Conversion of 4-(2,4-Dichlorophenoxy)butyric Acid to Homologs by Alfalfa

Mechanism of Resistance to This Herbicide

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After the application of 2,4-DB[4-(2,4-dichlorophenoxy)butyric acid] to alfalfa (*Medicago sativa* L.), the authors detected 2,4-dichlorophenoxycaproic and 2,4-dichlorophenoxydecanoic acids as well as limited amounts of 2,4-dichlorophenoxycrotonic and 2,4-dichlorophenoxyacetic acids (2,4-D). Similarly applied methyl ester of 2,4-dichlorophenoxycrotonic acid was both reduced to the methyl ester of 2,4-DB and de-esterified and reduced to 2,4-DB. All other metabolites mentioned were formed. The mechanism of the resistance of alfalfa to 2,4-DB may result from the synthesis of herbicidally inactive chlorophenoxy compounds having longer side chains than the parent herbicide. Thus, production of 2,4-D in lethal quantity by β-oxidation and subsequent translocation to sites of action may be prevented.

The ability of plants to degrade ω-substituted fatty acids by β-oxidation was demonstrated by Grace (1939) with a series of ω-(1-naphthyl) alkane carboxylic acids, and by Synerholm and Zimmerman (1947), who used a series of ω-(2,4-dichlorophenoxy)alkane carboxylic acids. These findings were confirmed by Wain and Wightman (1954b) and Fawcett *et al.* (1954) with ω-(4-chlorophenoxy)alkane carboxylic acids, as well as by Luckwill and Woodcock (1956), who used ω-(2-naphthoxy)alkane carboxylic acids. Wain and Wightman (1954a) reported that plants have different β-oxidation capabilities and were the first to suggest use of this property for selective weed control. If, for example, some plants but not others degraded 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) to 2,4-dichlorophenoxyacetic acid (2,4-D) by β-oxidation in lethal quantity, 2,4-DB would be an effective selective herbicide (Wain and Wightman, 1954a). Field research of Carpenter and Soundy (1954), Fryer and Evans (1956), and Shaw and Gentner (1957) demonstrated that 2-methyl-4-chlorophenoxybutyric acid (MCPB) and 2,4-DB did not harm forage legumes appreciably but at equivalent rates killed many weed dicots. Since the early research, numerous papers confirm the selectivity of 2,4-DB and MCPB in legumes; and today these herbicides find extensive use in commercial agriculture.

More recent research casts some doubt on the mechanism of selectivity of the chlorophenoxybutyric herbicides when applied to legumes. Wain (1964) had demonstrated that the split pea stem curvature test was positive with a number of chlorophenoxyalkane carboxylic acids having odd numbers of methylene groups. The exception was the 2,4,5-trichlorophenoxy series. In this case only the acetic acid derivative was active. Later, Kief (1961) indicated that acetone powder preparations of Alaska pea (*Pisum sativum* L.), plus additions of 2,4-DB, rapidly reduced a triphenyl tetrazolium chloride dye, but additions of 2,4-D did not. These studies indicated that peas have an active system for β-oxidation even though they are resistant to field applications of 2,4-DB. More currently, Lisk and Gutenmann (1963) detected 2,4-D after 2,4-DB applications to a legume-grass mixture, and Fertig *et al.* (1964) noted 2,4-D formation in bird's-foot trefoil (*Lotus corniculatus* L.) and pea plants. The authors many times (but not always) have detected 2,4-D concentrations of 1 to 2 p.p.m. or less in alfalfa and bird's-foot trefoil after 2,4-DB treatment. Evidently legumes have the enzymes necessary to convert 2,4-DB to 2,4-D through the process of β-oxidation. However, the question as to why this process does not produce lethal quantities in legumes remained.

Considering the importance of legumes and the selective action of the chlorophenoxybutyric herbicides, the authors considered that more research was needed on the mechanism of selectivity. Preliminary research in this laboratory, with autoradiographs of alfalfa treated with 2,4-DB-1-C^{14}, indicated that the C^{14} was immobilized in the leaves in a manner similar to that found by Weintraub *et al.* (1956) for 2,4-D-C^{14} and Robertson and Kirkwood (1966) for MCPA-C^{14} and MCPB-C^{14}. Prior to the initiation of the experiments reported in this paper, the authors observed that C^{14} washed from alfalfa leaves previously treated with 2,4-DB-1-C^{14} appeared in acids of high molecular weight (> 2,4-D) that were not 2,4-D, 2,4-DB, or

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2,4-dichlorophenoxycrotonic acid, and that the C^{14} content of these acids increased during 3 weeks. No C^{14} activity was found in assays of the major leaf lipids (chlorophyll and carotenes), long-chain *n*-alkanes, or *n*-alkanoic acids. These findings indicated that β-oxidation of the herbicide alkane moiety and incorporation of acetate into leaf lipids was not a significant process in alfalfa leaves, but suggested that additions to the alkanoic chain of 2,4-DB were possible. Studies reported herein were designed to test this hypothesis.

MATERIALS AND METHODS

Reagents. The following standards and reagents were used: 2,4-dichlorophenoxyacetic acid, crystalline (Eastman Organic Chemicals); 4-(2,4-dichlorophenoxy)butyric acid, crystalline (AmChem Products, Inc.); diethyl ether, U.S.P., redistilled, 33° to 33.5° C.; petroleum ether, 30° to 60° C. boiling range, analytical grade; 2-propanol, analytical grade; acetone, analytical grade; and *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide (precursor for diazomethane) (Eastman Organic Chemicals).

In addition, 4-(2,4-dichlorophenoxy)crotonic methyl ester was synthesized from 2,4-dichlorophenol (Eastman Organic Chemicals) and methyl 4-bromo-crotonate (Aldrich Chemical Co.) (Williamson ether synthesis). The methyl ester was crystallized from hexane and then sublimed. The yield was 88% of the theoretical. This compound had a melting point of 64–65° C. The compound, 2,4-dichlorophenoxycaproic acid, was synthesized from 2,4-dichlorophenol and 6-bromohexanoic acid (Eastman Organic Chemicals) (Williamson ether synthesis). The acid was crystallized from diethyl ether and recrystallized from methanol with a resulting yield of 62% of theoretical. The melting point was 90° C. The 10-(2,4-dichlorophenoxy)decanoic acid was prepared by reaction of 2,4-dichlorophenol (Eastman Organic Chemicals) and 10-chlorodecanoic acid [synthesized by monochlorination of 1,10-decanediol (Aldrich Chemical Co.) and subsequent oxidation to the acid with H_2SO_4–$K_2Cr_2O_7$]. Yield of the dichlorophenoxydecanoic acid was 30% of the theoretical, and the melting point was 108–10° C. All compounds synthesized for use as substrates and standards were pure as determined by both gas and thin-layer chromatography.

Apparatus. Equipment and apparatus were: Aerograph Hy-Fi gas chromatograph with concentric tube tritium detector, flame ionization and sodium thermionic detectors, temperature programmer and adaptor for sample splitting and collection; Honeywell-Brown Electronik 1-mv. recorder; Beckman Model 10 infrared spectrometer; Nalge Micro-melting point apparatus; and AEI-MS9 mass spectrometer.

The chromatograph parameters were: column, $1/8 \times 60$ inch coiled borosilicate glass; column packing, 60- to 80-mesh, HMDS-treated Chromosorb-W coated with ethyl acetate–fractionated Dow 11 silicone grease (10% by weight); oven and injector temperatures, 205° C.; nitrogen carrier gas, 40 ml. per minute.

Treatment and Analysis of Plant Material. Dense stands of Cayuga alfalfa were grown in metal flats in a greenhouse to a height of 5 to 6 inches. At this stage 4-(2,4-dichlorophenoxy)butyric acid or the methyl ester of 4-(2,4-dichlorophenoxy)crotonic acid was sprayed over the alfalfa with an all-glass sprayer at rates of $1\frac{1}{2}$ (study I A, B and study III) and $\frac{3}{4}$ pound per acre (study II A,B) (2,4-DB acid equivalent) in 30 gallons of an acetone-water (1 to 1) solution. Treated and control plants were cut at soil level, placed in polyethylene bags, and frozen with dry ice as soon as possible after sampling. In no case for the "initial" samples did the time interval between spraying and freezing exceed 5 minutes. Samples were taken immediately, 1, 2, and 3 days after spray application, and stored at −10° C. until analysis. All treatments were replicated four times. Water was supplied to the plants by subirrigation.

Chlorophenoxy compounds were determined by a gas chromatograph method previously reported (Hagin and Linscott, 1965). Briefly, the method required the immersing of frozen plant material in boiling water and heating until the water boiled again. Subsequently the material was extracted with acidified 2-propanol. Chlorophyll, carotenes, chlorophenoxy acids, and other organic acids were partitioned from the extract into petroleum ether (upper layer). Highly polar materials remained in 2-propanol–0.03*N* HCl (lower layer). Herbicide acids were methylated in petroleum ether with diazomethane. The excess diazomethane remaining after methylation was destroyed by brief exposure of the ester solution to 2*N* aqueous HCl. Ester and acid concentrations were determined by difference, before and after methylation.

The method was modified to separate surface and internal components in the following manner. External ether-soluble compounds were removed from the alfalfa leaf surfaces by brief immersion of the frozen plant tissues in 2 volumes of diethyl ether. Essentially all surface waxes were removed by this method without significant extraction of internal components (Martin and Batt, 1958). The two volumes of diethyl ether were combined, reduced in volume, and analyzed before and after methylation. Chlorophenoxy compounds found in this extract were designated "surface" materials. The remaining plant material was blanched, extracted, and analyzed as previously outlined, and the chlorophenoxy compounds detected were designated "internal" materials.

Herbicide concentrations were calculated as parts of herbicide per million parts of fresh plant material (p.p.m.). Limits of quantitative determination were: 2,4-D > 0.1 p.p.m., 2,4-DB and 2,4-dichlorophenoxycrotonic > 0.2 p.p.m., 2,4-dichlorophenoxycaproic acid > 0.4 p.p.m.

RESULTS

Gas chromatograph peaks of unknowns obtained from leaf surface extracts were compared with a nomogram of the retention volumes of the authentic methyl esters of 2,4-D, 2,4-DB, 6-(2,4-dichlorophenoxy)caproic acid, and 10-(2,4-dichlorophenoxy)decanoic acid. The unknown compounds had retention volumes very close to those for authentic 6-(2,4-dichlorophenoxy)caproic methyl ester and 10-(2,4-dichlorophenoxy)decanoic methyl ester, respectively. The relative retention volumes for authentic methyl esters at the column parameter previously described were: 2,4-D, 1.00; 2,4-DB, 2.22; 4-(2,4-dichlorophenoxy)crotonic acid, 2.89; 6-(2,4-dichlorophenoxy)caproic acid, 5.00; 10-(2,4-dichlorophenoxy)decanoic acid, 20.0.

Under standardized conditions, electron-capture flame ionization response ratios were determined to be 12.4 to 1 for 2,4-D methyl ester, 6 to 1 for 4-(2,4-dichlorophenoxy)-butyric methyl ester, and approximately 3 to 1 for 6-(2,4-dichlorophenoxy)caproic methyl ester. Electron-capture flame ionization response ratios for the alfalfa-derived metabolite agreed with ratios for authentic 6-(2,4-dichlorophenoxy)caproic methyl ester.

Samples of extracts containing the two unknown compounds in question were analyzed by a chromatograph and use of a sodium thermionic detector by varying parameters to produce a response for chlorine-containing compounds and a negative response for hydrocarbons and then the reverse. [Phosphorus-containing compounds would give a positive response under both conditions, according to Hartman (1966).] The observed detector responses indicated that both compounds contained chlorine.

Plant extracts were chromatographed, and fractions were collected at retention volumes of authentic 6-(2,4-dichlorophenoxycaproic acid). All absorption bands of medium and strong intensity in the infrared spectrum of the authentic compound were observed in the spectrum of the unknown compound. All evidence presented suggests that the two compounds in question were the methyl esters of 6-(2,4-dichlorophenoxy)caproic acid and 10-(2,4-dichlorophenoxy)decanoic acid, respectively.

The metabolism of 2,4-DB was compared directly to that of the methyl ester of 4-(2,4-dichlorophenoxy)crotonic acid in alfalfa. The methyl ester of 2,4-dichlorophenoxycrotonic acid was converted to the methyl ester of chlorophenoxybutyric acid and to chlorophenoxybutyric, crotonic, acetic, and caproic acids (Table I). A low resolution mass spectrum for the metabolite of the methyl ester of 4-(2,4-dichlorophenoxy)crotonic acid suspected to be 2,4-DB (Study III), obtained by use of a 70-volt ionization potential, gave an *m/e* value of 262. The ratio of parent ion to parent ion + 2 indicated the presence of Cl in the unknown and the fragmentation pattern obtained was that of the methyl ester of 4-(2,4-dichlorophenoxy)butyric acid. This finding coupled with chromatographic evidence leaves little doubt that the 2,4-DB was a reduction product of the parent chlorophenoxycrotonic methyl ester.

Metabolites of 2,4-DB as determined by gas chromatography included chlorophenoxycaproic, decanoic, crotonic, and acetic acids. In both treatments, 6-(2,4-dichlorophenoxy)caproic acid was found 1, 2, and 3 days

Table I. Chlorophenoxy Acids and Esters (PPM) in and on Alfalfa after Spray Treatment with 2,4-Dichlorophenoxybutyric Acid or the Methyl Ester of 2,4-Dichlorophenoxycrotonic Acid[a]

	Time after Treatment							
	Initial		1 Day		2 Days		3 Days	
Study	External	Internal	External	Internal	External	Internal	External	Internal
IA								
R-acetic	1.3	0.5	n	0.1	1.2	n	0.4	n
R-crotonic	n	8.5	n	7.6	9	1.3	n	6.1
R-butyric	157	85	77	51	18	33	9	15
R-caproic	n	n	x	n	x	n	x	n
R-decanoic	n	n	x	n	x	n	x	n
IB								
R-acetic	n	n	n	n	n	0.1	n	n
R-crotonic	17	11	9	5	0.8	3	0.7	1
R-crotonic methyl ester	26	17	7	11	3.5	9	3	6
R-butyric methyl ester	158	113	61	99	19	61	11	29
R-butyric	n	51	n	n	n	7	1	4
R-caproic	n	n	n	x	n	x	x	n
IIA								
R-acetic	n	n	n	n	n	n	n	n
R-crotonic	n	1.6	0.1	0.1	n	0.1	n	0.5
R-butyric	39	15	35	7	16	8	13	10
R-caproic	3	4	3	4	18	0.1	14	2
IIB								
R-acetic	n	4	n	0.7	n	0.1	n	n
R-crotonic	3	n	0.5	0.5	0.1	n	n	n
R-crotonic methyl ester	34	22	0.5	0.5	0.6	n	0.1	n
R-butyric methyl ester	10	12	n	n	n	n	n	n
R-butyric	4	6	n	9	n	3.7	0.5	n
R-caproic	n	31	n	6	1.1	8	n	n

[a] R. 2,4-Dichlorophenoxy radical.
x. Positive identification (nonquantitative).
Initial. Period of time after treatment (<5 min.) until plants were frozen with dry ice.
n. Negligible quantity.
Starting compounds underlined.

after treatment (Table I). In a second experiment, the chlorophenoxycaproic metabolite was found immediately after the application of either parent compound. Limited amounts of 2,4-D were also detected. But the concentration of chlorophenoxycaproic acid, the addition product, was greater than that of 2,4-D, the oxidation product, in all samples analyzed.

The 10-(2,4-dichlorophenoxy)decanoic acid was found in only one of the three studies (Tables I). However, chromatograph traces of other unknown compounds which had larger retention volumes than the decanoic acid derivative were found in all studies. This may indicate that chlorophenoxy compounds having longer side chains than decanoic acid were present. In study III, quantities of 2,4-dichlorophenoxycaproic and decanoic acid were too low for efficient collection. The authors obtained a mass spectrum for high molecular weight chlorophenoxy compounds (mass > 290) but could not make positive identification because of separation difficulties and subsequent interference by natural plant constituents. Unless samples are very pure, with the system used, positive identification by mass spectral analysis of chlorine-containing compounds from plant extractions is difficult.

The quantity of herbicide and metabolites found varies considerably. This amount of biological variation within experiments, though considerable, is not unusual for studies of this kind. If herbicide and metabolite concentrations (Table I) are converted to 2,4-DB equivalents and added for each sampling period, the pattern of herbicide disappearance is consistent with previous studies. Emphasis has been given to identification of the metabolites formed rather than to the absolute amounts found; however, a gross comparison of addition to degradation products shows a preponderance of synthetic over oxidative homologs.

DISCUSSION

All of the 2,4-dichlorophenoxyalkanoic acids with more than one methylene group are herbicidally inactive. Conversion of 2,4-DB by β-oxidation to the acetic acid derivative, 2,4-D, is required for the herbicide to become active. Therefore, the production by alfalfa of 2,4-dichlorophenoxycaproic and decanoic acids in quantity after 2,4-DB treatment is significant because it indicates the existence of an addition mechanism which depletes the concentration of the precursor of 2,4-D. Consideration of the quantity of these chlorophenoxy addition products produced from 2,4-DB by alfalfa indicates that the synthetic reactions use considerably more parent compound than the degrading reactions. These synthetic reactions apparently are more competitive for 2,4-DB than degrading reactions in this case; thus, lethal accumulations of 2,4-D in alfalfa may be prevented.

Evidently a considerable quantity of 2,4-DB and the methyl ester of 2,4-dichlorophenoxycrotonic acid penetrated the alfalfa leaf surface and entered the epidermal cells. Natural leaf surface waxes, long-chain fatty acids, alcohols, and alkanes originate primarily in the epidermis (Kolattukudy, 1968; Stumpf, 1965). Therefore, primary synthesis of the chlorophenoxycaproic and decanoic acids after 2,4-DB application may have occurred in the alfalfa epidermal cells. Probably, the malonate addition pathway described by Vagelos (1964), and confirmed for plant tissue by Overath and Stumpf (1964), was involved. Chlorophenoxybutyric-acyl carrier protein (ACP) might initiate 2-carbon additions by the malonate system. If true, the

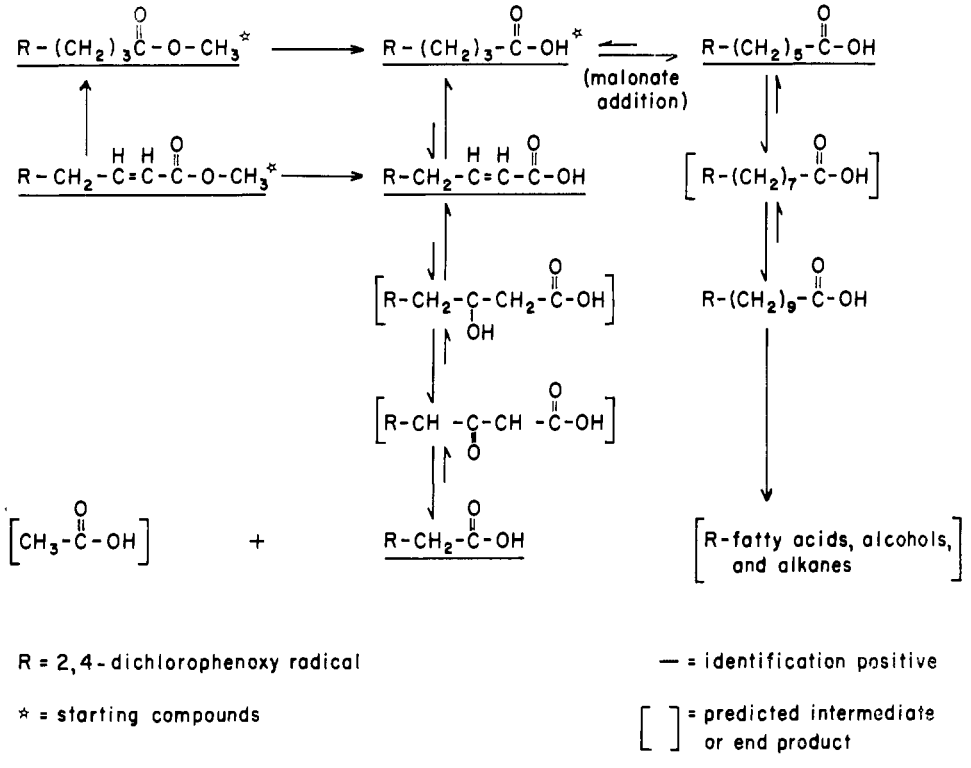

Figure 1. Fate of 2,4-dichlorophenoxybutyric acids and esters and 2,4-dichlorophenoxycrotonic esters after application to alfalfa

fate of 2,4-DB acid or ester when applied to alfalfa may be that shown in Figure 1. The methyl ester is hydrolyzed to the acid. The acid can be converted by β-oxidation through the crotonic, β-hydroxybutyric, and β-ketobutyric intermediates to 2,4-D. But fatty acid syntheses reactions predominate and form chlorophenoxycaproic and chlorophenoxydecanoic homologs and probably even longer chained chlorophenoxy acids, alcohols, and alkanes, as well. Similarly, the chlorophenoxycrotonic methyl ester is both hydrogenated to the butyric methyl ester and hydrolyzed to the crotonic acid. After the dichlorophenoxybutyric ester is hydrolyzed to butyric acid or the crotonic ester is reduced to dichlorophenoxybutyric acid, the previously described reactions are possible. The relatively high concentrations of 2,4-DB in relation to 2,4-D found after application of dichlorophenoxycrotonic methyl ester clearly establish the double bond hydrogenation capability of alfalfa. Subsequent identification of 2,4-dichlorophenoxycaproic acid confirms the synthesis theory.

Both acetate and butyrate serve as initiators for fatty acid synthesis (Majerus *et al.*, 1964; Stumpf, 1965). Alfalfa has some inherent resistance to 2,4-D, and this may be the result of additions to the 2,4-D molecule. We may then assume that 2,4-D–treated alfalfa is detoxified by a mechanism similar to that suggested for 2,4-DB. However, since 2,4-D is a metabolically active compound, synthesis mechanisms should not be expected to detoxify completely the dosages normally used for weed control.

A considerable volume of literature has accumulated on the physical properties of leaf surfaces (Holley, 1964), and from these studies a broad distinction between susceptibility and resistance of plants to chlorophenoxy herbicides can be drawn on the basis of leaf surface characteristics. Many resistant species have leaves covered by relatively heavy layers of waxes, while susceptible species have leaves covered by less and perhaps different kinds of waxes. Some of these compounds are species-specific and are used as chemical "markers" in plant taxonomy (Eglinton and Hamilton, 1967). There is little doubt that physical barriers to herbicides posed by leaf surface components of some plants are important factors in their resistance. However, the work reported herein indicates that metabolic reactions of the epidermal cells are responsible for both biophysical properties—i.e., wax production—and herbicide detoxication mechanisms, through a common pathway of biosynthesis. Recent research of Kolattukudy (1968) on the synthesis of surface lipids indicates the high speed of wax formation. Our data suggest that chlorophenoxy addition products are formed quickly in epidermal cells and that they partition through the epidermis to the leaf surface along with waxes, where they are ultimately removed by the action of wind and rainfall. Further research can be expected to elucidate the role played by epidermal cells in detoxifying other foliar-applied herbicides besides 2,4-DB.

To the best of the authors' knowledge, this research is the first to indicate that fatty acid synthesis reactions are important in herbicide-plant relations. The results lead to the suggestion that the mechanism of alfalfa resistance to 2,4-DB lies in lengthening of the side chain of 2,4-DB in the epidermal cells.

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