The results of the greenhouse tests are shown in Table II and expressed as a percent of yield or uptake of nutrients from the standard sources. The response to nitrogen or phosphorus of the standard fertilizers was two to five times that of the no-nitrogen or no-phosphorus controls, thereby assuring valid comparison among the different nutrient sources.

RESULTS AND DISCUSSION

These experiments clearly show that long-chain crystalline ammonium and potassium ammonium polyphosphates are effective sources of N and P. As shown in Table II, all of the long-chain polyphosphates were good sources of N, although N uptake tended to be slightly lower than from ammonium nitrate. The long-chain polyphosphates prepared from furnace acid gave responses equivalent to or higher than that of monoammonium orthophosphate, but those products made from wet-process acid were slightly less effective.

Most of the long-chain polyphosphates were low in available P (citrate + water soluble) and one sample contained only 27% of its total P in an available form, as shown in Table II. Thus, the conventional availability test indicates that these polyphosphates could not be useful sources of P for growing plants. In spite of their low rating in the availability test all were effective fertilizers, and the results from fine and granular sources showed the usual granule size response obtained from water-soluble P sources in Mountview soil. Apparently, the rate of dissolution in the soil was sufficient to give agronomic response typical of water-soluble sources. Therefore, the conventional availability test is not valid for these long-chain polyphosphates.

This investigation has shown that long-chain crystalline ammonium or potassium ammonium polyphosphates may be readily produced by thermal dehydration of orthophosphates or short-chain polyphosphates, and these highly condensed phosphates are effective sources of N and P.

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Received for review September 29, 1978. Accepted January 5, 1979.

Metabolism and Selectivity of Diclofop-methyl in Wild Oat and Wheat

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Diclofop-methyl (methyl 2-[4-(2′,4′-dichlorophenoxy)phenoxy]propanoate) was hydrolyzd rapidly to diclofop (2-[4-(2′,4′-dichlorophenoxy)phenoxy]propionic acid) in resistant wheat and susceptible wild oat. Neither compound accumulated in the tissues of either species. The major reactions in wheat are the oxygenation of the 2,4-dichlorophenyl moiety of diclofop, followed by conjugation to an acidic aryl glycoside. In wild oat, diclofop was conjugated as a neutral glycosyl ester. The sugar moiety has not been characterized positively in either conjugate. Only limited symplastic and apoplastic translocation of diclofop-methyl and its metabolites occurred in wheat and wild oat. Herbicide selectivity between wheat and wild oat may be a function of its placement on the plant and the rate of herbicide metabolism, but the ability of wheat to irreversibly detoxify the herbicide by aryl hydroxylation may be the primary selective factor.

The experimental herbicide diclofop-methyl shows considerable promise for control of wild oat (*Avena fatua* L.) and foxtails (*Setaria* spp.) in cereal crops (Friesen et al., 1976; Miller and Nalewaja, 1974). Diclofop-methyl is used mainly as a postemergence herbicide but it also has preemergence activity (Wu and Santlemann, 1976). Root growth was severely inhibited in wild oat but only slightly in wheat (*Triticum aestivum* L.) when intact plants were root-treated in nutrient solution (Hoerauf and Shimabukuro, unpublished data).

Postemergence application of diclofop-methyl inhibited wild oat growth and caused extensive ultrastructural damage to leaf cells. Growth of wheat was unaffected and ultrastructural damage was less severe than that observed with wild oat (Brezeanu et al., 1976). Diclofop-methyl was a stronger auxin antagonist than diclofop, but the free acid was more effective in inhibiting root growth than the ester (Shimabukuro et al., 1978). The presence of both forms may be necessary for herbicidal action in wild oat (Shimabukuro et al., 1977).

Little is known about the metabolism of diclofop-methyl in plants. Diclofop-methyl was hydrolyzed rapidly to diclofop in both wheat and wild oat (Shimabukuro et al., 1977). A major metabolite of diclofop-methyl in wheat was a conjugate of 2-[4-(2′,4′-dichloro-5′-hydroxyphenoxy)-phenoxy]propionic acid (Gorbach et al., 1977). The purpose of our study was to determine the differences in metabolism of diclofop-methyl between wild oat and wheat and relate the differences to herbicide selectivity. This paper also describes the isolation and characterization of

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metabolites of diclofop-methyl in wild oat and wheat reported earlier (Shimabukuro et al., 1977).

MATERIALS AND METHODS

Analytical Methods. Thin-layer chromatograms (TLC) were developed to a solvent front of 15 cm on glass plates coated with 250-μm layer of silica gel HF activated at 110 °C for 1 h. Chromatograms were developed in one of the following solvents: (A) benzene/acetic acid (25:4), (B) benzene/95% ethanol (2:1), (C) cyclohexane/ethyl acetate (17:3), (D) ethyl acetate/chloroform (9:1), (E) ethyl acetate/chloroform (1:1), (F) ethyl acetate/benzene (3:2).

Radioactivity in plant extracts, column effluent, partitioning solvent fractions, and TLC plates was quantitated by liquid scintillation spectrometry in Instagel (Packard Instrument Co.). Column effluent was monitored continuously with a radioactive flow monitor. Radioactivity on TLC plates was detected with radiochromatogram scanner or autoradiography on Kodak No-Screen X-ray film. The gel from radioactive zones on TLC plates was removed and counted in 10 mL of Instagel and 3 mL of water. The ^{14}C residue in extracted plant tissues was counted by liquid scintillation spectrometry after combustion to $^{14}CO_2$ in a Model 306 Packard Tri-Carb sample oxidizer.

Electron impact mass spectra (70 eV) were obtained with a Varian MAT CH-5DF mass spectrometer by using a solid sample probe. Reference mass spectra of two model compounds, MC-5127 (methyl 4-(2′,4′-dichlorophenoxy)-2-nitrobenzoate) and nitrofen (2,4-dichloro-4′-nitrodiphenyl ether), were also obtained with the above instrument.

Nonpolar metabolites and some derivatized samples were purified by gas–liquid chromatography (GLC) on a 1.52 m × 6.4 mm i.d. stainless steel column packed with 3% OV-1 on 60/80 mesh, acid-washed Gas-Chrom Q. Nitrogen at 60 cm^3/min was the carrier gas. A 10:1 stream splitter was used, and peaks detected with a flame ionization detector were trapped in a glass capillary for introduction into the mass spectrometer. The GC was temperature programmed from 125–270 °C at 4 °C/min, unless indicated otherwise. The inlet temperature was 250 °C.

Metabolites were methylated with diazomethane generated from Diazald (Aldrich Co.) (Schlenk and Gellerman, 1960). The metabolites were dissolved in 0.5 mL of methanol, 5 μL of acetic acid, and 4.5 mL of diethyl ether before derivatization. Metabolites were ethylated with dry HCl in chilled absolute ethanol for 15 min. The ethanol solution was kept in an ice bath for another 15 min before warming to room temperature. Water-soluble conjugates were acetylated in 10 mL of acetic anhydride and 0.1 g of fused zinc chloride catalyst (Wolfrom and Thompson, 1963). The reaction mixture was stirred continuously at 0 °C for 3 h and at room temperature for another 0.5 h. The mixture was cooled, stirred in 20 mL of ice and water, and neutralized, and the acetylated derivative was extracted with benzene (Wolfrom and Thompson, 1963) and isolated.

Water-soluble conjugates were hydrolyzed in 6 N HCl at 100 °C for 24 h under N_2. Excess HCl was removed by reducing the reaction mixture to dryness under a stream of N_2. The residue was dissolved in methanol and chromatographed by TLC in solvent A. The TLC plate was scanned for radioactivity, and ^{14}C components were quantitated as decribed.

Water-soluble conjugates were also hydrolyzed with β-glucosidase or hesperidinase in 0.1 mL of 0.01 M sodium actate, pH 5.2, containing 0.05 M KNO_3 and 0.1 mg of enzyme. The reaction mixture was incubated 2 h at 30 °C, frozen in dry ice–acetone bath, and lyophilized. The residue was redissolved in methanol and chromatographed by TLC in solvent A, and ^{14}C was quantitated as described.

Plant Material for Excised Shoots. Wheat ("Waldron") and wild oat were seeded in vermiculite in perforated metal trays and grown in a greenhouse. Wild oat was seeded 1 day before wheat so that both species were at the same growth stage when treated. The trays were subirrigated as required with one-third strength Hoagland's nutrient solution (Blankendaal et al., 1972).

Plants for Root Treatment. Wheat and wild oat seeds were germinated between wet paper towels in an incubator at 25 °C for 4 and 5 days, respectively. The germinated seedlings were transferred to one-third strength Hoagland's nutrient solution and placed in a growth chamber with a 14-h light period, 16-klux light intensity, 40 ± 5% relative humidity, and day–night temperatures of 24 and 20 °C, respectively.

Plant Treatment for Metabolite Identification. All plants were treated with methyl 2-[4-(2′,4′-dichlorophenoxy)phenoxy-*U*-^{14}C]propanoate (sp act., 3.26 mCi/mmol) diluted with unlabeled diclofop-methyl.

Wheat and wild oat shoots in the 1.5–2-leaf stages (11 days and 12 days old, respectively) were excised at the vermiculite level under water and inserted into 20-mL vials (approximately 22 wheat and 26 wild oat shoots per vial), each containing 15 mL of aqueous 10 μM [^{14}C]diclofop-methyl (sp act., 0.28 mCi/mmol) in 1% acetone. Excised wheat and wild oat seedlings were treated with a total of 3.1 and 3.4 μmol of diclofop-methyl, respectively. The vials with shoots were placed in a controlled environment chamber at 24 °C, 40% relative humidity, and continuous light of 14-klux light intensity. After 24 h, shoots were removed and plant parts immersed in [^{14}C]diclofop-methyl were rinsed in distilled water. Fresh weights were determined and shoots were frozen for subsequent analyses. The experiment was replicated six times.

Hydrolysis of Diclofop-methyl in Treatment Solution. Duplicate vials of excised wheat and wild oat shoots in the 1.5-leaf stage were treated as described above with 9 μM [^{14}C]diclofop-methyl (sp act., 2.2 mCi/mmol). Aliquots of the treatment solution containing about 5000 dpm were removed at 1, 2, 3, 4, 6, and 24 h and chromatographed by TLC in solvent B. Water was not added to the vials to replace transpiration losses. After 24 h, the treated shoots were rinsed and frozen for analyses. Eight wheat and wild oat shoots from the above treatment were freeze-dried and autoradiographed.

Hydrolysis of Diclofop-methyl by Endogenous Hydrolases or Microbial Contamination. Duplicate vials of excised wheat and wild oat shoots were treated as above with 15 mL/vial of 1% acetone in distilled water. The excised shoots were removed after 24 h and [^{14}C]-diclofop-methyl was added to the remaining solution to give 15 mL/vial of 9 μM [^{14}C]diclofop-methyl as above. The control was a vial containing similar [^{14}C]diclofop-methyl solution prepared with distilled water not exposed to excised shoots. The herbicide solution was assayed for metabolites in the same way as the treatment solution described above.

Root Treatment. Wheat and wild oat seedlings in nutrient solution were root-treated with 10 μM [^{14}C]diclofop-methyl (sp act., 0.49 mCi/mmol) in the two-leaf stage of growth (11 and 13 days old for wheat and wild oat, respectively). The roots of six plants/species were treated with 60 mL of 1% acetone solution of [^{14}C]diclofop-methyl. After 46 h, the roots were rinsed and plants were auto-

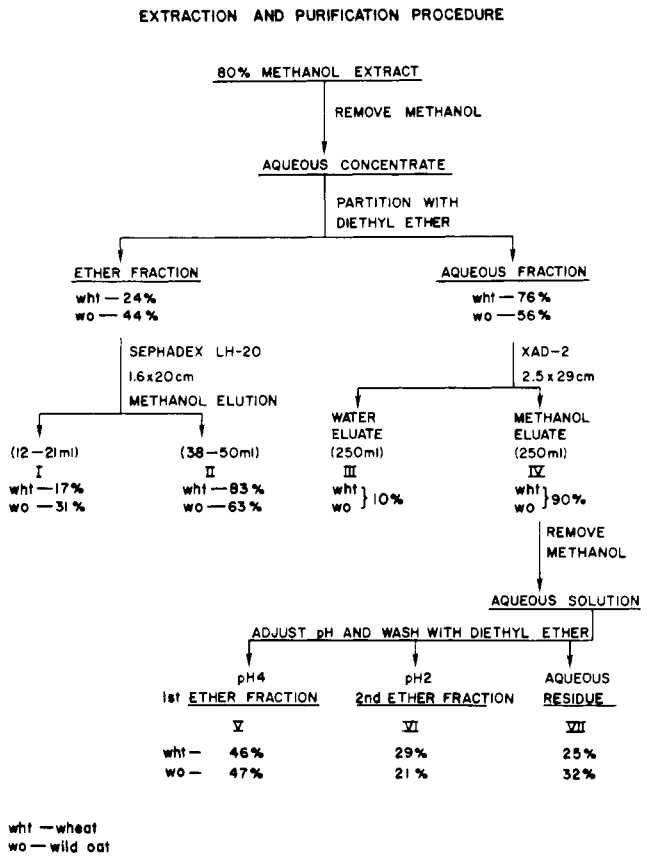


Figure 1. Separation of metabolites in excised shoots of wheat and wild oat.

radiographed. The roots and shoots of three plants from each species were separated and combusted for ^{14}C quantitation. The separated roots and shoots from the remaining plants were combined, extracted separately, and analyzed for metabolites of [^{14}C]diclofop-methyl.

Extraction and Purification. The extraction and purification procedures for metabolite isolation and characterization are shown in Figure 1. The frozen excised shoots of wheat and wild oat were homogenized in a blender with 80% methanol (5 mL/g fresh weight). The homogenate was filtered and the residue was extracted two more times. Methanol was removed from the combined extracts with a rotary evaporator at 35 °C. Chlorophyll and other nonpolar natural products precipitated as methanol was removed from the extract. The precipitated residue was redissolved later in diethyl ether and combined with the ether fraction from partitioning. The aqueous extract was diluted with distilled water to 50 mL and partitioned three times with 100 mL of diethyl ether. The ether fraction from each partitioning was washed with 25 mL of water. The aqueous and ether fractions were concentrated and assayed for ^{14}C. The ether fraction was reduced to dryness under N_2, redissolved in methanol, and counted.

The ether fraction (redissolved in methanol) was chromatographed on Sephadex LH-20 and separated into fractions I and II with 93–96% recovery of radioactivity applied to the column. Most of the radioactivity appeared in fraction II (Figure 1) for both wheat and wild oat.

The aqueous fraction was applied to an Amberlite XAD-2 column and eluted successively with water (III) and methanol (IV). Recovery was 65–75% of th radioactivity applied to the column. Ninety percent of the recovered radioactivity was in the methanol eluate for both species. The recovery of applied radioactivity was increased to 80–85% when acetone was used in place of methanol in two experiments.

Table I. Absorption of [^{14}C]Diclofop-methyl by Excised Wheat and Wild Oat Shoots in 24 h

species	appl ^{14}C absorb., %	distribution of absorbed ^{14}C: lower[a] ^{14}C, %	lower[a] fr. wt, g	upper[a] ^{14}C, %	upper[a] fr. wt, g
wheat	81	97	39	3	64
wild oat	87	83	36	17	50

[a] Lower stems of seedlings immersed in treatment solution; upper stems and leaves above treatment solution.

The methanol eluate (IV) was concentrated to dryness, redissolved in about 25 mL of water, and adjusted to pH 4.0 with dilute HCl. The solution was partitioned three times with 25 mL of diethyl ether. The pH of the aqueous solution was then adjusted to 2 and partitioned as above. The ^{14}C components in the diethyl ether fractions (V, VI) and aqueous residue (VII) were quantitated as described. Most of the radioactivity was present in the first ether fraction (V) for wheat and wild oat (Figure 1). The recovery of radioactivity in this fractionation procedure ranged from 91 to 97%.

In other wild oat experiments, the methanol eluate (IV) from XAD-2 chromatography (Figure 1) was rechromatographed as the aqueous solution on Sephadex LH-20 (1.6 × 50 cm) and eluted with 50% methanol in water. About 95% of the applied radioactivity was recovered and 70% of it eluted between 40 and 100 mL elution volume. This purified fraction was used for derivatization and characterization of the water-soluble metabolite in wild oat.

Fraction V from wheat was also hydrolyzed in HCl and the major hydrolysis product was methylated and subjected to mass spectroscopy. The major metabolite in fraction V of wheat was also methylated without hydrolysis, purified by TLC in solvents C and E, and subjected to mass spectroscopy.

Fraction V from wheat was further purified by rechromatography on Amberlite XAD-2 as above.

RESULTS

Absorption of Diclofop-methyl. The results from one representative experiment are discussed to present qualitative and quantitative differences between wheat and wild oat in their absorption and metabolism of diclofop-methyl. The results of five other replicate experiments were similar.

Most of the applied [^{14}C]diclofop-methyl was absorbed by excised wheat and wild oat shoots within 24 h (Table I). The recovery of applied ^{14}C was 102 and 81% for wheat and wild oat shoots, respectively. Approximately 10 mL of the treatment solution was recovered from each vial of wheat and wild oat shoots within 24 h. Despite the active transpiration in both species, very little translocation of ^{14}C components occurred in either wheat or wild oat. These results were unexpected since greater acropetal transport of ^{14}C components was anticipated by apoplastic translocation. Much of the [^{14}C]diclofop-methyl and/or its metabolites were absorbed and retained by the cells in the lower stems of both species.

Posttreatment Solution. Only two major radioactive components were detected in the posttreatment solutions of excised wheat and wild oat shoots. One major component (wheat, 98%; wild oat, 66%) cochromatographed with diclofop at R_f 0.55 in solvent A (Figure 2). The second component (wheat, 2%; wild oat, 34%) cochromatographed with diclofop-methyl at R_f 0.63 in the same

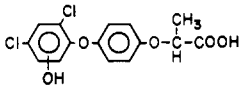
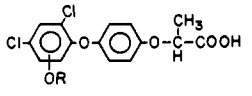
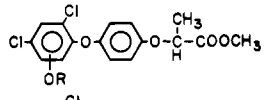
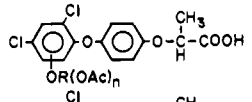
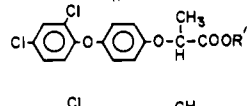
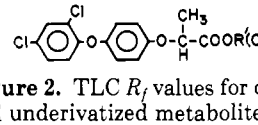
COMPOUNDS	R_f VALUES IN SOLVENT SYSTEMS: A	B	C
DICLOFOP-METHYL	0.63	0.83	0.30
DICLOFOP	0.55	0.33	0.0
DICLOFOP-ETHYL	—	0.85	0.37
	0.35	0.0	0.0
	0.0	0.0	0.0
	—	0.50	0.0
	0.20	0.75	—
	0.0	0.0	0.0
	—	0.80	0.0

Figure 2. TLC R_f values for diclofop-methyl and its derivatized and underivatized metabolites. The R and R′ indicated in the conjugates may or may not be the same glycosyl residues.

solvent (Figure 2). The amount of parent herbicide remaining in the treatment solutions for both wheat and wild oat shoots varied widely (3–30%) between experiments.

The metabolite that chromatographed at R_f 0.55 was derivatized in ethanolic HCl, purified by TLC in solvent B (R_f 0.85) (Figure 2) and chromatographed by GLC as described. The mass spectrum of a GLC peak eluting at 210 °C had a molecular ion ($M^{+\cdot}$) at 354 (Figure 3A). The spectrum was identical with that of authentic diclofop-methyl except for the $M^{+\cdot}$ at m/e 340 for diclofop-methyl. The mass spectra of MC-5127, nitrofen, and 2,4-D (2,4-dichlorophenoxyacetic acid) (Feung et al., 1973; Van Peteghem and Heyndrickx, 1976) all showed ions at m/e 162, 161, 145, and 109. These ions originated from the 2,4-dichlorophenoxy moiety common to each compound. The intense m/e 120 ion from diclofop-ethyl (Figure 3A) and diclofop-methyl was absent from the spectra of the other three model phenoxy compounds. The components were the same in the posttreatment solutions of both wheat and wild oat.

Extraction and Purification of Diclofop-methyl and Its Metabolites. Only the metabolites extracted from the immersed parts of excised shoots were purified. Extracts from wheat and wild oat shoots contained 2.4 and 1.9 μmol equiv of diclofop-methyl, respectively. The ether-soluble fractions from wheat and wild oat accounted for 24 and 44%, respectively, of the radioactivity in the aqueous concentrate (Figure 1).

Characterization of Diclofop-methyl and Its Metabolites. Radioactive components with the same R_f values were detected in fractions I and II from both wheat and wild oat. Polar material (R_f 0.0 in solvents A, B, C) accounted for a greater percentage of the radioactivity in fraction I than in fraction II. Components in fraction II

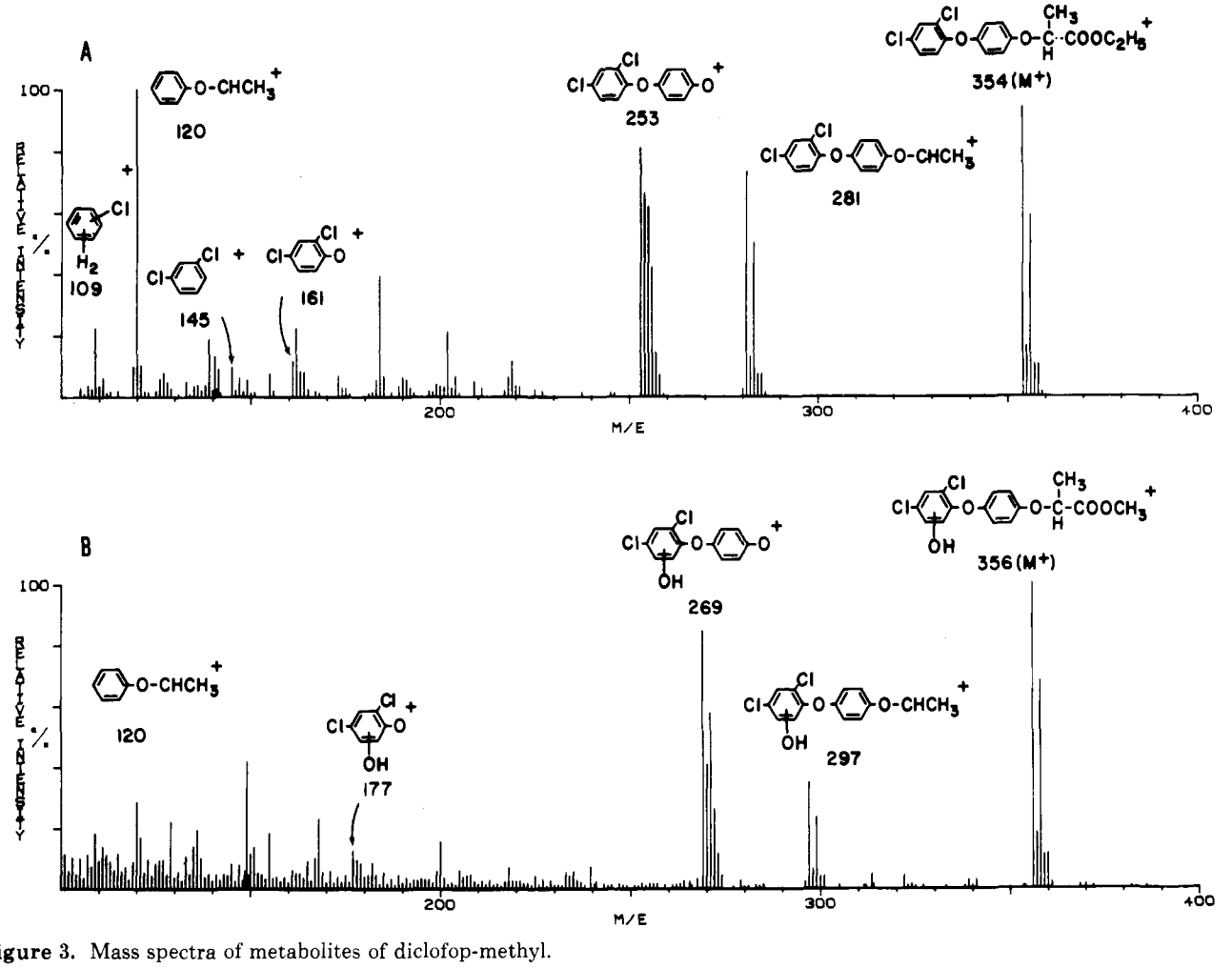


Figure 3. Mass spectra of metabolites of diclofop-methyl.

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

	distribution of ^{14}C on thin-layer chromatogram, %				
	R_f values (solvent A) [a]				
species	0.0	0.35	0.55	0.63	undefined
wheat	17	34	46	2	1
wild oat	15.6	2.4	64	18	0

[a] R_f, 0.35; ring-OH diclofop; 0.55, diclofop; 0.63, diclofop-methyl.

were separated and characterized since most of the ether-soluble radioactivity was present in this fraction for both species.

Fraction II from wheat contained two major metabolites that chromatographed at R_f 0.35 and 0.55 (solvent A). The only major metabolite in fraction II from wild oat chromatographed at R_f 0.55 (Table II). The metabolite (0.13 μmol) at R_f 0.35 from wheat was methylated with diazomethane. The R_f of this metabolite changed from 0.0 to 0.3 (solvent C) after methylation. The derivatized metabolite was chromatographed by GLC as described with temperature programmed from 100 to 300 °C at 5 °C/min. The peak eluting between 215 and 245 °C was subjected to mass spectrometry. The molecular ion with its relative abundance given in parentheses was m/e 370 (74). Other intense ions in the spectrum were m/e 311 (37), 283 (100), and 120 (76). The loss of $COOCH_3$ and $CH_3CHCOOCH_3$ from the molecular ion gave the ions at m/e 311 and 283 which were 30 amu greater than the analgous ions m/e 281 and 253 from diclofop-ethyl (Figure 3A). The results indicated that the derivative had a methoxy group in the diphenyl ether moiety of diclofop-methyl. The intense ion at m/e 120 in the spectra of the derivatized metabolite ($M^{+\cdot}$ 370) and diclofop-methyl ($M^{+\cdot}$ 340) indicated that the methoxy substituent was not in the phenoxypropionate moiety of the metabolite. Therefore, the derivatized metabolite was identified as the dimethyl derivative of 2,4-dichlorophenyl-ring-hydroxylated diclofop (ring-OH diclofop). The position of hydroxylation on the 2,4-dichlorophenyl ring was not determined.

The metabolite at R_f 0.55 (Table II) from wheat was further purified by TLC in solvent B (R_f 0.33, Figure 2) and methylated with diazomethane. The methylated metabolite cochromatographed on TLC with diclofop-methyl (Figure 2). The mass spectra of the derivatized metabolite and diclofop-methyl were also identical. Therefore, the metabolite at R_f 0.55 (solvent A) (Table II) was confirmed as diclofop. The metabolite at R_f 0.63 (solvent A) (Table II) was also confirmed as the parent diclofop-methyl by cochromatography on TLC and mass spectrometry.

The metabolites in fraction II of wild oat (Table II), other than the components at the origin, were identical with those of wheat as confirmed by cochromatography on TLC and mass spectrometry. Fraction II from wild oat contained more diclofop and unchanged diclofop-methyl and significantly less ring-OH diclofop than from wheat (Table II).

Water-Soluble Metabolites in Wheat and Wild Oat. Most of the metabolites eluted in fraction IV from Amberlite XAD-2 chromatography in wheat and wild oat were conjugates that were hydrolyzed by HCl (Table III). The major hydrolysis products were ring-OH diclofop for wheat and diclofop for wild oat. However, hydrolysis yielded both compounds in each species (Table III). Therefore, fraction IV from wheat and wild oat shoots were mixtures of

Table III. HCl Hydrolysis of Aqueous Solution from Diclofop-methyl Metabolism in Excised Shoots of Wheat and Wild Oat[a]

	R_f values (solvent A)	distribution of ^{14}C before and after HCl hydrolysis, %[b]			
		wheat		wild oat	
components		before	after	before	after
diclofop	0.55	0.8	17.5	0.3	58.5
ring-OH diclofop	0.35	0.8	58.3	0.7	13.9
conjugates	0.00	88.8	16.8	95.2	9.2
unidentified[c]		9.6	7.4	3.8	18.4

[a] Aqueous solution from methanol eluate (IV) of Amberlite XAD-2 chromatography. [b] Analyzed by TLC in solvent A. [c] Radioactivity in all other zones.

Table IV. Separation of ^{14}C Components in Fraction V by Thin-Layer Chromatography

	distribution of ^{14}C on thin-layer chromatogram, %				
	R_f values (solvent A)[a]				
species	0.00	0.35	0.55	0.63	undefined
wheat					
unhydrolyzed	98	1	1	0	0
hesperidinase	55	26	1	0	18
β-glucosidase	63	24	1	0	12
HCl[b]	Tr[c]	95	Tr	0	5
wild oat					
unhydrolyzed	95	0	3	0	2
hesperidinase[d]	95	1	3	0	1
β-glucosidase[d]					
HCl[e]	9	18	68	0	5

[a] Compounds at different R_f values are the same as in Table II. [b] Separation of ^{14}C (70–80%) that partitioned into ethyl acetate after HCl hydrolysis. [c] Tr, only trace amounts. [d] Same results for both enzymes. [e] Same as *b*.

conjugates of ring-OH diclofop and diclofop, respectively.

The pH fractionation procedure (Figure 1) was not successful in separating the water-soluble metabolites eluted in fraction IV and present in the aqueous solutions of wheat and wild oat. Fractions V, VI, and VII were all mixtures containing different proportions of conjugates and other metabolites. However, fraction V of both species was used for metabolite characterization since it contained higher concentrations of the conjugates than fractions VI and VII when analyzed by HCl hydrolysis and TLC (Table IV).

The water-soluble metabolites in fraction V of wheat were partially hydrolyzed with hesperinidase and β-glucosidase. Neither enzyme hydrolyzed the metabolites in fraction V of wild oat (Table IV). The radioactive component remaining at the origin after enzymatic hydrolysis probably contains unhydrolyzed metabolites. The major enzymatic hydrolysis product from fraction V of wheat appeared to be ring-OH diclofop.

The component at R_f 0.35 (solvent A) from HCl hydrolysis of fraction V (wheat) was methylated with diazomethane and purified by TLC in solvent C (R_f 0.3). The mass spectrum of the purified derivative gave a molecular ion m/e 370 and was identical with the dimethyl derivative of ring-OH diclofop found in fraction II.

Methylation of unhydrolyzed fraction V of wheat containing the phenolic conjugate gave a major derivatized component that remained at the origin in solvent C and had an R_f 0.2 in solvent E. The molecular ion of the conjugate was not detected in the mass spectrum of the

derivatized metabolite. The highest molecular weight ion (m/e 356) was the base peak (Figure 3B). The spectrum was similar to that of the parent diclofop-methyl ($M^{+\cdot}$ 340) and diclofop-ethyl (Figure 3A). The ions m/e 297 and 269 (Figure 3B) were 16 amu greater than the analogous ions m/e 281 and 253 from diclofop-ethyl (Figure 3A) and diclofop-methyl. This indicated the presence of oxygen in the diphenyl ether moiety of the metabolite. Both chlorine substituents were present in all the above ions as indicated by the chlorine isotopic peaks. The ions m/e 177 in Figure 3B and m/e 161 in Figure 3A and the presence of the ion m/e 120 in both spectra confirm the substitution of a hydroxyl group in the 2,4-dichlorophenoxy moiety (Figure 3B).

The derivatized compound in Figure 3B was identified as the methyl ester of a ring-OH diclofop conjugate. Only the free carboxyl group of the metabolite was derivatized by diazomethane. However, the hydrolyzed conjugate formed the dimethyl derivative ($M^{+\cdot}$ 370). Therefore, the water-soluble metabolite in fraction V of wheat is conjugated through the ring hydroxyl group which is not free for derivatization unless hydrolyzed. A molecular ion for the intact monomethyl conjugate was not observed but loss of the conjugated moiety with proton transfer in the mass spectrometer gave the monomethyl derivative (m/e 356) shown in Figure 3B.

The water-soluble conjugate from wheat was acetylated and purified by TLC in solvents A (R_f 0.2) and B (R_f 0.75), followed by methylation with diazomethane and purification by TLC in solvent B (R_f 0.8). The molecular ions were not observed in the mass spectra of either the acetylated or the acetylated and methylated derivatives of the water-soluble conjugate. The mass spectra of the two derivatized forms were similar with the following major ions and their relative intensities in parentheses: m/e 331 (24), 229 (10), 211 (7), 169 (49), 109 (35), and 43 (100). The fragmentation was similar to that of tetra-*O*-acetyl-D-glucosides (Paulson et al., 1973). The aglycon ion m/e 356 (Figure 3B) formed by the loss of an acetylated glycosyl moiety (m/e 331) of the conjugate was also not observed.

The mass spectral data and the results of the β-glucosidase hydrolysis suggest that the phenolic conjugate is an *O*-glucoside of 2,4-dichlorophenoxy-ring-OH diclofop. Further analysis will be required to positively identify the glycosyl moiety in the conjugate.

The major water-soluble metabolite in fraction V of wild oat was a conjugate of diclofop (Table IV). The component at R_f 0.55 (diclofop) increased from 3 to 68% after HCl hydrolysis. Methylation of the component at R_f 0.55 gave the same spectrum ($M^{+\cdot}$ 340) as the parent diclofop-methyl. Methylation of the component at R_f 0.35 gave a molecular ion m/e 370 and a spectrum similar to that of the dimethyl derivative of ring-OH diclofop. Therefore, fraction V of wild oat was a mixture of the phenolic conjugate found in wheat and an ester conjugate of diclofop.

The water-soluble conjugate from wild oat was acetylated and purified by successive TLC in solvents B (R_f 0.8), F (R_f 0.45), and D (R_f 0.65). The mass spectrum of the acetylated conjugate had an intense m/e 331 ion with fragmentation similar to that of a tetra-*O*-acetyl glycoside (Paulson et al., 1973). The molecular ion of the acetylated conjugate was not observed.

The major water-soluble metabolite of diclofop-methyl in wild oat appears to be a glycosyl ester of diclofop. The metabolite was hydrolyzed in HCl to diclofop but was not hydrolyzed with β-glucosidase or hesperidinase. The structure of the glycosyl moiety is not known.

Table V. Metabolism of [^{14}C]Diclofop-methyl in Excised Shoots of Wheat and Wild Oat [a]

components	distribution of ^{14}C, %[b]	
	wheat	wild oat
diclofop-methyl	1	3
diclofop	10	20
ring-OH diclofop	7	2
conjugates	77	72
insoluble residue	5	3

[a] Large-scale treatment for generation of metabolites. [b] ^{14}C distribution in lower stem extracted for metabolite purification.

Table VI. Hydrolysis of Diclofop-methyl to Diclofop in Treatment Solution of Excised Wheat and Wild Oat Shoots

time, h	diclofop-methyl, %[a]		diclofop	
	wheat	wild oat	wheat	wild oat
1	73	89	27	11
2	66	74	34	26
3	58	51	42	49
4	48	38	52	62
6	27	21	73	79
24	3	4	97	96

[a] Average of duplicate treatments of wheat and wild oat.

Diclofop-methyl was hydrolyzed and metabolized to glycosyl conjugates with similar aglycon moieties in excised shoots of wheat and wild oat (Table V). In both species very little unaltered diclofop-methyl remained after 24 h. More diclofop remained in wild oat than in wheat but more ring-OH diclofop was formed in wheat than in wild oat. The two metabolites accounted for only 17–22% of the total radioactivity in the plant tissues. Most of the radioactivity in both species was present as conjugates. However, the conjugates showed marked differences in their hydrolysis products between wheat and wild oat (Table III). Significant amounts of unhydrolyzed and unidentified products were present in both species.

Diclofop-methyl Hydrolysis in Treatment Solution. Diclofop-methyl was hydrolyzed to diclofop in the treatment solution of excised wheat and wild oat shoots (Table VI). The relative hydrolysis data do not account for the absorption of ^{14}C components by excised shoots between 1- and 6-h periods except for the 24-h period. At 24 h, only 16 and 11% of the applied radioactivity remained in the treatment solution of wheat and wild oat, respectively. Therefore, rapid uptake of dichlofop-methyl and/or diclofop by excised shoots of wheat and wild oat occurred simultaneously with an increase in the proportion of diclofop to diclofop-methyl in the treatment solution.

Diclofop-methyl Hydrolysis by Endogenous Hydrolases or Microbes. Treatment solutions from which excised shoots were removed after 24 h were capable of hydrolyzing diclofop-methyl. In wheat and wild oat, 17 and 22%, respectively, of the ^{14}C activity (6.0×10^5 dpm) in the posttreatment solution was diclofop after 24-h incubation. These tests differed slightly from the hydrolysis experiment described above in that the added ^{14}C in solution and the solution volumes were constant during the incubation period. Diclofop-methyl was not hydrolyzed when added to fresh 1% acetone solution over the same period. Therefore, the presence of diclofop in the treatment solution of excised shoots may result from secretion of the metabolite by plant cells and/or hydrolysis of diclofop-methyl by endogenous hydrolases released from the cut surfaces of wheat and wild oat shoots. Hydrolysis by microbial contaminants was negligible.

Ring-OH diclofop and conjugated metabolites were not

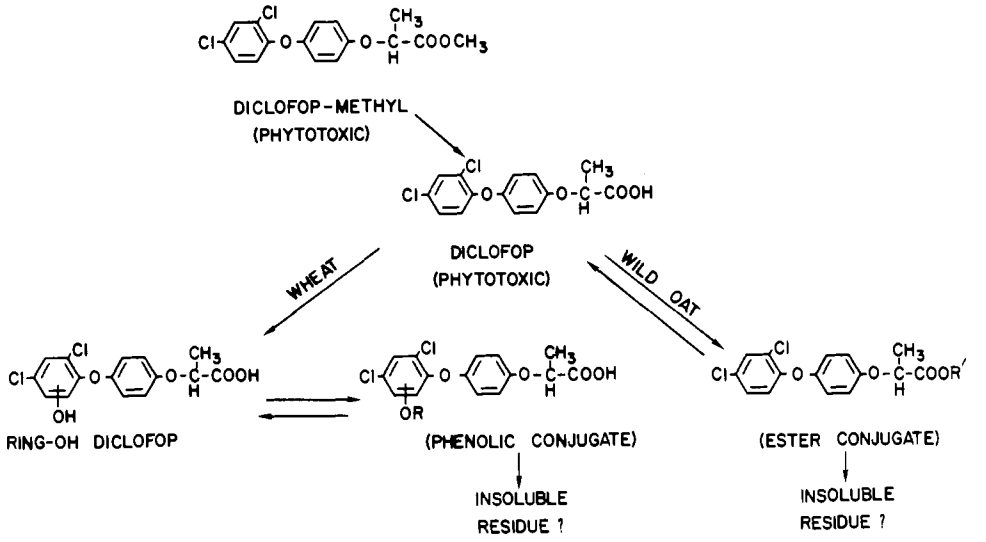


Figure 4. Proposed metabolic pathway for diclofop-methyl in wheat and wild oat. The identity of R and R′ is unknown.

Table VII. Metabolism and Translocation of [^{14}C]Diclofop-methyl in Excised Shoots of Wheat and Wild Oat

	distribution of ^{14}C in excised shoot, %[a]			
	upper		lower	
compounds	wheat	wild oat	wheat	wild oat
diclofop-methyl	Tr[b]	Tr	0.1	0.4
diclofop	Tr	2.9	2.3	12.0
ring-OH diclofop	0.2	0.3	10.7	2.8
conjugates	1.9	3.6	83.8	75.0
insoluble residues	0.9	0.2	0.1	2.8
total	3.0	7.0	97.0	93.0

[a] Five excised shoots combined for extraction and analysis. Upper 9-12 cm of leaves not immersed in treatment solution; lower 3-4 cm of excised shoot immersed in treatment solution. [b] Tr, trace amounts.

excreted or released from plant cells. They were not detected in the treatment solutions of excised wheat or wild oat shoots despite their presence in the plant tissues (Table V).

Translocation of Diclofop-methyl and Its Metabolites in Excised Shoots. The radioautographs of excised shoots treated with [^{14}C]diclofop-methyl showed that most of the radioactivity was in the lower stems that were immersed in the treatment solution. Only 3 and 7% of the radioactivity in excised wheat and wild oat shoots, respectively, were translocated to the upper leaf blades (Table VII). Only trace amounts of the translocated radioactivity in the upper leaves were diclofop-methyl in both species. Diclofop concentration was negligible in upper leaf blades of wheat but it was 2.9% of the translocated radioactivity in wild oat.

Metabolism and Translocation of Diclofop-methyl in Root-Treated Plants. Acropetal translocation of diclofop-methyl and/or its metabolites was limited. The roots of wheat and wild oat contained 92 and 89%, respectively, of the total radioactivity recovered from whole plants (Table VIII). No differences were observed in the distribution and localization of radioactivity between wheat and wild oat plants. Diclofop-methyl equivalents in the plant tissues (μg/g dry weight) were: shoot, 0.05 (wheat) and 0.02 (wild oat); root, 0.5 (wheat) and 0.3 (wild oat).

Very little of the absorbed and/or translocated radioactivity in roots or shoots of wheat and wild oat was diclofop-methyl (Table VIII). Aftr 46 h, the posttreatment solution contained 32 and 63% of the applied radioactivity in wheat and wild oat, respectively. Only diclofop was recovered from the treatment solutions of both plant species. More of the translocated radioactivity in the shoots of wild oat (3.3%) was diclofop than in wheat (1.0%). Higher amounts of diclofop were detected in roots (Table VIII) than in lower excised shoots of both species (Table VII). Less [^{14}C]diclofop-methyl was metabolized to conjugates in wheat and wild oat roots (49 and 36%, respectively; Table VIII) than in their shoots (84 and 75%, respectively; Table VII). Water-soluble conjugates in the roots of wheat and wild oat were qualitatively similar to the conjugates in their shoots when analyzed by HCl hydrolysis and TLC.

Table VIII. Metabolism and Translocation of [^{14}C]Diclofop-methyl in Root-Treated Wheat and Wild Oat

	distribution of ^{14}C in treated plants, %[a]			
	shoot		root	
compounds	wheat	wild oat	wheat	wild oat
diclofop-methyl	Tr[b]	Tr	0.4	0.6
diclofop	1.0	3.3	22.0	38.4
ring-OH diclofop	0.6	0.2	5.6	0.6
conjugates	5.9	6.9	49.0	36.2
insoluble residue	0.4	0.6	15.0	13.2
total, %	8.0	11.0	92.0	89.0

[a] Forty-six hour treatment period; combined extracts of three plants/species. [b] Tr, trace amounts.

DISCUSSION

A proposed metabolic pathway for diclofop-methyl in wheat and wild oat is shown in Figure 4. Aryl hydroxylation and phenolic conjugation are predominant reactions in wheat. In wild oat, glycosyl ester conjugation is a major reaction. The high concentrations of diclofop in the treatment solutions of excised shoots and in roots of intact wheat and wild oat indicate that hydrolysis of diclofop-methyl to diclofop is the initial degradation reaction.

Ring-OH diclofop in wheat was characterized by electron impact mass spectrometry and the results agreed with those of Gorbach et al. (1977) who characterized the same metabolite by chemical ionization mass spectrometry. The fragmentation of the dimethyl derivative (M^+· 370) differed

considerably between the two methods, but the hydroxylation of the 2,4-dichlorophenyl moiety of diclofop-methyl was confirmed by the mass spectra. However, the position of hydroxyl substitution was not determined in our work.

The glycosyl moieties in the phenolic and ester conjugates (Figure 4) have not been positively identified. Similar *O*-glucosides of ring-hydroxylated chlorpropham (isopropyl *m*-chlorocarbanilate) (Still and Mansager, 1973) and glucose esters of chloramben (3-amino-2,5-dichlorobenzoic acid) (Frear and Swanson, 1978) and flamprop [(±)-2-(*N*-benzoyl-3-chloro-4-fluoroanilino)propionic acid] (Dutton et al., 1976) have been reported. The hexose ester conjugate of flamprop was characterized as the malonylglucose ester (Dutton et al., 1976).

The diphenyl ether bond of diclofop-methyl was not cleaved. This resistance to cleavage was similar to that of nitrofen, a diphenyl ether herbicide with an analogous structure (Frear and Swanson, 1973), but contrasts to fluorodifen (2,4′-dinitro-4-trifluoromethyl diphenyl ether) (Shimabukuro et al., 1973; Frear and Swanson, 1973), a diphenyl ether herbicide that was readily cleaved. Diclofop was the major metabolite of diclofop-methyl in soil and only trace quantities of the phenolic derivatives, 4-(2,4-dichlorophenoxy)phenetole, and 4-(2,4-dichlorophenoxy)phenol, were detected in soil (Smith, 1977). The phenolic derivatives were not detected in wheat or wild oat plants.

Metabolism of diclofop-methyl is probably the basis for herbicidal selectivity between wheat and wild oat. This selective mechanism was not evaluated as readily as with some other herbicides (Frear et al., 1972) since the biologically active forms, diclofop-methyl and diclofop (Shimabukuro et al., 1978), accumulated in low concentrations in both resistant wheat and susceptible wild oat. Irreversible hydroxylation of diclofop to ring-OH diclofop (Figure 4) is probably a detoxication mechanism since it is the predominant reaction in resistant wheat. Conjugation of ring-OH diclofop or in vivo hydrolysis of the phenolic conjugate may have no influence on phytotoxicity.

Diclofop-methyl was a stronger auxin antagonist than diclofop but diclofop was a stronger inhibitor of root growth than diclofop-methyl (Shimabukuro et al., 1978). Diclofop-methyl applied postemergence inhibited growth and resulted in ultrastructural damage particularly to susceptible wild oat (Brezeanu et al., 1976). It was proposed that growth inhibition may be caused primarily by diclofop-methyl and ultrastructural cell damage may be caused by diclofop (Shimabukuro et al., 1978).

The inhibition of growth by diclofop-methyl must occur soon after application since hydrolysis to diclofop is very rapid in plants. However, it takes 4–5 days before visible differences in growth appear. The rapid metabolism of diclofop-methyl and its limited translocation in both the symplast (Brezeanu et al., 1976) and apoplast (Tables VII and VIII) suggest that placement of the herbicide near the apical and meristematic regions of the plant is critical for growth inhibition. The discrete and limited area of chlorosis observed on wheat leaves (Brezeanu et al., 1976) is probably due to the rapid metabolism of diclofop, one of the active forms of the herbicide, to ring-OH diclofop coupled with limited translocation. In wild oat, chlorosis was severe and occurred even in newly expanded leaf tissue (Brezeanu et al., 1976). If diclofop is primarily responsible for ultrastructural damage resulting in chlorosis, a difference in diclofop concentrations should be observed between wheat and wild oat. The low concentrations of diclofop in both wheat and wild oat do not appear to account for the observed selective action between the species. However, the ester conjugate of wild oat is a large, readily hydrolyzable potential diclofop pool. Chloramben appears to be regenerated by in vivo hydrolysis of its glucose ester (Frear et al., 1978). Therefore, if the limited translocated component is the ester conjugate, damage to newly expanded leaf tissue in wild oat may occur from regenerated diclofop. The response of resistant wheat and susceptible wild oat to diclofop-methyl supports the results on metabolism which show that wheat irreversibly detoxifies diclofop-methyl more than wild oat.

Herbicidal mode of action of diclofop-methyl involves growth inhibition and ultrastructural cell damage. The role of each in the effectiveness of wild oat control in the field must be further evaluated. Barban (4-chloro-2-butynyl *m*-chlorocarbanilate) (Friesen, 1961; Shimabukuro et al., 1976) and benzoylprop-ethyl (ethyl (±)-2-(*N*-benzoyl-3,4-dichloroanilino)propionate) (Jeffcoat and Harries, 1973) inhibited growth of wild oat but caused no ultrastructural damage comparable to that of diclofop-methyl. The inhibited wild oat plants survived unless overcome by crop competition. It is likely that inhibition of growth by diclofop-methyl may be sufficient to control wild oat. However, ultrastructural damage together with growth inhibition should increase the effectiveness of diclofop-methyl in controlling wild oat.

The effect of root-applied diclofop-methyl on root growth (Wu and Santlemann, 1976; Shimabukuro et al., 1978) may be due to the action of diclofop since very little diclofop-methyl remains in roots (Table VIII). Diclofop-methyl applied preemergence to soil may hydrolyze rapidly to diclofop (Smith, 1977). Therefore, diclofop may be the form that is absorbed from soil.

ACKNOWLEDGMENT

The authors thank R. G. Zaylskie for his expert assistance in mass spectrometry and C. Graham for computer-assisted graphic display of spectra. The [^{14}C]diclofop-methyl and unlabeled diclofop-methyl and diclofop were provided by Hoechst AG; MC-5127 and nitrofen were provided by Mobil Chemical Co. and Rohm and Hass Co., respectively.

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Received for review November 10, 1978. Accepted February 2, 1979. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Recent Progress in the Study on the Mechanism of Action of Soybean Lipoxygenase[1]

Johannes F. G. Vliegenthart,* Gerrit A. Veldink, and Jan Boldingh

Soybean lipoxygenase-1 (EC 1.13.11.12; M_r 98500) is a mononuclear non-heme iron dioxygenase catalyzing the regio- and stereospecific oxygenation of polyunsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene system. A review is given of some recent results. A new ^{1}H NMR method to analyze the enantiomeric composition of fatty acid hydroperoxides is described. Electron paramagnetic resonance studies of the NO complex of the enzyme indicate that the native enzyme contains Fe(II)–O_2. Kinetic studies of the anaerobic reaction in which both fatty acid hydroperoxides and fatty acids occur as substrates point to a substituted-enzyme mechanism with double substrate inhibition.

Soybean lipoxygenase-1 (EC 1.13.11.12) is a dioxygenase containing 1 mol of non-heme iron per mole of protein (M_r 98500). The dioxygenase function of this enzyme has been extensively studied. In recent years the enzyme has been characterized with a number of spectroscopic techniques. In addition to its dioxygenase properties, under certain conditions lipoxygenase also displays hydroperoxidase activity. [For reviews, see Axelrod (1974), Veldink et al. (1977), Vliegenthart and Veldink (1977), Eskin et al. (1977).] The present paper summarizes some recent results, mainly from the authors' laboratory.

The lipoxygenase-catalyzed oxygenation of polyunsaturated fatty acids, containing a 1,4-*cis*,*cis*-pentadiene system, is shown in Figure 1. The soybean enzyme mainly produces the 13-L-hydroperoxide, whereas the corn germ enzyme gives rise to the formation of predominantly the 9-D isomer. Frequently, questions arise as to which molar ratio of the 13 and 9 isomer is produced and to what extent these hydroperoxides are formed in a stereospecific way. Several methods have been proposed for the determination of the ratio of the positional isomers. Most of the literature data are based on mass spectrometry of the hydroxydienoates or hydroxystearates or by thin-layer chromatography (TLC) of ^{14}C-labeled hydroxy fatty acids. At this moment the best method seems to be high-performance liquid chromatography of the hydroxydienoates or the hydroxydienoic acids, followed by integration of the chromatograms (Aoshima, 1977; Verhagen et al., 1978a). This procedure can also be carried out on a preparative scale. The determination of the enantiomeric composition of the positional isomers is more complicated. Two methods are currently in use; the determination of the optical rotation which in view of the low specific rotation of the hydroxy acids has a rather low accuracy. Furthermore, this method requires the availability of standards of high enantiomeric purity.

A second method has been described by Hamberg (1971) and is based on the separation by gas–liquid chromatography of the menthylchloroformate derivatives of compounds obtained after reduction of the hydroperoxy group, followed by ozonolysis and esterification.

Recently, we developed a new and simple nuclear magnetic resonance (NMR) method based on the lanthanide-induced shift of protons in a chiral derivative of hydroxydienoates (Van Os et al., unpublished data). The derivatization is carried out with one of the enantiomers of the acid chloride of α-methoxy-α-(trifluoromethyl)-phenylacetic acid (MTPA ester). This gives rise to diastereomers for the D and L hydroxydienoate (Figure 2).

For the determination of the ratio of enantiomers, it is essential that the NMR signals are well resolved. This can be achieved by the addition of a lanthanide shift reagent like Eu(fod)$_3$. The signal of the OCH_3 group of the fatty acid moiety shifts strongly, whereas the OCH_3 signal of the MTPA group broadens and shifts only slightly. Increasing amounts of shift reagent afford a splitting of this signal. When the signal is well separated in two peaks, integration of these peaks gives the molar ratio of the enantiomers (Figure 3). With a spectrometer operating in the Fourier transform mode, less than 1 mg of starting material is sufficient.

MECHANISM OF THE ANAEROBIC REACTION

A few years ago we proposed a scheme for the mechanism of the reactions catalyzed by soybean lipoxygenase-1 (De Groot et al., 1975). Figure 4 summarizes the mech-

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[1]Dedicated to Professor Dr. E. Havinga on the occasion of his 70th birthday.

0021-8561/79/1427-0623$01.00/0 © 1979 American Chemical Society