# Metabolism of Diclofop-methyl in Root-Treated Wheat and Oat Seedlings

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Diclofop-methyl [methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate] was hydrolyzed initially to diclofop [2-[4-(2,4-dichlorophenoxy)phenoxy]propanoic acid] in roots of resistant wheat and susceptible oat. Diclofop was detoxified rapidly in wheat roots by aryl hydroxylation of the 2,4-dichlorophenyl moiety to at least three isomeric aryl-hydroxylated compounds and conjugation to acidic aryl *O*-glycosides. Virtually no diclofop accumulated in wheat roots, but in oat roots, approximately 35% of the radioactivity recovered was phytotoxic diclofop. The primary pathway in oat roots produced a water-soluble metabolite that was identified by mass spectroscopy as a neutral glucose ester conjugate of diclofop. Small quantities of the three hydroxylated isomers of the acidic aryl *O*-glycoside were products of a secondary metabolic pathway in oat roots. The ability of wheat roots to detoxify diclofop-methyl completely by aryl hydroxylation and subsequent conjugation is probably the basis for herbicidal selectivity.

Diclofop-methyl is a selective herbicide that controls a number of annual grass weeds in cereal crops (Anderson, 1976; Miller and Nalewaja, 1974). Root and shoot growth in oat and wild oat is inhibited by diclofop-methyl, whereas wheat growth is unaffected (Shimabukuro et al., 1978; Hoerauf and Shimabukuro, 1979). Absorption and translocation of diclofop-methyl are not important factors in selectivity (Brezeanu et al., 1976; Shimabukuro et al., 1979; Jacobson and Shimabukuro, 1982).

Gorbach et al. (1977) and Shimabukuro et al. (1979) found a water-soluble conjugate in diclofop-methyl-treated wheat shoots that was acid hydrolyzed to ring-hydroxylated diclofop. They suggested that the conjugate was an aryl *O*-glycoside. In wild oat shoots, the major water-soluble conjugate was hydrolyzed to the free acid, diclofop, suggesting that the conjugate was a neutral glucose ester (Shimabukuro et al., 1979). This difference in metabolism between resistant wheat and susceptible oat suggests that metabolism is the basis for herbicidal selectivity. This report examines the metabolism in diclofop-methyl in roots of resistant wheat and susceptible oat for two reasons: (1) to compare the metabolism in root and shoot tissues of both species and (2) to further characterize and identify the water-soluble metabolites found in both species.

## MATERIALS AND METHODS

**Chemicals.** [$^{14}$C]Diclofop-methyl (2,4-dichlorophenoxy-*U*-$^{14}$*C*) was purchased from New England Nuclear (specific activity 2 mCi/mmol). Unlabeled diclofop-methyl was obtained by extraction and purification from the formulted emulsifiable concentrate. Unlabeled diclofop was obtained by refluxing diclofop-methyl for 3 h in 3 N NaOH containing sufficient acetonitrile to dissolve the compound. Purity of the compounds was checked by thin-layer chromatography (TLC) and UV and IR spectroscopy. The 5′-hydroxylated diclofop was a gift from Hoechst Chemical Co. (Sommerville, NJ).

**Chromatographic Procedures.** Thin-layer chromatograms (silica gel HF) were developed and radioactive compounds were detected and quantitated as described previously (Jacobson and Shimabukuro, 1982). The solvent systems used were (A) toluene–absolute ethanol–acetic acid (150:7:7 v/v/v), (B) chloroform–methanol–water (65:25:4 v/v/v), (C) chloroform–methanol–water–acetic acid (65:25:4:2 v/v/v/v), (D) toluene–95% ethanol (2:1 v/v), and (E) cyclohexane–ethyl acetate (17:3 v/v).

High-performance liquid chromatography (HPLC) was performed with a Waters HPLC system (Model M6000 pumps, a Model 660 solvent programmer, a U6K injector, and either a 3.9 mm × 30 cm μBondapak C-18 column or an 8 mm i.d. RCM C-18 column). Ultraviolet absorption at 254 nm (Chromatronix Model 210 detector) and $^{14}$C activity (CAI Instruments radioactive flow monitor) were monitored. The solvent flow rate was 2 mL/min with a linear gradient of water–methanol–acetic acid (49:50:1 to 9:90:1 v/v/v) or isocratically with methanol–water, 85:15 (v/v).

**Plant Material and Treatment.** Wheat (*Triticum aestivum* L. cv. Waldron) and oat (*Avena sativa* L. cv. Garry) seedlings were grown on moist paper toweling as described previously (Jacobson and Shimabukuro, 1982). Twenty to thirty seedlings from 3-day-old wheat or 4-day-old oat were placed in glass vials (25 mm × 40 mm) containing 5 mL of 10 μM aqueous [$^{14}$C]diclofop-methyl in 1% acetone. After 24 h the seedlings were removed from the treatment solution, and their roots were rinsed, excised, and frozen for subsequent extraction.

The remaining treatment and rinse solutions were combined into a single posttreatment solution. The experiment was replicated 8 times.

**Metabolite Extraction and Purification.** The extraction and purification procedures for metabolite isolation and characterization appear in Figure 1. Frozen excised roots were homogenized at 4 °C in a Sorvall omnimixer for 2 min with methanol–water–acetic acid (80:19:1 v/v/v). The homogenate was centrifuged at 10000*g* for 10 min and the insoluble residue reextracted 2 more times. Methanol was removed from the soluble extracts with a rotary evaporator, and the resulting acidic aqueous extract was partitioned 3 times with dichloromethane. The aqueous and dichloromethane phases were concentrated and assayed for radioactivity. An aliquot of the dichloromethane-soluble fraction was chromatographed (TLC) in solvent A, and radioactive components were detected with a radiochromatogram scanner.

The water-soluble phase was acidified (pH 2) and extracted 3 times with ethyl acetate. The aqueous and ethyl acetate phases were concentrated and assayed for radioactivity.

The ethyl acetate soluble phase was further analyzed by successive TLC in solvents B and C. Radioactive zones

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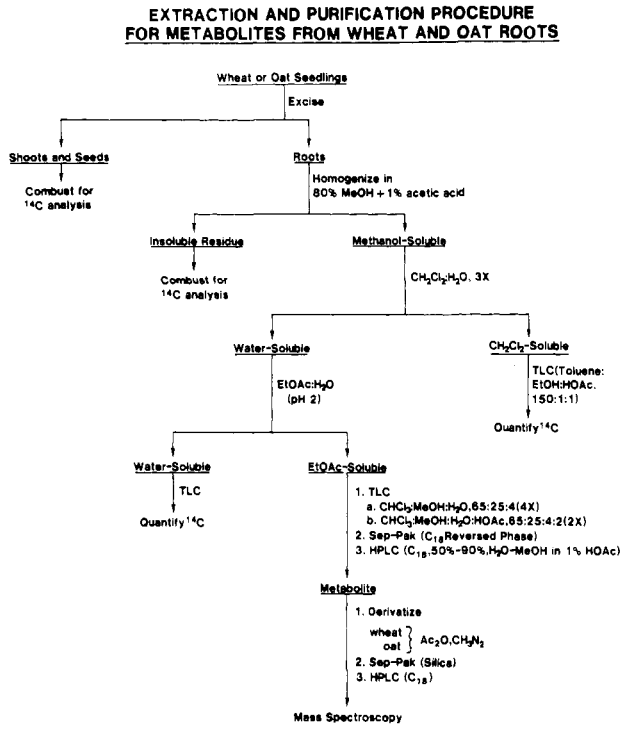


**Figure 1.** Extraction and purification of metabolites from wheat and oat roots treated with diclofop-methyl.

were detected by either radioautography or radiochromatogram scanning. The silica gels from these zones were removed, and the radioactivity was eluted with 80% methanol. The eluate was further purified on a C-18 Sep-PAK cartridge (Waters Associates, Inc.), concentrated, and chromatographed by HPLC using a linear gradient as described previously.

**Derivatization and Mass Spectroscopy.** The more polar of the water-soluble conjugates from both wheat and oat were derivatized for mass spectroscopy by methylation with diazomethane (Shimabukuro et al., 1979) followed by acetylation with acetic anhydride in pyridine (4:1 v/v) at 40 °C for 12–16 h. The less polar metabolite isolated only from oat root (retention time 20 min) was acetylated as above. The acetylated conjugates were dried under a stream of $N_2$, redissolved in chloroform, passed through a silica Sep-PAK, and chromatographed on a C-18 HPLC column (isocratic elution).

Derivatized metabolites were analyzed by electron impact mass spectrometry (70 eV) on a Varian MAT CH-5DF using a solid sample probe.

**Hydrolysis of Water-Soluble Conjugates.** Acid (6 N HCl) and enzymatic ($\beta$-glucosidase) hydrolyses of conjugates were performed as described previously (Shimabukuro et al., 1979).

**Synthesis of 1-*O*-[2-[4-(2,4-Dichlorophenoxy)phenoxy]propanyl]-$\beta$-D-glucose Tetraacetate.** The acetylated derivative of the neutral glucose ester of diclofop was prepared according to the method of Hiraga et al. (1974).

## RESULTS

**Absorption of Diclofop-methyl.** The absorption and translocation of [$^{14}$C]diclofop-methyl in roots of wheat and oat were examined previously (Jacobson and Shimabukuro, 1982). In this study the average absorption of $^{14}$C in nine replicate experiments was 62.1 ± 7.4% for wheat and 45.7 ± 5.4% for oat after 24 h. Recovery of $^{14}$C was in excess of 92% of the initially applied radiolabel for both species.

**Initial Characterization of Metabolites.** The quantitation of components within the water- and dichloromethane-soluble fractions and characterization of the

**Table I. Initial Characterization and Distribution of $^{14}$C Metabolites Isolated from Root Tissues Treated with [$^{14}$C]Diclofop-methyl**

| compound | $R_f$[a] | distribution of $^{14}$C, % wheat | oat |
|---|---|---|---|
| diclofop-methyl | 0.65 | 0.6 | 4.2 |
| diclofop | 0.43 | 0.5 | 34.6 |
| ring-OH diclofop | 0.22 | 3.1 | |
| conjugate A | 0.11 | 2.8 | |
| conjugate B | 0.00[b] | 84.9 | |
| conjugate C | 0.00[b] | | 53.8 |
| unknown | | 0.7 | 4.4 |
| insoluble residue | | 7.4 | 3.0 |

[a] Solvent system A. [b] Conjugates B and C were identified as two different conjugates from hydrolysis data.

**Table II. Separation of $^{14}$C Components in Dichloromethane-Soluble Fraction by Thin-Layer Chromatography**

| compound | $R_f$[a] | distribution of $^{14}$C, % wheat | oat |
|---|---|---|---|
| diclofop-methyl | 0.65 | 7.6 | 8.5 |
| diclofop | 0.43 | 79.9 | 81.6 |
| ring-OH diclofop | 0.22 | 3.8 | |
| polar conjugates | 0.00 | 8.7 | 9.9 |

[a] $R_f$ values in solvent A.

dichloromethane-soluble components were accomplished in the initial experiments. Approximately 1 g of root tissue treated previously with diclofop-methyl was extracted with 80% methanol in a glass tissue homogenizer, and the metabolites were isolated and characterized by TLC.

Two water-soluble conjugates (conjugates A and B) are the predominant metabolites in wheat (Table I). The major metabolite, conjugate B, hydrolyzes to ring-hydroxylated diclofop in either HCl or $\beta$-glucosidase. Conjugate A hydrolyzes to the ring-hydroxylated diclofop in HCl but no hydrolysis occurs with $\beta$-glucosidase. Very little diclofop-methyl or diclofop remains in wheat tissue.

Very little diclofop-methyl remains also in oat roots after 24 h (Table I). The major metabolite is a water-soluble conjugate (conjugate C) but appreciable quantities of diclofop are also present. Acid hydrolysis of conjugate C yields predominantly diclofop with minor amounts of ring-hydroxylated diclofop present.

The methanol-insoluble residue from root tissues following extraction contained 7.4% of the radioactivity in wheat and 3.0% in oat (Table I).

**Posttreatment Solution.** In the posttreatment solution from wheat, two polar conjugates A and B accounted for 70% and diclofop accounted for 19% of the $^{14}$C activity. In the posttreatment solution from oat, diclofop accounted for 90% of the $^{14}$C activity. Diclofop-methyl was detected in only minor amounts in the posttreatment solutions from wheat and oat.

In another experiment, wheat or oat roots were placed in water and removed after 24 h. [$^{14}$C]Diclofop-methyl was added to the root-free posttreatment solution. Hydrolysis of diclofop-methyl to dichlofop was detected in the root-free solution after 24 h. [$^{14}$C]Diclofop-methyl added to fresh water showed no significant hydrolysis to diclofop over the same period. Thus, a soluble esterase that is capable of hydrolyzing [$^{14}$C]diclofop-methyl either may be excreted by plant roots or may be of microbial origin.

**Dichloromethane-Soluble Metabolites.** The results from the separation and quantitation of dichloromethane-soluble metabolites are shown in Table II. Di-

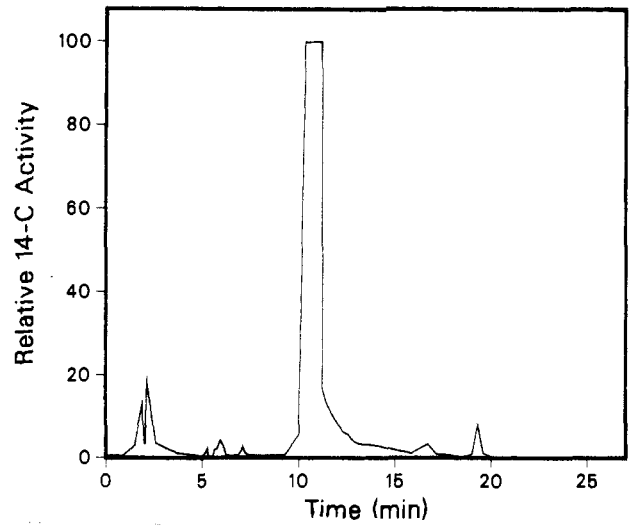


**Figure 2.** HPLC ($C_{18}$) of water-soluble metabolites of diclofop-methyl from wheat roots. Metabolite at a retention time of 10 min is conjugate B.

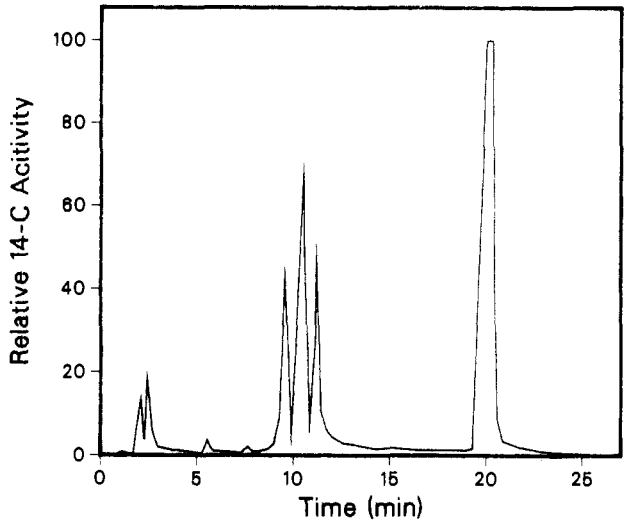


**Figure 3.** HPLC ($C_{18}$) of water-soluble metabolites of diclofop-methyl from oat roots. Major metabolites are at a retention time of ca. 10 min (conjugate B′) and a retention time of 20 min (conjugate C).

clofop was the major $^{14}C$ compound present in the dichloromethane fraction from wheat and oat (ca. 80%). This amounted to less than 1% and 35% of the total $^{14}C$ recovered from wheat and oat tissue, respectively (Table I). A small percentage of [$^{14}C$]diclofop-methyl (ca. 8%) and [$^{14}C$]polar conjugates (ca. 10%) was observed in the dichloromethane-soluble fraction from both species. The large lipophilic group present in diclofop probably permitted a small amount of $^{14}C$ polar metabolites to partition with extensive washings, into the dichloromethane. The amounts of $^{14}C$ polar conjugates detected in the dichloromethane-soluble fraction was less than 0.1% and 4.0% of the total radioactivity recovered from wheat and oat tissue, respectively.

**Water-Soluble Metabolites.** Water-soluble metabolites resulting from dichloromethane/water partitioning accounted for approximately 90% and 55% of $^{14}C$ activity recovered from the wheat and oat roots, respectively. The polar products were further purified by partitioning with ethyl acetate. The ethyl acetate fraction from wheat roots chromatographed (TLC, solvent A) into two $^{14}C$ bands: a major band at the origin containing more than 90% of the radioactivity and another band ($R_f$ 0.23) that cochromatographed with ring-hydroxylated diclofop. The ethyl acetate fraction from oat roots gave only one detectable $^{14}C$ band at the origin.

**Table III. Acidic and Enzymatic Hydrolysis Products of Polar Metabolites from Wheat and Oat Roots Treated with [$^{14}C$]Diclofop-methyl**

| reactant | HCl | β-glucosidase |
|---|---|---|
| wheat | | |
| conjugate A | ring-OH diclofop | unchanged[a] |
| conjugate B | ring-OH diclofop | ring-OH diclofop |
| oat | | |
| conjugate B′ | | |
| 1[b] | ring-OH diclofop | ring-OH diclofop |
| 2 | ring-OH diclofop | ring-OH diclofop |
| 3 | ring-OH diclofop | ring-OH diclofop |
| conjugate C | diclofop | polar[c] |

[a] Remained at $R_f$ 0.11 in solvent A. [b] 1, 2, and 3 represent the three peaks of the triplet with 1 having the lowest retention time and 3 the highest. [c] Remained at the origin in solvent A.

**Table IV. Identification of Primary Oat Metabolite Isolated from Roots by Thin-Layer Chromatography**

| compounds | $R_f$ values solvent A | solvent D | solvent E |
|---|---|---|---|
| diclofop | 0.43 | 0.38 | 0.17 |
| acetylated primary oat metabolite[a] | 0.85 | 0.89 | 0.77 |
| glucose tetraacetate ester of diclofop | 0.85 | 0.89 | 0.77 |

[a] HPLC retention time of 20 min prior to derivatization.

The polar metabolites from both species were chromatographed by HPLC following TLC and C-18 Sep-PAK purification (Figures 2 and 3). In wheat, practically all of the $^{14}C$ activity was in a peak with a retention time of 10 min (conjugate B) (Figure 2). In oat the $^{14}C$ activity was in a triplet peak with a retention time of ca. 10 min (conjugate B′) and a peak with a retention time of 20 min (conjugate C) (Figure 3).

**Characterization and Identification of Water-Soluble Metabolites.** A portion of the ethyl acetate soluble fraction from wheat (prior to HPLC) was spotted on a 500-μm silica gel HF TLC plate and developed in solvent A. The silica gel zone ($R_f$ 0.11), corresponding to the moderately polar conjugate A, was removed and eluted with 80% methanol.

Incubation of the methanol eluant (concentrated and dissolved in water beforehand) with β-glucosidase did not hydrolyze conjugate A since the $R_f$ before and after enzyme incubation was the same. However, treatment of conjugate A with HCl yielded ring-hydroxylated diclofop (Table III, 60–80% yield). Since conjugate A is a moderately polar metabolite ($R_f$ 0.11 in solvent A), it is probably a nonglucosidic conjugate of arylhydroxylated diclofop. No attempt was made at further identification.

The HPLC purified metabolites from wheat (conjugate B) and oat (conjugates B′ and C) were treated with HCl and β-glucosidase (Table III). Conjugate B from wheat was hydrolyzed to ring-hydroxylated diclofop by both treatments. Similarly, the three individual peaks of conjugate B′ from oat were hydrolyzed to ring-hydroxylated diclofop. Thus, conjugates B and B′ are probably conjugates of the 2,4-dichlorophenoxy ring-hydroxylated diclofop. Conjugate C from oat was acid hydrolyzed to diclofop, but no enzymatic hydrolysis of the conjugate occurred. This is consistent with a tentative identification of conjugate C as a neutral glucose ester.

Conjugate C was acetylated and chromatographed in solvents A, D, and E. The acetylated conjugate chroma-

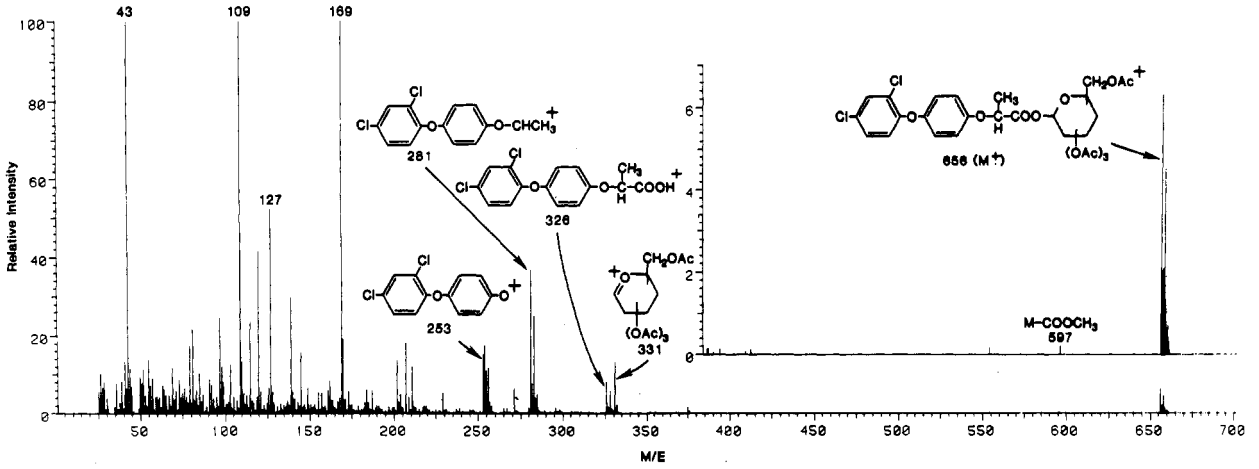


**Figure 4.** Mass spectrum of acetylated conjugate C from oat roots.

tographed in all three solvent systems with the synthesized glucose tetraacetate ester of diclofop (Table IV). The hydrolysis and TLC results (Tables III and IV) are consistent with the suggestion that conjugate C is a neutral glucose ester of diclofop.

Conjugates B, B′, and C were derivatized for mass spectral analysis. Conjugate C was acetylated, and conjugates B and B′ were first methylated and then acetylated. The molecular ions of conjugates B and B′ were not detected but each spectrum had a base ion of $m/e$ 331, which corresponds to the tetraacetylglucose ion ($Ac_4$-glucose) (spectra not presented). No peak appeared at $m/e$ 356 (ring-hydroxylated diclofop) (Shimabukuro et al., 1979), and other identifiable fragments were not detected. The molecular ion of conjugate C corresponding to the tetraacetate glucose ester was observed at $m/e$ 656. Other key fragments were observed at $m/e$ 597 (M – $COOCH_3$), $m/e$ 331 ($Ac_4$-glucose), $m/e$ 326 (M – [4Ac-glucose]), and $m/e$ 281 [M – ($CO_2$-$Ac_4$-glucose)]. The mass spectrum of derivatized conjugate C identified the metabolite as the neutral glucose ester of diclofop (Figure 4). The mass spectrum of the synthetic 2-[4-(2,4-dichlorophenoxy)-phenoxy]propanoate β-D-glucose tetraacetate was the same as that in Figure 4.

## DISCUSSION

A proposed metabolic pathway for diclofop-methyl in wheat and oat roots is presented in Figure 5. The initial hydrolysis of dichlofop-methyl to diclofop occurs rapidly, as evidenced by the low concentrations of the parent ester in both the posttreatment solution and the tissues. The roots also appear to contain a stable esterase capable of hydrolyzing diclofop-methyl. Evidence for the presence of a similar esterase has been reported elsewhere (Shimabukuro et al., 1979). Many carboxylic acid ester herbicides are hydrolyzed readily to their free acids. KK-80 [ethyl 4-[4-[4-(trifluoromethyl)phenoxy]phenoxy]-2-pentenoate] (Shimabukuro and Walsh, 1982), chlorfenprop-methyl [methyl 2-chloro-3-(4-chlorophenyl)propanoate] (Fedtke and Schmidt, 1977), and flamprop-methyl [methyl (±)-2-(*N*-benzoyl-3-chloro-4-fluoroanilino)propanoate] (Dutton et al., 1976) are initially hydrolyzed in vivo to their respective acids. Active esterases that hydrolyze chlorofenprop-methyl have been isolated from wheat and oat (Fedkte and Schmidt, 1977).

Wheat appears to form predominantly the acidic aryl *O*-glycoside of ring-hydroxylated diclofop. We were unsuccessful in obtaining a mass spectrum of the intact metabolite, but hydrolysis with acid and β-glucosidase gave ring-hydroxylated diclofop as a product. Similar results were obtained by Gorbach et al. (1977) in shoots of summer

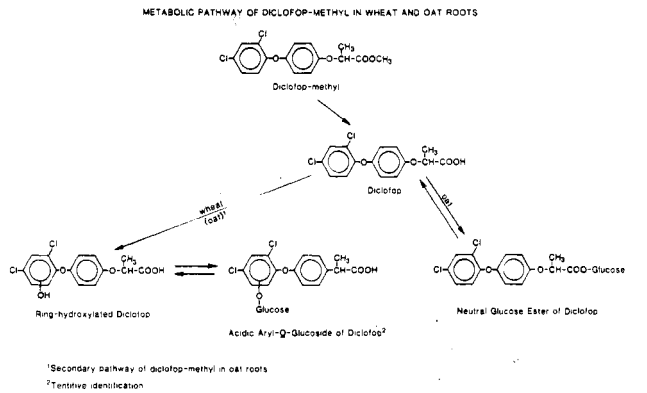


**Figure 5.** Proposed metabolic pathway for diclofop-methyl in wheat and oat roots.

wheat, by Shimabukuro et al. (1979) in shoots and roots of Waldron wheat, and by Dusky et al. (1980) in cell suspension cultures of *Triticum monococcum*.

Two water-soluble metabolites of diclofop-methyl were isolated from oat roots. These metabolites appear to be similar to those found in suspension cultures of *Avena sativa* L, cv. Garry (Dusky et al., 1982). The more polar of the two conjugates (retention time 10 min) appears to be chromatographically similar to the water-soluble conjugate from wheat roots, the major difference being the appearance of a triplet for the oat conjugate and only a single peak for wheat conjugate upon HPLC. The triplet was probably due to three positional isomers resulting from hydroxylation of the 2,4-dichlorophenyl ring. Evidence for this include (1) hydrolysis of the three peaks individually with β-glucosidase and HCl results in the formation of ring-hydroxylated diclofop and (2) three positional isomers of ring hydroxylated diclofop were isolated previously from wheat shoots with the 5′-OH metabolite as the major isomer (Gorbach et al., 1977). The chromatographic difference (triplet vs. singlet) between the metabolites from oat and wheat probably results from using two different HPLC columns to isolate the conjugates. The conjugate from wheat was isolated on a 30-cm steel column packed with μBondapak, whereas the conjugate from oat was isolated on a Waters Radial Compression Module (RCM).

The major metabolite in oat was identified positively as the neutral glucose ester of diclofop. The identification was confirmed by the hydrolysis and TLC data and mass spectral analysis of the metabolite and the synthetic product. Shimabukuro et al. (1979) suggested previously that one of the metabolites isolated from wild oat shoots treated with diclofop-methyl was a neutral glucose ester

of diclofop. This was based primarily on hydrolysis data. Boldt and Putnam (1981) reported that the susceptibile species, barnyardgrass and proso millet, formed a water-soluble conjugate that was hydrolyzed to diclofop with acid.

Herbicides with the diphenyl ether moiety are metabolized in different ways. Aryl hydroxylation of KK-80 does not occur (Shimabukuro and Walsh, 1982). Acifluorfen [sodium 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate] (Frear et al., 1980) and fluorodifen [4-[2-nitro-4-(trifluoromethyl)phenoxy]nitrobenzene] (Shimabukuro et al., 1973) are cleaved at the diphenyl ether bond. Acifluorfen is metabolized by soybean to the *S*-(3-carboxy-4-nitrophenyl)homoglutathione and [2-chloro-4-(trifluoromethyl)phenoxy]malonylglucose conjugates (Frear et al., 1980). The diphenyl ether bond in diclofop-methyl is apparently not cleaved in plants.

Metabolism appears to be the basis for diclofop-methyl selectivity in plants. Resistant plants (wheat) form ring-hydroxylated diclofop by a reaction that is probably not reversible. Ring hydroxylation of 2,4-D appears to be a detoxication mechanism (Feung et al., 1973, 1974). This may also apply to aryl hydroxylation of diclofop. In susceptible plants (oat), the major conjugated metabolite is a neutral glucose ester. Activation of 2,4-D appears to involve the formation of amino acid amides (Feung et al., 1974). Thus, the neutral glucose ester of diclofop is probably nonphytotoxic, but it may be hydrolyzed readily to phytotoxic diclofop.

Only small amounts of the water-soluble conjugate of ring-hydroxylated diclofop are detected in oat. Unlike wheat, oat is not able to rapidly detoxify the herbicide by aryl hydroxylation. As ultrastructural (Brezeanu et al., 1976) and physiological (Shimabukuro et al., 1978) damage occur, a decrease in the enzymatic activity of both the oxygenase and the glucosyl transferase may occur, resulting in an accumulation of diclofop. In cotton hypocotyls, injury (excision) results in the production of a low molecular weight fraction that inhibits the microsomal *N*-demethylase activity and thus reduces detoxication of substituted 3-phenyl-1,1-dimethylurea herbicides (Rusness and Frear, 1973). Thus, after 24 h of treatment, the concentration of diclofop-methyl and diclofop is greater in oat (39%) than in wheat (1%). Additionally, in a bioassay, the synthetic tetraacetyl glucose ester of diclofop was as phytotoxic as diclofop and diclofop-methyl on oat root growth (Jacobson et al., 1984).

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