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Cross-Resistance to Herbicides in Annual Ryegrass (*Lolium rigidum*)[1]

III. On the Mechanism of Resistance to Diclofop-Methyl

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ABSTRACT

Annual ryegrass (*Lolium rigidum*) biotype SLR 31 is resistant to the postemergent graminicide methyl-2-[4-(2,4-dichlorophenoxy)phenoxy]-propanoate (diclofop-methyl). Uptake of [^{14}C](U-phenyl)diclofop-methyl and root/shoot distribution of radioactivity in susceptible and resistant plants were similar. In both biotypes, diclofop-methyl was rapidly demethylated to the biocidal metabolite diclofop acid which, in turn, was metabolized to ester and aryl-*O*-sugar conjugates. Susceptible plants accumulated 5 to 15% more radioactivity in diclofop acid than did resistant plants. Resistant plants had a slightly greater capacity to form nonbiocidal sugar conjugates. Despite these differences, resistant plants retained 20% of ^{14}C in the biocidal metabolite diclofop acid 192 hours after treatment, whereas susceptible plants, which were close to death, retained 30% in diclofop acid. The small differences in the pool sizes of the active and inactive metabolites are by themselves unlikely to account for a 30-fold difference in sensitivity to the herbicide at the whole plant level. Similar high-pressure liquid chromatography elution patterns of conjugates from both susceptible and resistant biotypes indicated that the mechanisms and the products of catabolism in the biotypes are similar. It is suggested that metabolism of diclofop-methyl by the resistant biotype does not alone explain resistance observed at the whole-plant level. Diclofop acid reduced the electrochemical potential of membranes in etiolated coleoptiles of both biotypes; 50% depolarization required 1 to 4 μM diclofop acid. After removal of diclofop acid, membranes from the resistant biotype recovered polarity, whereas membranes from the susceptible biotype did not. Internal concentrations of diclofop acid 4 h after exposing plants to herbicide were estimated to be 36 to 39 micromolar in a membrane fraction and 16 to 17 micromolar in a soluble fraction. Such concentrations should be sufficient to fully depolarize membranes. It is postulated that differences in the ability of membranes to recover from depolarization are correlated with the resistance response of biotype SLR 31.

Diclofop-methyl[2] is an aryloxyphenoxypropionate graminicide which, in Australia, is used as a postemergent herbicide to remove grass weeds, mainly wild oats (*Avena fatua* and *Avena sterilis*) and annual ryegrass (*Lolium rigidum*), from wheat, barley, and legume crops. In its commercially available form, diclofop-methyl is a 1:1 mixture of *R*(+)- and *S*(−)-enantiomers which do not exhibit the same toxicity (16). The herbicide is a potent inhibitor of the enzyme ACCase (19) and can depolarize membranes (18, 22, 29). In animals and humans, similar compounds have been reported to inhibit ACCase (3), disrupt lipid metabolism (7), and interfere with membrane transport (1).

In the southern Australian cereal and grain-legume cropping regions, several hundred populations of *L. rigidum* have developed resistance to diclofop-methyl following selection with this herbicide. Many of the diclofop-resistant biotypes also exhibit resistance to other herbicides, including the aryloxyphenoxypropionate graminicides fluazifop-butyl, haloxyfop-methyl, quizalofop-ethyl, fenoxaprop-ethyl, and chlorazifop-propynil; the cyclohexanedione graminicides sethoxydim, cycloxydim, alloxydim-sodium, and tralkoxydim; the sulfonylurea herbicides chlorsulfuron, metsulfuron-methyl, and triasulfuron; the dinitroaniline herbicide trifluralin; and, to a lesser extent, the triazinone herbicide metribuzin (6, 10–12, 17). Resistance to herbicides other than diclofop-methyl is biotype dependent. A population resistant to a number of triazine and phenylurea herbicides has been also described (4).

The herbicides against which there is resistance do not exhibit the same modes of action. Like the aryloxyphenoxypropionates, the cyclohexanediones inhibit ACCase from grasses (5, 19, 20). At least one cyclohexanedione, sethoxydim, also has the capacity to depolarize membranes (30). The

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[2] Abbreviations: diclofop-methyl, methyl-2-[4-(2,4-dichlorophenoxy)phenoxy]-propanoate; fluazifop-butyl, (±)-butyl 2-[4-[(5-(trifluoromethyl)-2-pyridinyl)oxy]-phenoxy]propanoate; ACCase, acetyl coenzyme A carboxylase; alloxydim, 2-(1-alloxyaminobutylidene)-5,5-dimethyl-4-methoxycarbonyl-cyclohexane-1,3-dione; chlorsulfuron, 2-chloro-*N*-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]-benzene-sulfonamide; haloxyfop-methyl, (±)-methyl-2-[4-((3-chloro-5-(trifluoromethyl)-2-pyridinyl)oxy)-phenoxy]propanoate; sethoxydim, [1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one; trifluralin, a,a,a-trifluoro-2,6-dinitro-*N*-*N*-dipropyl-*p*-toluidine; MFO, mixed-function oxidase.

sulfonylureas are potent inhibitors of acetolactate synthase (2), trifluralin inhibits tubulin polymerization (27), and metribuzin is an inhibitor of PSII activity. Resistance in *L. rigidum* biotype SR4/84 to aryloxyphenoxypropionate and cyclohexanedione graminicides is not correlated with either the amount or the kinetic properties of the target enzyme ACCase (14). Cross-resistance to the sulfonylurea herbicide chlorsulfuron is not correlated with any properties of acetolactate synthase (14) but is correlated with the ability to metabolize the herbicide (6).

Several mechanisms of resistance or tolerance to diclofop-methyl have been reported in other species. ACCase that exhibits reduced sensitivity to the herbicide has been extracted from tolerant dicotyledon species (5), the tolerant grass *Festuca rubra* (25), and from a resistant biotype of *Lolium multiflorum* (8). Wheat, like resistant ryegrass, possesses a herbicide-sensitive ACCase (14) yet tolerates diclofop-methyl (13, 21). This tolerance is due, at least in part, to the high capacity of wheat to detoxify the biocidal metabolite diclofop acid (23). Wheat contains an active MFO which catalyzes the physiologically irreversible aryl-hydroxylation of diclofop-acid (15). The aryl-hydroxyl group is glucosylated to form a sugar conjugate with little or no herbicidal activity. Plasma membranes from wheat coleoptiles are depolarized by diclofop acid but can, given favorable conditions, recover transmembrane potentials (29). The overall tolerance of wheat to diclofop-methyl is, therefore, the result of a combination of properties, including the relatively high activity of the detoxifying system, the unidirectional nature of the initial detoxifying reaction, the biocidal inactivity of the detoxification products, and the ability of the plasma membranes to recover polarity.

We report here the ability of the leaves of susceptible ryegrass biotype SLR 2 and resistant ryegrass biotype SLR 31 to absorb, translocate, and metabolize diclofop-methyl. The effects of diclofop acid on electrochemical membrane potentials in the two biotypes are also described.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Susceptible ryegrass (*Lolium rigidum* biotype SLR 2 and resistant biotype SLR 31) were characterized originally by Heap and Knight (11) who referred to them as populations 1 and 12, respectively. The latter biotype is resistant to herbicides including diclofop-methyl, haloxyfop-methyl, haloxyfop-ethoxyethyl, fluazifop-butyl, quizalafop-ethyl, fenoxaprop-ethyl, trifluralin, alloxydim, sethoxydim, chlorsulfuron, metsulfuron methyl, and triasulfuron. It is susceptible to triazines and phenylureas. Wheat (*Triticum aestivum*) cv Millewa and oats (*Avena sativa*) cv Echidna which are tolerant and susceptible to diclofop-methyl, respectively, were also used.

Seeds were germinated on 0.8% agar, transplanted at the one-leaf stage, and grown in sterilized potting soil. Growth conditions were 20°C, 12 h, 330 μmol photons m^{-2} s^{-1} light period/16°C, 12 h dark period. Plants were used for experiments when at the two-leaf stage, the developmental stage at which they would normally be sprayed in the field.

Application of [^{14}C](U-Phenyl)Diclofop-Methyl

Typically, 2.5 mM [^{14}C](U-phenyl)diclofop-methyl (592 Bq μL^{-1}) in 1 μL of a solution that contained, with the exception of herbicide, the ingredients present in the commercial herbicide formulation Hoegrass (Hoegrass 36 EC, Hoechst Australia), was deposited on leaf axils of two-leaved ryegrass plants in the light. The amount of diclofop-methyl applied per plant is similar to that deposited on the axils of plants when the herbicide is sprayed at recommended rates (about 11 mM) under agricultural conditions. In some experiments, 1 μL of 70% (v/v) acetone containing 2.5 mM [^{14}C]diclofop-methyl (592 Bq μL^{-1}) and 0.02% (v/v) Triton X-100 was deposited on the leaf axils.

Extraction, Separation, and Identification of Metabolites

After varying periods of exposure, tissue between the zone of root initiation and 2 to 3 cm above the leaf axil was separated from the roots and tops. In some experiments the plant fractions were extracted separately; in others they were pooled. Each extraction contained material from 24 to 30 plants. Tissue was washed quickly and vigorously in 100 mL of 20% methanol containing 0.02% (v/v) Triton X-100. The washes were retained and the tissue segments blotted dry, weighed, and frozen in liquid N_2. Following pulverization in a mortar, the tissue powder was extracted with 5 mL of 80% methanol at −20°C. The brei was centrifuged at 9000*g* for 20 min at 4°C. The pellet was resuspended in 2 mL of 80% methanol, reextracted, and recentrifuged. The supernatants were pooled.

HPLC Analyses

Radiolabeled metabolites were separated using gradient reverse-phase HPLC with a Brownlee Labs ODS-5 Spherisorb-5 column (250 × 4.6 mm i.d.). Radioactivity was detected with a flow-through scintillation detector (Radiomatic A140, Canberra Instruments).

Solvents used were 10% (v/v) acetonitrile:89% (v/v) H_2O:1% (v/v) acetic acid (solvent A) and 90% (v/v) acetonitrile:9% (v/v) H_2O:1% (v/v) acetic acid (solvent B). Elution conditions involved a 10-min linear gradient from 30 to 35% solvent B, followed by a 12-min linear gradient from 35 to 50% solvent B, followed by a 3-min linear gradient from 50 to 100% solvent B. The column was then eluted with 100% solvent B for 10 min. At all times, the combined flow rates of solvents A and B were 1.5 mL min^{-1}.

Extracts were reduced in volume under vacuum and resuspended in sufficient 50% methanol to give specific radioactivities suitable for the radioactivity detector. Samples were filtered through a 0.22-μm Teflon filter before injection.

TLC Analyses

Aliquots were spotted onto TLC plates (Kieselgel 60, Merck GmbH), dried, and developed in toluene:ethanol:acetic acid, 150:7:7. The TLC plates were sprayed with a fluorescent radioactivity-intensifying compound (Amplify, Amersham), overlain with Kodak XAR 5 x-ray film and exposed at -80°C for 3 d. The labeled herbicide and metabolites were identified

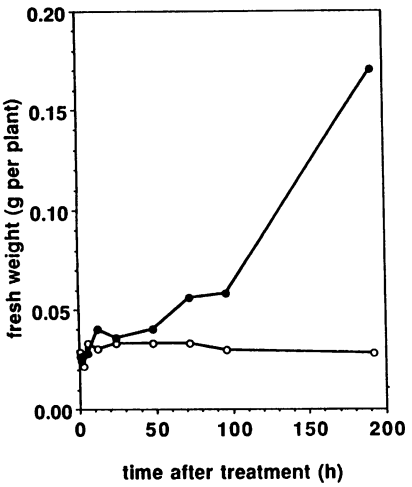

Figure 1. Fresh weights of susceptible (○) and resistant (●) *L. rigidum* following the application to the leaf axil of two-leaf plants of 1 μL of 2.5 mM [^{14}C]diclofop-methyl dissolved in a commercial herbicide blank. Each value is the average for 30 plants.

following comparison with authentic labeled and unlabeled diclofop-methyl and diclofop acid and with labeled conjugates from wheat and oats.

Radioactivity in extracts, TLC spots, washes, and pellets was determined by scintillation counting. Counts were converted to disintegrations following corrections for background radioactivity, quenching, and counting efficiency.

Measurement of Herbicide Contents in Plant Fractions

Ryegrass plants, labeled with [^{14}C]diclofop-methyl for 4 h, were washed in 10% methanol and extracted with acid-washed sand, using a mortar and pestle, in ice-cold 10 mM Hepes, pH 7.2. Cell walls, cuticle, and other coarse-insoluble matter were filtered out with a 2-mm mesh strainer. The filtrate was washed with a small volume of extraction buffer and spun in a microfuge for 2 min. The pellet was rewashed and respun. The combined eluate and supernatants were spun for 30 min at 100,000*g* and 4°C. The pellet was resuspended in a small volume of extraction buffer and recentrifuged for 15 min at 100,000g. The pellet was designated membranous material, and the pooled supernatants were designated the soluble fraction.

A 4-h labeling time was used because it was recognized that the mild extraction procedure would not stop esterase-catalyzed demethylation of diclofop-methyl from taking place during the extraction. At 4-h postlabeling, nearly all diclofop-methyl is already converted to diclofop acid; yet, there is little to no formation of subsequent metabolites. In an attempt to determine whether the membranous fraction removed diclofop acid from the extract, or vice versa, labeled membrane or labeled soluble fractions were diluted with varying amounts of unlabeled extract or unlabeled membrane and incubated at 4°C for 30 min before separation by centrifugation. No loss of ^{14}C from membranes into the soluble fraction occurred, nor did membranes absorb [^{14}C]diclofop acid from the soluble fraction.

Measurement of Membrane Potentials

The membrane potentials of cells in etiolated coleoptiles were measured as described by Häusler *et al.* (9).

RESULTS

Effect of [^{14}C]Diclofop-Methyl on Growth of Ryegrass

The amount of [^{14}C]diclofop-methyl applied to the susceptible and resistant plants killed the susceptible but not the resistant plants. Some leaf elongation was observed in the susceptible plants, but in no cases did a third leaf emerge. Growth in resistant plants was slowed for about 48 h (Fig. 1).

Uptake of [^{14}C]Diclofop-Methyl

The uptake kinetics for [^{14}C]diclofop-methyl dissolved in a commercial formulation (Fig. 2) were similar for both susceptible and resistant ryegrass. The extent of uptake was maximal 24 h after application with 50% of uptake having occurred by about 4 h. Although the total amount of ^{14}C recovered during the period of the experiment shown in Figure 2 decreased with time, the loss was not from inside the tissue. It is probable that the loss of ^{14}C was due to volatilization of the herbicide from the leaf surface.

The kinetics of uptake of [^{14}C]diclofop-methyl were formulation dependent (J.A.M. Holtum, unpublished data). Uptake in both biotypes was more rapid when herbicide was dissolved in 70% acetone containing 0.02% Triton X-100. Nevertheless, for each formulation, uptake by the susceptible and resistant biotypes was similar.

The root/shoot distribution of ^{14}C from diclofop-methyl was the same in both biotypes (Table I). More than 90% of the radiolabel remained in the shoots and meristematic region for at least 48 h after treatment. A similar distribution has been observed in *A. fatua* (28).

Radioactivity in Diclofop-Methyl and Diclofop Acid

In susceptible and resistant tissues, radioactivity was lost from diclofop-methyl at an equal rate (Fig. 3A). Radioactivity rapidly accumulated in diclofop acid; 45 min after treatment, 55% of the label in the tissue was in diclofop acid (Fig. 3B).

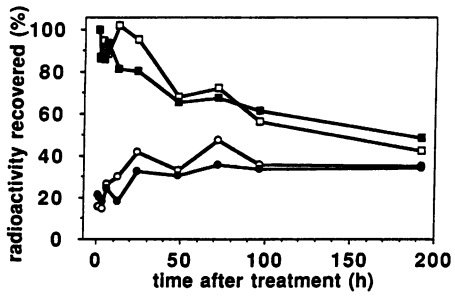

Figure 2. Radioactivity recovered during a 192-h treatment period from washed (○, ●) and unwashed (□, ■) susceptible (○, □) and resistant (●, ■) *L. rigidum* treated with 1 μL of 2.5 mM [^{14}C]diclofop-methyl dissolved in a commercial herbicide blank. Each value is the average for 30 plants.

Table I. *Recovery and Distribution of Radioactivity from [^{14}C] Diclofop-Methyl Deposited on the Leaf Axils of Two-Leaved Plants of Susceptible and Resistant Annual Ryegrass*

Exposure Period	Radioactivity in		Recovery of Radioactivity
	Shoots	Roots	
h	%		% ^{14}C *applied*
Susceptible			
0.5	100	ND[a]	82.1
9	96.3	3.7	91.7
48	97.1	2.9	81.7
Resistant			
0.5	100	ND	78.3
9	97.3	2.7	78.3
48	95.3	4.7	94.3

[a] Not detected.

The amounts of radioactivity in diclofop acid were not the same in the two biotypes; the difference shown in Figure 3B was seen both when the herbicide was fed in a commercial formulation and when given in 70% acetone. From 6 to 192 h postapplication, the susceptible tissue consistently contained 5 to 15% more ^{14}C in diclofop acid than did resistant tissue. At 6 h postapplication, the time at which the radioactive content of the diclofop acid pools was at a maximum, 85% of the radioactivity in the susceptible tissue was in diclofop acid compared with 80% in the resistant tissue. Assuming a similar specific activity for radioactive diclofop acid in both tissues, this difference indicates that the pool size of diclofop acid in the susceptible tissue was 1.5 nmol per plant compared with 1.2 nmol per plant in the resistant tissue, a difference of 1.26-fold. After 96 h exposure, the amounts of diclofop acid had decreased in both biotypes, but the diclofop acid pool in the susceptible plants (30%) was 1.5-fold that of the pool in the resistant plants.

Radioactivity in Metabolites

Resistant tissue accumulated more radioactivity in metabolites than did susceptible tissue (Fig. 3C). The rate of accumulation was 1.3 times greater in the resistant tissue during the 24 h immediately postapplication; the subsequent slower rate of accumulation was similar for both tissues. After 72 h, the net transfer of ^{14}C from diclofop acid to other metabolites ceased in both biotypes.

The metabolites, which exhibited zero mobility on TLC plates developed in toluene:ethanol:acetic acid, 150:7:7, were separated by reverse-phase HPLC (Fig. 4). The ^{14}C elution patterns of extracts from the susceptible and resistant ryegrass biotypes 96 h after exposure to diclofop-methyl were similar. The compound containing the most radioactivity, even 96 h posttreatment, was diclofop acid in both biotypes. A substantial, but similar, portion of radioactivity was present in a number of more polar metabolites which could be separated into two groups, *viz.* polar compounds with elution times of <11 min and those less polar compounds with elution times of 11 to 25 min. Although the individual metabolites have not been identified, the elution profiles shown in Figure 4 are similar, which indicates that, if there are differences in the metabolites produced by the two biotypes, the differences are small.

The ryegrass elution patterns differed from those of wheat (Fig. 5A), which is tolerant to diclofop-methyl, and oats, which is susceptible to diclofop-methyl (Fig. 5B). Twenty-four hours after exposure to [^{14}C]diclofop-methyl, wheat had converted nearly all the ^{14}C into polar compounds which eluted from the column in <10 min. Only 8% of the ^{14}C was present as diclofop acid and only 1.6% as diclofop-methyl. These polar compounds have been identified by others as mainly aryl-*O*-sugar conjugates (23, 26). In contrast, oat, which is susceptible to the herbicide diclofop-methyl, exhibited a limited capacity to metabolize the herbicide beyond diclofop acid. Small amounts of polar metabolites were formed, as were larger amounts of metabolites of intermediate polarity which are probably ester sugar conjugates (23). In oats 24 h after exposure to the herbicide, 30% of the radioactivity was present as diclofop acid.

Effect of Diclofop Acid on Electrochemical Membrane Potential

In the presence of 50 μM diclofop acid, membranes in etiolated coleoptiles from both susceptible and resistant ryegrass depolarized during a 5- to 10-min period (Fig. 6). After removal of diclofop acid, membranes from the resistant biotype recovered polarity, but membranes from the susceptible

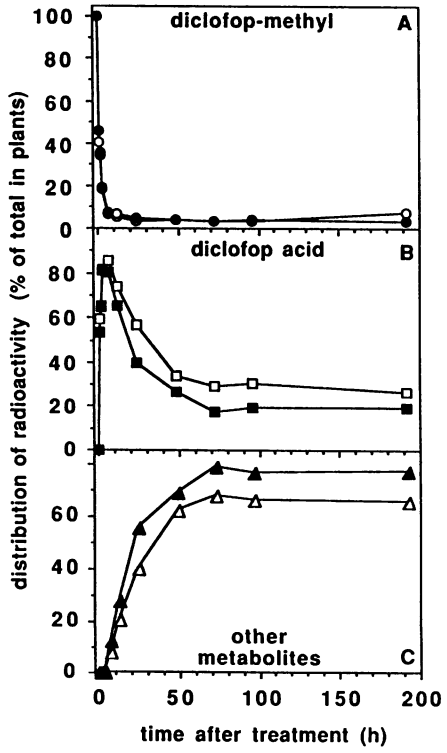

Figure 3. Distribution of radioactivity in diclofop-methyl (A), diclofop acid (B), and other metabolites (C) up to 192 h after treatment with 1 μL of 2.5 mM [^{14}C]diclofop-methyl dissolved in a commercial herbicide blank. Open symbols, susceptible; closed symbols, resistant. Each value is the average for 30 plants.

biotype did not. The extent of depolarization but not repolarization was concentration dependent (Fig. 7). The concentration of diclofop acid required to reduce membrane potentials by 50% was approximately 4 μM for the susceptible tissue and 1 μM for the resistant tissue. Depolarization responses have been observed also for excised root tips from ryegrass and in coleoptiles from wheat, oats, and wild oats (22, 29).

Estimation of the Internal Concentration of Diclofop Acid

Diclofop acid was associated with crude membrane fractions extracted from susceptible and resistant ryegrass (Table II). Four hours after exposure of the tissue to [^{14}C]diclofop-methyl, 3.6 and 3.9 nmol of ^{14}C were present in membranous fractions from susceptible and resistant tissues, respectively. If one makes the conservative assumption that membranes constitute about 10% of the volume of young growing cells, then it can be estimated that the average concentrations of diclofop-acid in susceptible and resistant ryegrass were 35.8 and 38.7 μM, respectively, for membrane fractions and 16 and 17 μM, respectively, for the soluble fractions.

DISCUSSION

Uptake, Translocation, and Metabolism of [^{14}C]Diclofop-Methyl

Resistant *L. rigidum* biotype SLR 31 does not possess a mechanism that retards the rate of entry of diclofop-methyl into the plants (Fig. 2), nor do they exhibit different root/shoot distributions of diclofop-methyl or its catabolites (Table I). The extent of disappearance of radioactivity from absorbed diclofop-methyl indicates that at least 95% of the diclofop-methyl in the tissues was located in one or more pools readily accessible to degradative metabolic processes. The rate of loss of radiolabel from absorbed diclofop-methyl was rapid and similar in both susceptible and resistant ryegrass. These data indicate that resistance is not related to differential uptake, metabolism, or interorgan translocation of diclofop-methyl.

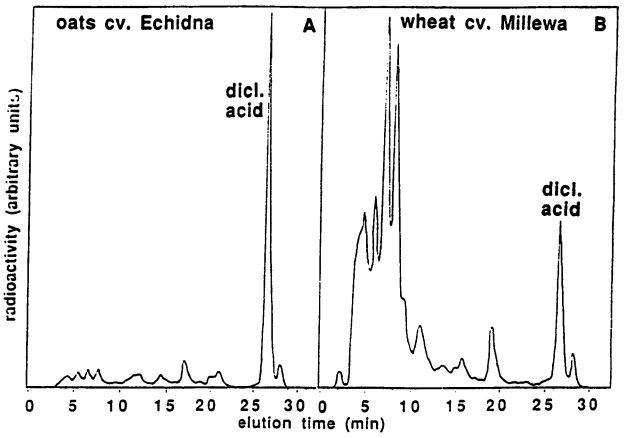

Figure 5. HPLC elution profiles of extracts from wheat cv Millewa (A) and oat cv Echidna (B) 24 h after exposure to [^{14}C]diclofop-methyl dissolved in a commercial herbicide blank.

Biotypic differences in the capacity to metabolize diclofop acid and/or in the internal compartmentation of the compound are most likely responsible for the greater accumulation of radioactivity in diclofop acid in the susceptible plants than in the resistant plants (Fig. 3B). Although the differences in the amounts of radioactivity, and presumably the pool sizes, represent only 5 to 15% of the whole plant radioactivity, on a whole plant basis they could reflect, depending upon the intracellular location, substantial differences in concentrations *in vivo*.

Metabolism of Diclofop Acid

Both ryegrass biotypes maintained significant, but not equal, internal pools of the toxic metabolite diclofop acid (Fig. 3B). The pool sizes were greater than those in wheat, which tolerates the herbicide because it can rapidly degrade it, but smaller than in oats, which is susceptible. Despite the

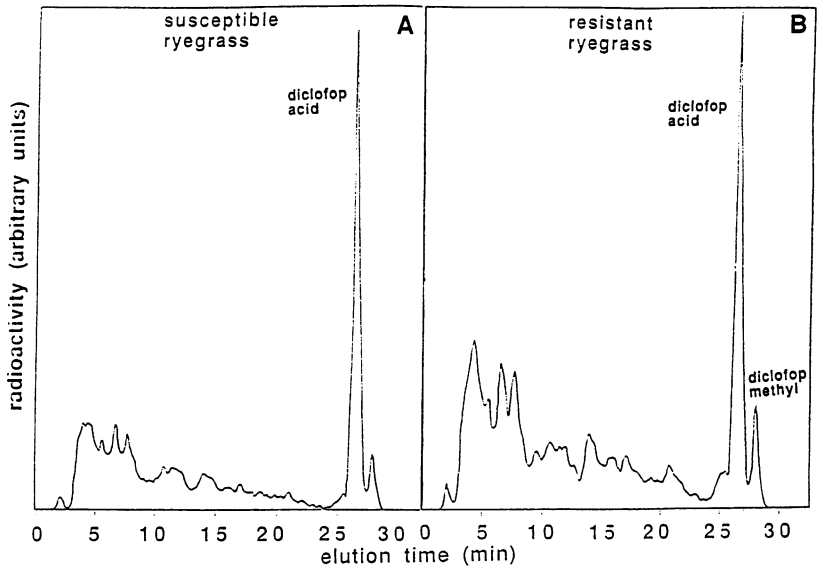

Figure 4. HPLC elution profiles of extracts from susceptible (A) and resistant (B) ryegrass plants 96 h after exposure to [^{14}C]diclofop-methyl dissolved in a commercial herbicide blank.

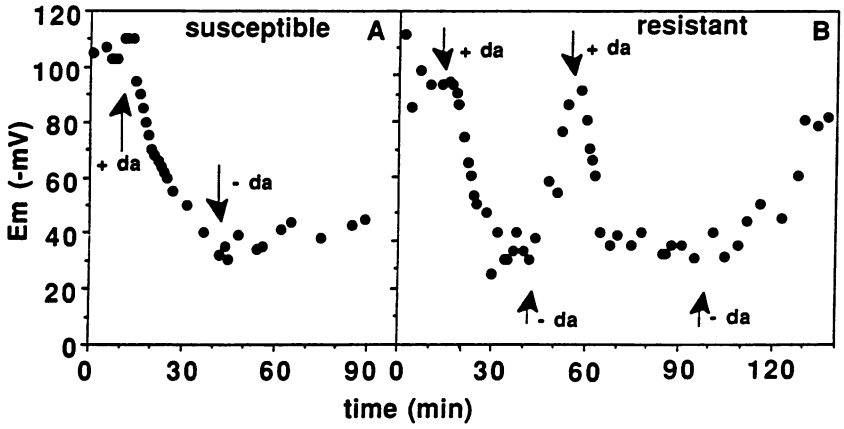

Figure 6. Electrochemical potentials measured in cells of etiolated coleoptiles of susceptible (A) and resistant (B) ryegrass. Arrows, times when 50 μM diclofop acid was added to or removed from the bathing solution.

presence of considerable pools of diclofop acid and the possession of herbicide-sensitive ACCase (14), growth continued in the resistant biotype but was inhibited in the susceptible biotype.

The similarity of the distribution of radioactivity in HPLC profiles of extracts from susceptible and resistant ryegrass (Fig. 4) indicates that, in the biotypes tested, when diclofop-methyl is applied to the leaf surface in a manner known to give herbicidal activity, similar pathways are involved in its metabolism. Either the metabolites are not toxic to the resistant plants or the intra- or intercellular locations of the metabolites are not the same.

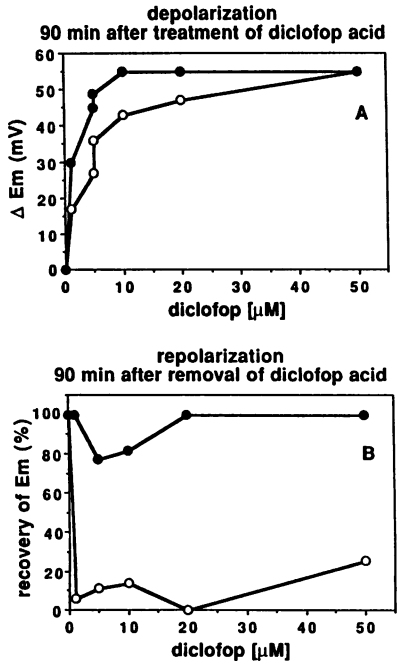

Figure 7. Depolarization (A) and repolarization (B) of electrochemical membrane potential in cells of etiolated coleoptiles of susceptible (○) and resistant (●) ryegrass. Values are steady-state potentials measured 90 min after the addition or removal of diclofop acid. Repolarization is expressed as the percentage recovery of ΔE_m of depolarization.

The metabolism of diclofop acid and the nature of metabolites in susceptible and resistant ryegrass were intermediate between that of diclofop-tolerant wheat and diclofop-susceptible oats. These observations do not support the postulate that resistance in SLR 31 is the product of an enhanced capacity of MFO to detoxify diclofop acid (17). Despite this observation, enhanced MFO-catalyzed detoxification may be involved in the ability of biotype SLR 31 to resist the sulfonylurea herbicide chlorsulfuron (6). It is possible that exposure to diclofop in the field selects for the increased activity of a MFO which has some affinity for diclofop but which has a greater affinity for chlorsulfuron. However, because SLR 31 contains substantial pools of chlorsulfuron even 12 h after exposure (6), it is also possible that the biotype may exhibit an increased capacity to sequester the herbicide and that the higher rate of chlorsulfuron metabolism observed in the resistant plants is due to there being less cellular damage. Leakage of the herbicide from the compartment could be detoxified by the intrinsic activity of detoxifying enzymes.

Effect of Diclofop Acid on Electrochemical Membrane Potentials

The responses to diclofop acid of the electrochemical potentials in coleoptiles of susceptible and the resistant ryegrass

Table II. *Estimated Concentrations of Diclofop Acid in Soluble, Membranous, and Residue-Containing Fractions from Susceptible and Resistant Annual Ryegrass Labeled with [^{14}C]Diclofop-Methyl for 4 Hours*

The values were estimated assuming that 1 g of tissue was equivalent to 1 mL and that membranes constitute 10%, residue fraction 20%, and soluble fraction 70% of the cell volume of dividing tissues. After extraction, 1 g of susceptible tissue contained 19.8 nmol of ^{14}C of which 16.8 nmol was in diclofop acid; 1 g of resistant tissue contained 20.7 nmol of ^{14}C of which 16.7 nmol was in diclofop acid.

Biotype	Plant Fraction		
	Membrane	Soluble	Residue
		μM	
Susceptible	35.8	17.1	6.0
Resistant	38.7	16.1	7.7

were not the same (Fig. 6). Coleoptiles of resistant ryegrass recovered full membrane potentials only if diclofop acid was removed from the bathing solution. The membrane potentials in susceptible coleoptiles did not recover full membrane potentials (Figs. 6 and 7). Because both the reduction and the recovery of membrane potential depended upon the treatment concentration of diclofop acid, the recovery response will only be of importance to resistance in ryegrass if, first, the internal concentrations of diclofop acid are high enough to depolarize membranes in both biotypes and, second, if resistant plants possess a mechanism that reduces the concentration of herbicide in the vicinity of the affected membranes. The internal concentrations of diclofop acid appear sufficient to depolarize membranes because the concentrations of diclofop acid that are required for 50% inhibition of ACCase, a target site of diclofop acid, and for 50% membrane depolarization are similar (14, 19; Fig. 7).

One can calculate the average concentration of diclofop acid in the tissues. Figure 2 shows that 4 h after treatment 18% of the 2.45 nmol of [^{14}C]diclofop-methyl applied per plant was taken up by the tissues with 70% present as diclofop acid; with the conservative assumption that the acid is uniformly distributed throughout the meristematic and shoot region (fresh weight of about 0.03 g plant^{-1}), then the average concentration of acid would be approximately 10 μM in both the resistant and susceptible tissue. This concentration is sufficient for 100% depolarization of membranes (Fig. 7) and inactivation of ACCase (14, 19). However, our experiments with isolated membrane fractions indicate that the distribution of [^{14}C]diclofop acid among membranous and soluble and insoluble fractions is not uniform (Table II). The concentration of [^{14}C]diclofop associated with membranes is approximately 37 μM 4 h after treatment. These concentrations are far in excess of those required for full depolarization of membranes from both susceptible and resistant biotypes and for irreversible depolarization of membranes from susceptible plants. Therefore, our data indicate that membrane depolarization occurs when diclofop-methyl is applied at normal agricultural rates and supports the suggestion of Shimabukuro (21) who concluded that membrane depolarization may be involved in the toxic action of diclofop-methyl. Indeed, membranes in intact ryegrass coleoptiles sprayed with a recommended rate of diclofop-methyl are depolarized and remain so 45 h after treatment (RE Häusler, unpublished data). It is stressed, however, that the total concentration of diclofop acid in the soluble fraction was in the region of 16 to 17 μM (Table II). Such concentrations, if present in the chloroplasts, would be sufficient to fully inhibit ACCase from both ryegrass biotypes. *In vitro,* ACCase is inhibited 50% by <1 μM diclofop acid (14, 19).

In ryegrass biotype SLR 31, both plasma membrane and ACCase appear to be sites of diclofop activity. The continued blockage of either site will retard growth and ultimately result in plant death. Inhibition of ACCase is reversible and is, therefore, dependent upon the local concentration of the herbicide and the duration of exposure. The physiological mechanism that permits the recovery of membrane polarity is unknown, but the initial observations indicate that the ability to recover polarity is not an intrinsic feature of all plants (9, 21). A more extensive survey of this phenomenon in susceptible and tolerant species is required.

Mechanism of Resistance to Diclofop-Methyl in Ryegrass Biotype SLR 31: A Hypothesis

Any hypothesis for resistance in SLR 31 must accommodate the following observations: both resistant (SLR 31) and susceptible (SLR 2) biotypes possess ACCase that is inhibited by low concentrations of diclofop acid (14), membranes that are depolarized by low concentrations of diclofop acid (Figs. 6 and 7), and internal pools of diclofop acid that persist for at least 192 h after exposure to the herbicide diclofop-methyl (Figs. 3 and 4). In addition, the rates of entry of herbicide into susceptible and resistant plants are similar (Fig. 2), the distribution of herbicide between roots and shoots is similar (Table I), and the amounts and nature of herbicide catabolites are also similar (Fig. 4). Despite these similarities, the rate of herbicide required to kill 50% of a population of SLR 31 is at least 30-fold greater than that required for SLR 2 (14). If resistant plants grow despite having target sites sensitive to the herbicide and having active herbicide in the tissue, then it must be concluded that the concentration of herbicide at the target sites in the resistant plants is either always low or that an initially high concentration is reduced and any damage

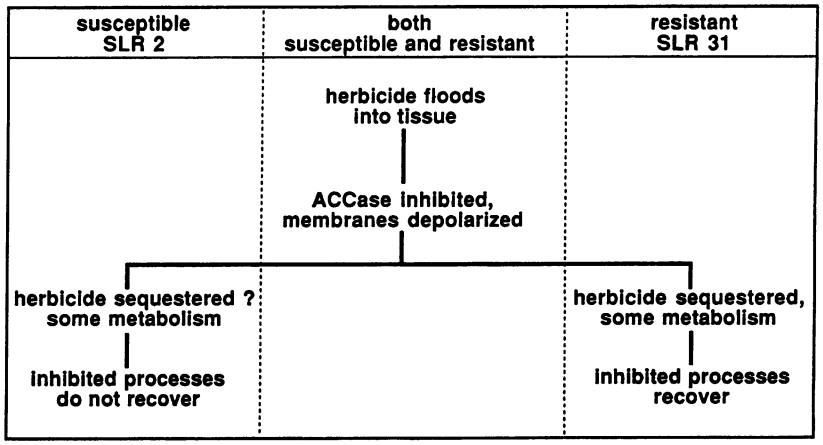

Figure 8. A model that describes the postulated sequence of events that lead to resistance to diclofop-methyl in *L. rigidum* biotype SLR 31.

is reversible. This observation that resistant and susceptible plants differ in their ability to recover from herbicide-induced membrane damage forms the basis for the mechanism proposed for resistance presented in Figure 8 and described below.

After herbicide is sprayed, it floods the tissues in both biotypes (Fig. 3), inhibiting ACCase activity and depolarizing membranes. It is suggested that diclofop acid is then sequestered, perhaps in the vacuole or the apoplastic space, such that the concentration of the biocidal components in the vicinity of the membranes and ACCase is reduced. Although we have no data that demonstrate sequestration *per se*, such a postulate would account for the substantial pools of diclofop acid in the tissues and the observation that the resistant plants continue to grow in the presence of these pools.

Sequestration would reduce the cytosolic concentration of herbicide. At sufficiently low concentrations the inhibition of ACCase, which is reversible (19), would be lifted in both biotypes but only the membranes in resistant plants would repolarize (Fig. 6). If herbicide leaks from the sequestration compartment, the rate of leakage in resistant plants would have to be one that can be adequately handled by the detoxification system which, in comparison to wheat but not oats, is slow (*cf.* Figs. 4 and 5). Because the metabolism of diclofop acid is effectively complete after 72 h (Fig. 3), all remaining herbicide must either be unavailable to the metabolic processes or those processes are inactivated. The latter possibility is unlikely to be the case for the resistant plants.

It is not clear from our data whether both biotypes have a similar capacity to sequester herbicide or whether biotypic differences in compartmentation occur. Although the susceptible plants maintain a slightly larger pool of diclofop acid, both biotypes maintain diclofop acid pools even 192 h after treatment. A less effective sequestration process in the susceptible plants, although not required by the model, would increase their susceptibility to the herbicide. The postulate that resistance to diclofop in ryegrass biotype SLR 31 is endowed by differences in the properties of one or more membrane-associated proteins is being tested, as is the ability of ryegrass to sequester herbicides.

It should be noted that our studies have been conducted with only one of hundreds of biotypes of resistant ryegrass. Because most cases of resistance have evolved separately, the mechanism of resistance will not necessarily be the same in all populations. It is also probable that populations will contain individuals with different mechanisms of resistance. In a population of a closely related species, *L. multiflorum* or Italian ryegrass (24), with resistance to diclofop-methyl the sensitivity of ACCase to inhibition by the herbicide is less in resistant than in susceptible plants (8). The *L. multiflorum* biotype is not cross-resistant to other herbicides. To our knowledge, the capacity of membranes from susceptible and resistant *L. multiflorum* to recover polarity following exposure to diclofop-methyl has not been tested.

In conclusion, the only diclofop-related physiological and biochemical differences yet reported between susceptible ryegrass biotype SLR 2 and resistant ryegrass biotype SLR 31 are the differences in the ability to recover membrane polarization, the 15% higher pool sizes of diclofop acid in shoots of susceptible plants, and a 15% higher capacity to form conjugates in the shoots of resistant plants. At present, the simplest interpretation of the data is that given in Figure 8.

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