nonextractable residues	81/70	70/55	58/62	67/82	73	57
total recovered	107/110	120/82	95/108	100/104	100	76

[a] 30-day TRR/139-day TRR; all TRR values in ppm of 2,4-D ae. [b] 139-day TRR value only. [c] 30-day TRR value only. [d] ND, not detected. The limit of detection was ≤0.004 ppm of 2,4-D ae. [e] The aqueous phase from the matrix with aqueous phase from Figure 2.

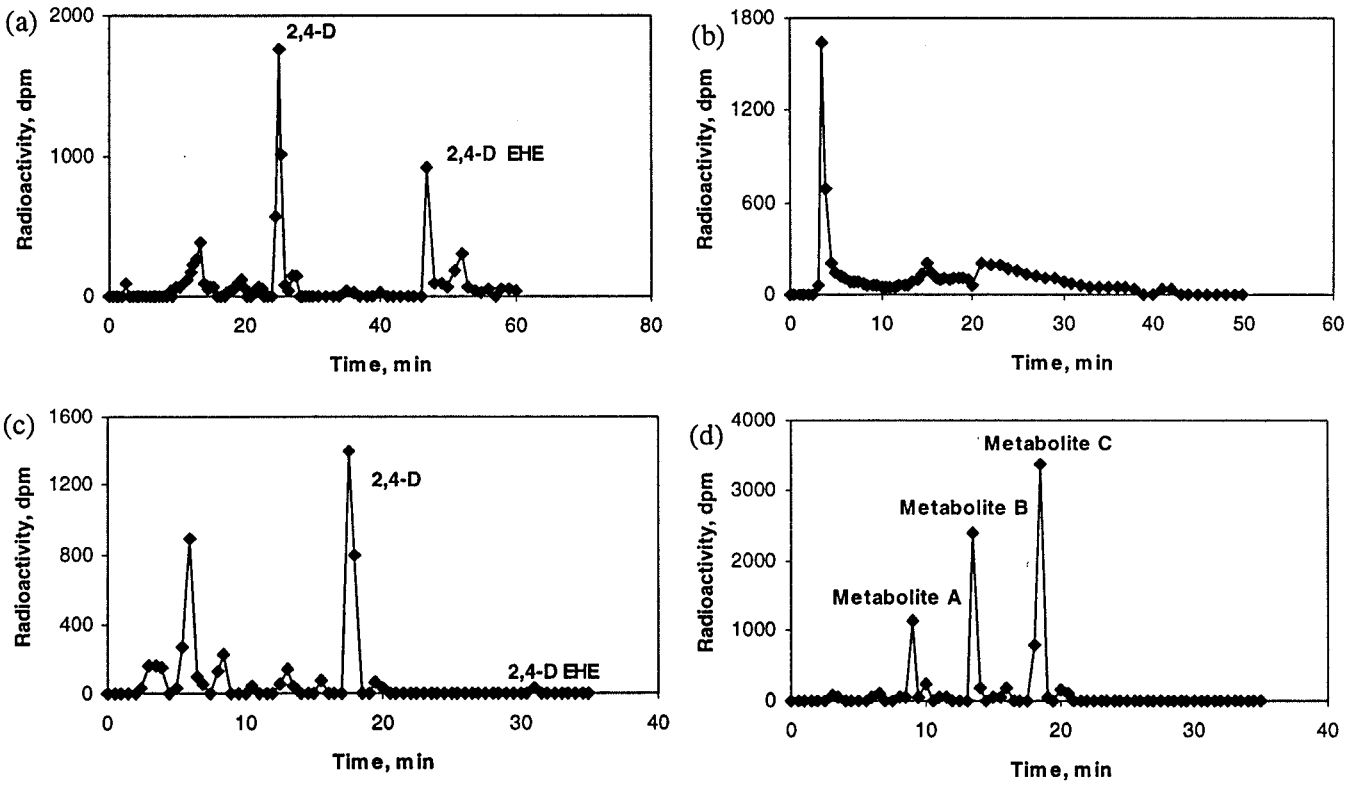

Figure 3. HPLC radiochromatogram of (a) the ether extract of wheat straw, (b) the aqueous ethanol extract of wheat straw, (c) the acetonitrile extract of potato, and (d) the mild acid hydrolysate from the acetonitrile extract of potato tubers.

residue profile in the mild acid hydrolysate of the neutral acetonitrile extract is shown in Figure 3d. The ^{14}C components in Figure 3d were labeled metabolites A−C.

Metabolites A−C were isolated by HPLC using method 2. The ^{14}C isolates were derivatized and their identities were tentatively suggested to be the methyl esters of 4-OH-2,5-D, 4-CPAA, and 2,4-D for metabolites A, B, and C, respectively, by comparison of the chromatographic mobility of the derivatives with that of the nonradiolabeled standards for these methyl esters. GC-MS confirmed the identities as tentatively proposed.

The results of the extraction and characterization procedures used for the extractable ^{14}C residues from potato tuber are presented in Table 7. Mild acid hydrolysis was sufficient for hydrolyzing any conjugates observed in the neutral acetonitrile extract.

Crops (Soil). The ^{14}C residue in the ether extract was ≤0.005 ppm for all crop fractions, which had detectable ^{14}C residues (Table 8). Experiments to evaluate the partitioning behavior of 2,4-D by diethyl ether from the crop matrices were conducted. Untreated matrix was fortified at approximately the TRR value with [^{14}C]-2,4-D and extracted according to the procedure outlined in Figure 2. Recovery of 2,4-D in the ether extract ranged from 89 to 101% of the fortified value. Thus, if 2,4-D residues were present in the crops grown in treated soil, they were not present at >0.005 ppm under the application conditions of this study (Tables 2 and 3). As was true for the foliar applications, the major pool of extractable ^{14}C residue was the aqueous ethanol extract; however, the absolute quantity of ^{14}C activity was very low and not characterized further.

Characterization of the ^{14}C Nonextractable Residues. *Wheat Straw and Forage (Foliar).* For wheat straw and forage from the foliar application, the amount of nonextractable residue was ≤23% of TRR. Table 5 summarizes the results of the strong base hydrolyses and enzyme digestions for both tissues. The majority of the organo/aqueous nonextractable residue was released by the strong base hydrolysis procedure, with the majority of the residue being 2,4-D. This finding supports the suggestion that the large number of 2,4-D conjugates found in the aqueous ethanol extract were base-labile glycosyl derivatives incorporated into the plant matrix.

Table 9. Distribution of ^{14}C-Labeled Nonextractable Residues in Crops Planted 30 and 139 Days after Soil Application of [*phenyl*(U)-^{14}C]-2,4-D[a]

	% TRR			
fraction	radish, top	lettuce	wheat, forage	wheat, straw
nonextractable residue	36	58	73	67/82[b]
mild acid hydrolysates	20	24	30	17/24
crude cellulose	6	5.3	10	12/16
crude lignin	6	5.3	6.7	17/19
alkaline hydrolysate, aqueous	12	16	6.7	12/17
alkaline hydrolysate, ether	4	5.3		
total recovered	48	55	53	57/75

[a] TRR values were as follows: radish top, 0.043; lettuce, 0.019; wheat forage, 0.030; wheat straw, 0.060/0.084 ppm of 2,4-D ae. [b] 30-day value/139-day value.

Wheat Grain (Foliar). Strong base hydrolysis and acid hydrolysis were the most effective means of releasing the organo/aqueous-nonextractable ^{14}C activity (Table 6). Again, the percentage distributions of the ^{14}C residues were very similar for both treated and untreated grain. The high level of fractionation of the grain residue with the multiple-step extraction procedure used could obscure the presence of a component found at low levels in several fractions. Thus, a strong base hydrolysis was conducted on unextracted aliquots of treated and untreated grain. The results are shown in Table 6 in parentheses. Chromatographically, a ^{14}C residue with retention behavior similar to that of 2,4-D was noted in the untreated grain sample. The level was very low (0.003 ppm) and could not be confirmed. The requirement for basic hydrolytic conditions to release the majority of the 2,4-D residue again suggests glycosyl conjugates, although simply a solubilization of the matrix cannot be discounted as the source for the base-released 2,4-D.

Characterization of the incorporation of ^{14}C residues into natural cell constituents for both treated and untreated (in parentheses) grain gave % TRR values of 17 (12), 8 (3), and 20 (21) for the protein, starch, and cellulose fractions, respectively. The close correspondence of the treated and untreated grain ^{14}C residue profiles suggests the majority of the residues in wheat grain are due to natural incorporation of ^{14}C activity.

Potato Tuber. Mild acid hydrolysis was sufficient to release the majority of the organo/aqueous-nonextractable ^{14}C activity from the solvent PES (Table 7).

Crops (Soil). The results for a standard bound residue analysis are presented in Table 9 for those tissues that had >0.010 ppm of nonextractable residue. Wheat straw showed a fairly uniform distribution of ^{14}C activity over the various fractions; the succulent tissues had a higher portion of residues recovered in the acid and base hydrolysates. For radish, top, and wheat, forage, the total residue recovered, as calculated by summing the individual fractions, differed significantly from the initial bound residue present. The discrepancies are attributed to the procedural difficulties of carrying very low residues (~0.02 ppm initial bound residue) through a multistep fractionation procedure.

The standard procedure for the isolation of cellulose, starch, and protein from wheat grain gave results comparable to the distribution observed in the foliar study. To further support the hypothesis that the majority of the residues in wheat grain was due to incorporation of $^{14}CO_2$, starch was isolated from the grain and hydrolyzed, and the released glucose was derivatized with phenylhydrazine to form the glucosazone. The specific activity of the glucosazone was consistent with the amount of starch in the grain.

Comparative Degradative Routes. Table 10 summarizes the total amount of each identified 2,4-D moiety for the individual crop matrices for the foliar application. The importance of the pathway is calculated relative to the total amount of ^{14}C residue identified as containing a 2,4-D moiety.

Wheat (Foliar). The degradative routes observed in wheat, with relative importance in parentheses, were ester hydrolysis (97%), base-labile conjugation (70%), ring hydroxylation (10%), acid-labile conjugation (4%), and side-chain degradation (<1%). The primary degradative route other than ester hydrolysis (i.e., base-labile conjugation) was assumed to be glycosidic esterification of 2,4-D on the basis of the diversity of products and their mild base lability. The distribution of the ring-hydroxylated degradates was 4-OH-2,5-D > 4-OH-2,3-D > 5-OH-2,4-D. As noted in other studies of whole plant metabolism of 2,4-D (*3*), amino acid conjugation was not observed in these whole plant experiments. Amino acid conjugation, up to this time, has been observed only in nonwhole plant explorations of metabolic activity on 2,4-D (*4*).

Potato. The degradative routes observed for 2,4-D in potato were ester hydrolysis, dehalogenation, ring hydroxylation, and glycosidic carboxylic and phenolic esterification. Aside from ester hydrolysis, the primary pathway for 2,4-D in potato was dehalogenation. Con-

Table 10. Total Amount of ^{14}C Residues Identified as 2,4-D Moieties after the Foliar Application of [*phenyl*(U)-^{14}C]-2,4-D to Spring Wheat and Potato

	wheat, forage		wheat, straw		wheat, grain		potato, tuber	
total degradate	% TRR	rel pathway[a]	% TRR	rel pathway[a]	% TRR	rel pathway[a]	% TRR	rel pathway[a]
total 2,4-D	**74**	**0.8**	**70**	**0.8**	**6**	**1**	**42**	**0.5**
free	10		6		1			
conjugated	64		64		5			
total ring hydroxylation	**11**	**0.1**	**13**	**0.15**			**16**	**0.2**
free	7							
conjugated[b]	4							
4-OH-2,5-D	8		9	0.1			16	0.2
4-OH-2,3-D	2		3	0.03				
5-OH-2,4-D	1		2	0.02				
total 2,4-DCP	**0.5**	**<0.01**	**0.9**	**0.01**				
total 2,4-D EHE	**3**	**0.03**	**2.0**	**0.02**				
total 4-CPAA							**26**	**0.3**
total identified	**89**		**86**		**6**		**84**	

[a] Relative to the total identified. [b] Total of the ether-MAH fractions in Table 5.

jugation reactions were not as important in potato as in wheat for foliar applications.

Apple. Because of the low levels of residues detected in apple fruit from the soil application, metabolic pathways are not proposed for 2,4-D DMA salt in apple.

Wheat, Lettuce, and Radish (Soil). Soil degradation of the applied 2,4-D was the dominant pathway. Residues in the plant were related to the uptake of highly metabolized 2,4-D residues.

ABBREVIATIONS USED

2,4-D, 2,4-dichlorophenoxyacetic acid; ae, acid equivalent; ai, active ingredient; appln, application; DAT, days after treatment; DMA, dimethylamine; EHE, 2-ethylhexyl ester; GC-MS, gas chromatography−mass spectroscopy; HPLC, high-performance liquid chromatography; LSC, liquid scintillation counting; MAH, mild acid hydrolysis; MBH, mild base hydrolysis; PES, post-extracted sample; SBH, strong base hydrolysis; TLC, thin-layer chromatography; TRR, total radioactive residue.

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