2,4-D Metabolism in Resistant Grasses

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3-(2,4-Dichlorophenoxy)propionic acid [3-(2,4-DP)] was recovered from bromegrass (*Bromus inermis* Leyss.), timothy (*Phleum pratense* L.), and orchardgrass (*Dactylis glomerata* L.) after application of 2,4-dichlorophenoxyacetic acid (2,4-D). Identification of the metabolite of 2,4-D was accomplished by mass spectroscopy. As much as 70 ppm of 3-(2,4-DP) was found in bromegrass and 20 ppm in timothy and orchardgrass, indicating that it is a major metabolite of 2,4-D. The conversion of 2,4-D to herbicidally inactive 3-(2,4-DP) may be a primary mechanism of resistance of grass species to 2,4-D.

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is used extensively in agriculture as a selective broadleaf weed killer. Most grasses are resistant to this herbicide; therefore grassed areas, both agricultural and nonagricultural, may receive treatments to control broadleaf weeds.

A considerable body of literature has accumulated relative to the metabolism of sublethal dosages of 2,4-D in susceptible plants. However, few studies report on the metabolism of 2,4-D in resistant plants. The physiological or biochemical basis for the resistance of grasses to 2,4-D is not known.

Previous work in our laboratory (Linscott *et al.*, 1968) indicated that the selective herbicide 4-(2,4-dichlorophenoxy)-butyric acid (2,4-DB) when applied to the resistant legume, alfalfa (*Medicago sativa* L.) was converted to homologs with longer side chains. Therefore, wax biosynthesis was implicated in the metabolism of 2,4-DB. Similarly, application of ester derivatives of 2,4-D, 4-(2,4-dichlorophenoxy)crotonic acid, 4-(2,4-dichlorophenoxy-β-hydroxy)butyric acid as well as 2,4-DB resulted in 2-carbon additions to the aliphatic portion of the applied compounds (Linscott and Hagin, 1970). The works of Horning *et al.* (1961) and Kolattukudy (1968) show that diverse fatty acids can serve as chain initiators in long chain fatty acid synthesis. Peters *et al.* (1960) demonstrated that fluoroacetate can serve as a chain initiator for long chain fatty acid synthesis in *Dichaptetalum toxicarium* Engl. These studies led to the hypothesis that 2,4-D might also be a chain initiator and encouraged us to examine this herbicide and its reaction with resistant grasses.

MATERIALS AND METHODS

In Experiment I, 1.1 kg per hectare of authentic 2,4-D as the acid (Eastman Organic Chemicals), in 1 to 1 (v/v) acetone-H_2O was applied to stands of bromegrass (*Bromus inermis* Leyss.), 10 to 13 cm tall, growing in metal flats. The chemical was applied with a sprayer in which all parts in contact with the spray solution were glass or Teflon. The plants were grown under incandescent and fluorescent lights ($\simeq$1200 fc) (16 hr day) and were subirrigated. Control samples and treated samples were taken initially and at 24, 28, and 72 hr after treatment. Samples were harvested, frozen with dry ice, and stored in a freezer at $-20°$ C before analysis. The treatments were replicated eight times in randomized blocks and the study repeated once. Greenhouse temperature was 75° F in the day and 60° F at night. Bromegrass was 20 to 25 cm tall at the time of treatment in the repeat study (IA). Samples were composited across replications to assure sufficient material for identification and analysis. Therefore, statistical treatment of the data is not possible.

Experiments II and III involved two other grass species, timothy (*Phleum pratense* L.) and orchardgrass (*Dactylis glomerata* L.), respectively. These plants were grown in the field in dense, nearly pure stands to a height of 20 to 25 cm before treatment. In mid-June, single strips 2 by 18 meters of each species were sprayed with a portable sprayer activated by CO_2 gas. Authentic 2,4-D acid in 1 to 1 (v/v) acetone-H_2O was applied at a rate of 2.2 kg per hectare in each case. Samples of each species, representing sprayed and control treatments, were taken at random from several areas in the strips just after spraying and at 24 and 48 hr later. No rainfall occurred during the study. Temperature maximum during the period was 85° F and the minimum 62° F.

In all studies, purity of the 2,4-D was verified by gas chromatography. In no cases were metabolites reported herein found in the original solutions or in the solutions after passing through the sprayers. All samples were analyzed for external and internal 2,4-D by electron capture and flame ionization gas chromatography using previously published methods (Hagin and Linscott, 1966; Linscott *et al.*, 1968). Frozen samples (10 to 20 g green wt) were dipped in two 100-ml portions of redistilled diethyl ether to remove leaf surface components after methods of Martin and Batt (1958). The ether solutions were pooled, reduced in volume to less than 10 ml, methylated with diazomethane, and made up to 10 ml after destroying excess diazomethane with 100 μl of concentrated acetic acid. These solutions were analyzed by electron capture and flame ionization gas chromatography. Herbicide and metabolite determined from these extracts are considered to be external or that associated with the leaf surface and the epidermal cells. After external component separation, the plant material was blanched with hot water, and extracted with isopropanol. The isopropanol extract was partitioned between 0.03*N* HCl and light petroleum ether. The petroleum ether fraction containing the chlorophenoxy acids was reduced in volume to less than 10 ml, methylated with diazomethane as before, and analyzed by electron capture gas chromatography. Herbicide concentrations found in these extracts are designated internal. Results were checked by flame ionization gas chromatography of solutions concentrated 10 to 100 times those used for electron capture detection. The concentrates were prepared by partitioning the isopropanol extract between 0.03*N* HCl and light petroleum ether as before. The petroleum ether phase was extracted with 5% $NaHCO_3$. The bicarbonate extracts were acidified, extracted with diethyl ether, reduced to near dryness, and methylated with diazomethane.

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Table I. Chlorophenoxy Acids (ppm, Fresh Weight) in and on Bromegrass, Timothy, and Orchardgrass After a Spray Treatment with 2,4-Dichlorophenoxyacetic Acids[a]

Study			Time After Treatment: Initial External ppm	Initial Internal ppm	24 Hr External ppm	24 Hr Internal ppm	48 Hr External ppm	48 Hr Internal ppm	72 Hr External ppm	72 Hr Internal ppm
Bromegrass	I	R—ACETIC	320	128	204	146	212	98	180	94
		R—propionic	70	X	67	X	83	X	50	X
	IA	R—ACETIC	75	28	41	21	24	13	25	13
		R—propionic	4	1	N	1	1	N	N	1
Timothy	II	R—ACETIC	200	54	160	48	57	30		
		R—propionic	20	12	6	7	6	2		
Orchardgrass	III	R—ACETIC	148	50	90	46	83	42		
		R—propionic	22	14	8	5	10	5		

[a] External = those chlorophenoxy components removed by a brief immersion of intact plant in diethyl ether. Internal = those compounds determined after ether rinsing and isopropanol extraction of homogenized plants. R = 2,4-dichlorophenoxy radical. Capital letters indicate applied material; lower case letters indicate metabolite. Initial = period of time after treatment until plants were frozen with dry ice (<10 min). N = negligible quantity. X = traces of unknown electron capturing compound very close to the retention time of 3-(2,4-DP).

Determinations for 2,4-D and 3-(2,4-DP) were quantitative above 0.1 ppm and 0.2 ppm, respectively.

Authentic 3-(2,4-dichlorophenoxy)propionic acid [3-(2,4-DP)] was synthesized by the method outlined by Synerholm and Zimmerman (1947). Mass spectra from bromegrass isolates were determined on an Associated Electrical Industries (Manchester, England) MS-9 mass spectrometer. Mass spectra from timothy and orchardgrass isolates were determined on a Perkin-Elmer Model 270 mass spectrometer. Both spectrometers were equipped with a direct inlet system and were operated at an ionization potential of 70 eV. Quantitative analysis of 3-(2,4-DP) methyl ester was performed by comparing peak heights with standard electron capture response curves prepared after all samples had been analyzed. Thus, possible contamination of the chromatograph system was avoided. The avoidance of contamination is vital in the determination of metabolites. Subsequent to electron capture analysis, extracts were concentrated 10 to 100 times and analyzed by flame ionization detection for verification of results.

RESULTS AND DISCUSSION

Chromatograph peaks with a retention relative to 2,4-D methyl ester (1.00) of 1.46 were recorded for most treated samples of all three species. These peaks correspond to that of the methyl ester of 3-(2,4-dichlorophenoxy)propionic acid [3-(2,4-DP)]. Methylated fractions from each treated grass were collected by gas chromatography at the retention volume of authentic 3-(2,4-DP) methyl ester and subjected to mass spectrometry. These unknown fractions from all three grass species yielded mass spectra identical to that of 3-(2,4-DP) methyl ester.

Since plants were treated at different stages of growth and since the data are expressed in ppm fresh wt, initial differences in 2,4-D concentrations are largely the result of differences in plant weight (Table I). For instance, Experiment IA had approximately six times as much plant material per unit areas as Experiment I. The 3-(2,4-DP) levels were considerably higher in Experiment I relative to Experiment IA. This difference can best be attributed to metabolic differentials between the plant growth stages. Disappearance of 2,4-D during a 72 hr period followed a pattern often observed in other herbicide residue studies—a rapid decline after application. The pattern for the 3-(2,4-DP) metabolite decrease was similar.

Quantities of the 3-(2,4-DP) metabolite found varied from traces to as much as 75 ppm, depending on the species, stage of growth, and the time after application (Table I). The 3-(2,4-DP) found in initial samples (<10 min after spraying and before action was stopped by freezing) ranged from 5 to more than 20% of the 2,4-D detected. In view of these quantities, it appears that 3-(2,4-DP) is a major metabolite of 2,4-D in grasses. Quantities of the metabolite decreased in subsequent samplings, indicating that metabolite was acted on further by the plant. An attack on the ring moiety or additions to the side chain may have been involved. Either process could have produced reaction products difficult to detect by the methods used.

Although our data indicated that 3-(2,4-DP) was further metabolized, conversion of 2,4-D to 3-(2,4-DP) is sufficient in itself for herbicidal inactivation. The work of Synerholm and Zimmerman (1947) using a homologous series of seven ω-(2,4-dichlorophenoxy)alkanecarboxylic acids and the tomato leaf epinasty test indicated that the acetic, butyric, caproic, and octanoic homologs were active, while the propionic, valeric, and heptanoic homologs were biologically inactive. β-Oxidation of side chains with even numbered carbons led to formation of 2,4-D (active). Similarly, 2,4-dichloroanisole (unstable) and 2,4-dichlorophenol (inactive) resulted from oxidation of compounds with odd numbered side chains. Fawcett *et al.* (1954) found a similar mechanism operable with the split pea test and a series of ω-(4-chlorophenoxy)alkanecarboxylic acids. Wain (1954) expanded this work to spray applications of ω-(2-methyl-4-chlorophenoxy)alkanecarboxylic acids. Annual nettle (*Urtica urens* L.) and Canada thistle (*Cirsium arvense* L.) treated with ω-2,4-dichlorophenoxy propionic, valeric, and heptanoic derivatives remained unaffected. Shaw and Gentner (1957) showed that 2-(2,4-DP) was very active on several legumes, while in comparison the 3-(2,4-DP) derivative had much less effect. Oats were little affected by 2,4-D, 2-(2,4-DP), or 3-(2,4-DP). Thus, it is evident that a 3-(2,4-dichlorophenoxy)-propionate derivative has little if any herbicidal properties. We suggest that conversion of 2,4-D to 3-(2,4-DP) may be a primary step in inactivating 2,4-D in the grasses studied. The resistance of grasses to 2,4-D may be related directly to its ability to detoxify the applied herbicide.

At this time we cannot explain the mechanisms of 3-(2,4-DP) production from 2,4-D. No precedent that we know of exists in plant biochemistry for the formation of propionate directly from acetate. Stumpf (1965) believes that although propionic acid is rarely found in plants, it may arise from an initial α-oxidation of long chain even numbered carbon acids, followed by a series of β-oxidations that eventually result in propionic acid. A modified β-oxidation system could then

convert propionate to acetate and CO_2. It is doubtful that we can explain the formation of 3-(2,4-DP) by such mechanism in grass because the action was so rapid and because no traces of longer chained chlorophenoxy homologs were found. Our findings suggest that propionate may be produced directly from acetate in grass and that propionate might serve as a chain initiator in wax biosynthesis mechanisms of these plants.

The appearance of 3-(2,4-DP) (Table I) at relatively high levels in surface extracts of initial samples and in lesser quantities internally indicates that reactions are occurring on or near the leaf surface, possibly within the epidermal cells or cell walls. Very little extraction of subepidermal tissues by the ether dip method could have occurred, because chlorophylls did not appear in the external extracts. The ether wash evidently contained extracts from only surface or epidermal tissues.

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