Comparative Metabolism of 2,4-D by Bean and Corn Plants

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Three routes of metabolism of 2,4-D were demonstrated in resistant and susceptible plants. These pathways are simple conjugation, conjugation and hydroxylation, and oxidation of the side chain. In the process of hydroxylation the chlorine atom in the 4 position is shifted to the 3 or 5 position. The primary shift is to the 5 position so that major metabolic residue is 4-hydroxy-2,5-dichlorophenoxyacetic acid with lesser amounts of 4-hydroxy-2,3-dichlorophenoxyacetic acid being formed. Only very small amounts of 2,4-D are detoxified through oxidation of the side chain. Essentially all of the absorbed 2,4-D is rapidly conjugated in corn and bluegrass. In beans the conjugation is much slower, with free 2,4-D being present at each of the harvest times. These findings strongly indicate that inactivation through conjugation is an important factor in selectivity.

One of the most extensively used herbicides in agricultural production is 2,4-dichlorophenoxyacetic acid (2,4-D). However, there is only limited information on the metabolite residues which may occur in resistant crop plants as a result of the use of this herbicide. The primary effort has been directed toward establishing the metabolic fate of 2,4-D in beans.

The major metabolic reaction of 2,4-D in beans involves conjugation with peptides and sugar derivatives (Bach and Fellig, 1961; Klambt, 1961). The parent compound is released from its conjugated form by acid hydrolysis (Crosby, 1964). In addition to conjugation, oxidation to more polar products has been demonstrated. Holley (1952) suggested that a conjugated metabolite which he found was an hydroxylated derivative of 2,4-D. It was subsequently shown by Thomas *et al.* (1964) that in beans 2,4-D is hydroxylated in the 4 position with an accompanying shift of the chlorine atom to the 3 or 5 position. Both of the above metabolites were present as plant conjugates.

The nature of 2,4-D metabolites found in resistant crop plants has received limited investigation. Fang and Butts (1954) studied the translocation and metabolism of 2,4-D in corn and wheat. However, no attempt was made to hydrolyze the conjugated metabolites. Glaze and Wilcox (1966) did not find any metabolism of 2,4-D in the excised roots of corn, wheat, or oats. More recently, Hagin *et al.* (1970) found that in certain grasses 2,4-D undergoes chain elongation, giving rise to 3-(2,4-dichlorophenoxy)propionic acid. The studies reported here were carried out to determine the comparative rates of 2,4-D metabolism in beans and corn and to establish the nature of metabolite residues in beans, corn, and bluegrass.

EXPERIMENTAL

The plants used in these studies were beans (*Phaseolus vulgaris*, var. Top Crop), corn (*Zea mays*, var. Tendermost), and bluegrass (*Poa praten*, var. Newport). The plants were grown in the greenhouse in soil and allowed to attain a height of about 6 in. prior to treating with 0.1 ml of a 250-ppm solution of ^{14}C-carboxyl labeled 2,4-D (3.03 mCi per mmol). The treatment solution contained 0.1% Tween 20 to enhance penetration. A 0.5-ml aliquot of the same solution was applied randomly to the foliage of grass growing in small greenhouse pots. Corn and bean plants were harvested 2, 7, and 11 days following treatment. The grass was harvested 7 days following treatment.

Upon harvest the plants were rinsed with 1% sodium bicarbonate solution to remove unabsorbed herbicide and then blended with 80% ethanol in a food blender. The macerate was heated for 1 hr on a steam bath to extract 2,4-D and its metabolites and filtered. The residue was washed with 80% ethanol and the combined extracts were concentrated to 100 ml.

The amount of 2,4-D which was absorbed was determined by scintillation counting of the alcohol extracts. The nature of the labeled compounds in the crude extract was determined by paper chromatography using butanol saturated with 1.5 *M* ammonium hydroxide as the developer. The developed chromatograms were scanned with a Vanguard 4TT, windowless chromatogram scanner.

Since it is known that 2,4-D and its metabolites form conjugates with plant constituents, the crude extracts were hydrolyzed by first evaporating the alcohol from an aliquot of the crude extract and then heating the residue with 50 ml of 1 *M* hydrochloric acid on a steam bath for 1 hr. The 2,4-D and metabolites were recovered from the hydrolysis mixture by extracting three times with 25-ml portions of diethyl ether. The number of metabolites was determined by thin-layer chromatography on silica gel G plates using petroleum ether:diethyl ether:formic acid (50:50:2). Radioactive components were determined using a Vanguard windowless plate scanner.

2,4-D metabolites were formed by all of the plants tested. Thus it was necessary to synthesize the various hydroxylated dichlorophenoxyacetic acids. The compounds synthesized were 6-hydroxy-2,4-dichlorophenoxyacetic acid (Cavill and Ford, 1954), 5-hydroxy-2,4-dichlorophenoxyacetic acid (Moszew and Wojciechowski, 1954), 3-hydroxy-2,4-dichlorophenoxyacetic acid, 4-hydroxy-2,5-dichlorophenoxyacetic acid (Faulkner and Woodcock, 1965), and 2,3-dichloro-4-hydroxyphenoxyacetic acid (Thomas *et al.*, 1964). The R_f values of these synthetic products were compared to the hydrolyzed metabolites using thin-layer chromatography. The location of the synthetic materials on the plates was determined by spraying with *p*-nitrobenzenediazonium fluoborate (Thomas *et al.*, 1964).

The metabolites and synthetic products were also analyzed by gas chromatography. Since these compounds are not sufficiently volatile to be chromatographed directly, they were esterified with diazomethane in ether, which methylates both hydroxyl and carboxyl groups. The excess diazomethane, which is highly toxic, was dispelled by boiling off about 50%

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Table I. Rate of 2,4-D Metabolism by Bean and Corn Plants

Days following exposure		Percentage of radioactivity present as: Free 2,4-D	Conjugated products	Hydrolyzed products: 2,4-D	Major	Minor
Corn plant						
3	Crude extract[a]	0	100			
	Hydrolyzed[b]			29	53	18
7	Crude extract	0	100			
	Hydrolyzed			58	36	6
11	Crude extract	0	100			
	Hydrolyzed			57	39	4
Bean plant						
3	Crude extract	40	60			
	Hydrolyzed			37	48	15
7	Crude extract	30	70			
	Hydrolyzed			57	37	6
11	Crude extract	12	88			
	Hydrolyzed			64	33	3
Bluegrass plant						
7	Crude extract	0	100			
	Hydrolyzed			55	39	6

[a] 80% alcohol extract. [b] Ether extract of acid hydrolyzate.

of the ether solution containing the methylated compounds. Although a number of different column packings were tested, resolution of all of the methylated compounds could not be achieved. This problem was overcome by also preparing the propyl derivatives by treatment with diazopropane (Glaze and Wilcox, 1966).

The gas chromatographic analyses were performed on a Dohrmann gas chromatograph having a microcoulometric detector. The column which was used was 0.25-in. glass, 4 ft long, packed with 7% OV-1 on Gas Chrom Q. The flow rate was approximately 40 ml per min. The column temperature was 140 or 160° C, depending on which derivative was being analyzed.

To determine the extent of decarboxylation, bean and corn plants were treated with 0.1 ml of a solution containing 200 ppm of ^{14}C-carboxyl labeled 2,4-D (12.1 mCi per mmol). The plants were placed in a 3-l. desiccator modified to serve as a respiratory chamber. A continuous flow of incoming air was passed through 5% sodium hydroxide to remove carbon dioxide and the outgoing air was passed through carbonate-free sodium hydroxide to trap carbon dioxide given off by the plants.

At the end of a 72-hr collection period, the plant leaves were rinsed with 1% sodium bicarbonate to remove surface residues. The plants were extracted as previously described to determine how much 2,4-D has been absorbed. The carbon dioxide given off by the plants was precipitated as barium carbonate and the radioactivity was determined.

RESULTS AND DISCUSSION

The rates of absorption of 2,4-D by bean and corn plants were quite similar. There appeared to be a more rapid absorption by corn initially, but there were no significant differences at the end of 7 and 11 days. Thus, for beans, the percent absorption at 3, 7, and 11 days was 22, 60, and 65% for the same time intervals, respectively. For corn, the absorption was 42, 60, and 65%.

Paper chromatographic analysis of the crude extracts showed that beans, corn, and bluegrass metabolize 2,4-D. In the chromatograms of bean extracts there were two radioactive areas. One, at R_f 0.75, was 2,4-D, while the other was a broad band from 0 to 0.1. In corn and bluegrass there was just a single radioactive area from 0 to 0.1. These low R_f bands appeared to be conjugates such as were found in the metabolism of dicamba (Broadhurst *et al.*, 1966). Small amounts of free 2,4-D must have been present in the corn and grass extracts since the compound was continually being absorbed. However, these concentrations were too low to be detected.

Thin-layer chromatography of the hydrolyzed extract indicated the presence of three radioactive compounds, one of which was 2,4-D. The other two compounds will be referred to as major and minor metabolites since one was present in much higher concentration. The rates of metabolism are presented in Table I.

It is apparent from Table I that initially there is extensive metabolism of 2,4-D to major and minor metabolites. In both corn and beans there is greater than 60% conversion of absorbed 2,4-D to metabolites during the first 3-day exposure period. However, with increasing periods of exposure the metabolic process seems to favor simple conjugation. This could possibly be explained by overloading of the detoxication enzymes, although the amount of chemical involved is relatively small. In any event, conjugation is the major process in that all of the metabolites and most of the 2,4-D are present in a conjugated form.

To obtain a tentative identification of the metabolites, the hydrolyzed extracts and synthetic compounds were analyzed

Table II. R_f Values of 2,4-D, 2,4-D Metabolites and Various Hydroxydichlorophenoxyacetic Acids in Thin-Layer Chromatography

Compound	R_f values[a]
2,4-D	0.85
6-OH-2,4-D[b]	0.88
5-OH-2,4-D	0.66
3-OH-2,4-D	0.70
4-OH-2,5-D	0.75
4-OH-2,3-D	0.53
Conjugates	0.0
Hydrolyzed bean extract	0.54,[c] 0.74,[d] 0.85
Hydrolyzed corn extract	0.54, 0.75, 0.86

[a] Silica gel G plates developed in petroleum ether:diethyl ether:formic acid (50:50:2). [b] First number denotes position of hydroxyl group, second numbers denote position of chlorine atoms on aryl portion of phenoxyacetic acid. [c] Minor metabolite. [d] Major metabolite.

Table III. Retention Times of Methyl and Propyl Derivatives of 2,4-D, 2,4-D Metabolites, and Various Hydroxydichlorophenoxyacetic Acids in Gas Chromatography

Compound	Derivative: methyl[a]	Propyl[b]
2,4-D	2.3	2.1
6-OH-2,4-D	4.5	6.4
5-OH-2,4-D	5.7	7.6
3-OH-2,4-D	5.1	7.9
4-OH-2,5-D	5.7	8.2
4-OH-2,3-D	7.6	10.9
Bean extract	2.3, 5.7,[c] 7.6[d]	2.1, 8.2,[c] 10.9[d]
Corn extract	2.3, 5.7, 7.6	2.1, 8.2, 10.9
Grass extract	2.3, 5.7, 7.6	2.1, 8.2, 10.9

[a] Analysis performed at 140° C. [b] Analysis performed at 160° C. [c] Major metabolite. [d] Minor metabolite.

Table IV. 3-Day Cumulative Release of $^{14}CO_2$ from Bean and Corn Plants Exposed to ^{14}C-Carboxyl-Labeled 2,4-D

	cpm	
	Beans	Corn
$^{14}CO_2$ release	2.78×10^4	2.38×10^4
^{14}C in plants	8.34×10^5	1.59×10^6
% conversion to $^{14}CO_2$	3.3	1.5

by thin-layer chromatography. The results of these analyses are shown in Table II. The R_f values of the metabolites indicated that the metabolic products in all three plants were the same as those found by Thomas *et al.* (1964) in beans. Thus the major metabolite appeared to be 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D) and the minor metabolite appeared to be 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D).

In order to confirm the identity of the metabolites, they were methylated and analyzed by gas chromatography. As can be seen in Table III, the retention time of the minor metabolite agreed with that for synthetic 4-OH-2,3-D. The major metabolite could not be identified under these conditions since 5-OH-2,4-D and 4-OH-2,5-D had the same retention times. This problem was resolved by the preparation and analysis of the propyl derivatives of these compounds, showing the major metabolite to be 4-OH-2,5-D rather than 5-OH-2,4-D.

The above studies demonstrated what labeled metabolites were formed through conjugation and modification of the ring structure of 2,4-D. However, any reactions involving decarboxylation would result in loss of the label and production of $^{14}CO_2$. Therefore, plants were treated with ^{14}C 2,4-D and the amount of 2,4-D absorption and release of $^{14}CO_2$ was measured over a 3-day period. The results of this study are shown in Table IV.

Small amounts of $^{14}CO_2$ are given off by treated plants, showing the decarboxylation is operative but only to a limited extent. This is illustrated by the data in Tables I and IV. In beans 60% of the absorbed 2,4-D is converted to conjugated metabolites in a 3-day period. Of the 60% which is conjugated 63% is present as the hydroxylated metabolites. Thus about 38% of the absorbed 2,4-D is hydroxylated in a 3-day period. During this same time period only 3.3% of the absorbed radioactivity is given off as $^{14}CO_2$. $^{14}CO_2$ evolution is even less in the corn plants.

We feel that these observations are significant, both to residue methodology and to the understanding of selectivity, at least in part. First, residue methods must include a hydrolytic step in order to measure total 2,4-D residues. The herbicide must be released from its conjugated form so that it can be methylated and analyzed by gas chromatography. Also, residue methods should include analysis for the hydroxylated metabolites since they may represent a significant amount of the absorbed herbicide.

With respect to selectivity, it would appear that conjugation is an important factor. In the resistant plants, essentially all of the 2,4-D is conjugated as fast as it is absorbed. In beans, which are quite susceptible, the conjugation is much slower, so that there is free 2,4-D present. It seems quite likely that the conjugation process is a very important factor in determining selectivity by regulating the amount of free herbicide.

After the submission of this manuscript, two papers on the metabolism of 2,4-D by plants were published in this journal. Feung *et al.* (1971) found that in soybean callus tissue 2,4-D is metabolized to 2,4-dichlorophenoxyacetyl glutamic acid and the glycosides of 4-OH-2,5-D, 4-OH-2,3-D and 5-OH-2,4-D. Hamilton *et al.* (1971) reported that in bean plants the metabolites of 2,4-D are the aglycones of 4-OH-2,5-D and 4-OH-2,3-D. Our findings are consistent with these results.

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Received for review March 22, 1971. Accepted June 7, 1971. This work was supported in part by NIEHS Grant No. ES 0004-05 to the Environmental Health Sciences Center, Oregon State University, Oregon Agricultural Experiment Station, Technical Paper No. 3053.