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REMOVAL OF THE ACETATE-MOIETY OF 2,4-DICHLOROPHENOXYACETIC ACID IN *RIBES SATIVUM**

J. R. FLEEKER

Department of Biochemistry, North Dakota State University, Fargo, ND 58102, U.S.A.

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Key Word Index—*Ribes sativum*; Grossulariaceae; 2,4-dichlorophenoxyacetic acid; metabolism; glyoxylic acid; glycine.

Abstract—Ten minutes after uptake of 2,4-dichlorophenoxyacetic acid-1-^{14}C(2,4-D-1-^{14}C) by excised *Ribes sativum* leaves, 37·8% of the radioactivity in water-soluble metabolites was in glyoxylic acid. When 2,4-D-2-^{14}C was supplied under the same conditions, 23·0% of the radioactivity of the water-soluble metabolites was in glyoxylic acid. Radioactive glycine and glyoxylic acid, isolated from *Ribes sativum* 6 hr after uptake of 2,4-D-1-^{14}C, contained essentially all of the ^{14}C in the carboxyl-carbon atoms. When 2,4-D-2-^{14}C was the precursor, the glycine isolated contained 64·8% of its radioactivity in C_2, while 60·0% of the radioactivity in glyoxylic acid was in C_2. The side-chain label of 2,4-D-2-^{14}C-4-^{36}Cl was more efficiently incorporated into ethanol-insoluble plant residue than the ring-label. The metabolism of glyoxylic acid-1-^{14}C and 2,4-D-1-^{14}C in excised *Ribes sativum* leaves were compared. The data suggest a cleavage of the acetate-moiety of 2,4-D resulting in a C_2 compound, perhaps glyoxylate.

INTRODUCTION

ONE of the first detoxication pathways of 2,4-dichlorophenoxyacetic acid (2,4-D) discovered in plants was the oxidation of the side-chain moiety.[1] The oxidation occurs in most species at a rate which does not give the plant resistance to the herbicide.[2–6] However, a few dicotyledonous plants, such as *Stellaria media, Syringa vulgaris, Ribes sativum* and certain *Malus* and *Fragaria* species show resistance to 2,4-D that appears to be related to an ability to rapidly oxidize the acetate-group to carbon dioxide.[7–9]

The mechanism of the side-chain cleavage is not known. Based primarily on the rate of $^{14}CO_2$ produced when carboxyl- or methylene-labeled 2,4-D is supplied to plants, it has been suggested the acetate-carbons are removed as a C_2 unit.[5,10] In the majority of plant species studied, labeled carbon dioxide is released from 2,4-D-1-^{14}C at a greater rate than

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with 2,4-D-2-^{14}C.[3,5,8] Leafe[11] found that only small amounts of the side-chain from the herbicide 2-methyl-4-chloro-phenoxyacetic acid (MCPA) was oxidized to carbon dioxide in MCPA-resistant *Galium aparine*. However, ^{14}C from the side-chain labeled herbicide was incorporated into protein and nucleic acid fractions to a much greater extent than ring-labeled MCPA. This suggested a cleavage at the ether-linkage was a major route for inactivation of the herbicide in this species.

TABLE 1. TOTAL RADIOACTIVITY IN GLYCINE, GLYOXYLIC ACID, AND THE AQUEOUS EXTRACT* OF EXCISED *Ribes sativum* LEAVES AT VARIOUS INTERVALS AFTER UPTAKE OF 2,4-D-1-^{14}C AND 2,4-D-2-^{14}C

Time after uptake (min)	Radioactivity in aqueous extract (mμCi)		Radioactivity in glyoxylic acid (mμCi)		Radioactivity in glycine (mμCi)
	2,4-D-1-^{14}C	2,4-D-2-^{14}C	2,4-D-1-^{14}C	2,4-D-2-^{14}C	2,4-D-1-^{14}C
10	18·0	17·4	6·8	4·0	4·1
30	30·7	43·2	4·6	9·8	5·3
60	65·5	48·6	4·6	7·9	7·1
90	122	129	8·9	15·2	4·9

* The aqueous extract remained after concentrating an 80% EtOH-extract of leaf tissue to an aqueous solution, adjustment of the pH to 2, and extraction with CH_2Cl_2.

Ribes sativum was chosen for this study because it was reported to rapidly oxidize the acetic acid moiety of 2,4-D.[8] High levels of activity were necessary in order to incorporate isotope label from the herbicide side-chain into the oxidation products in sufficient amount for their isolation and identification.

RESULTS

In preliminary experiments with *Ribes sativum* leaves, glyoxylic acid and glycine were found to contain ^{14}C a short time after uptake of side-chain labeled 2,4-D. Table 1 shows the results of an experiment where glycine and glyoxylic acid were isolated from leaf tissue after uptake of 2,4-D-1-^{14}C and 2,4-D-2-^{14}C. Ten minutes after exposure to 2,4-D-1-^{14}C, 60·6% of the radioactivity in the water-soluble components of the leaves was in glycine and glyoxylic acid. After 30, 60 and 90 min, the amount of radioactivity in these two acids did not increase in proportion to the total ^{14}C in the water-soluble constituents. The data in Table 1 suggest a C_2 fragment such as glycolate or glyoxylate resulted from the cleavage of the herbicide side-chain. However, at least one other route is possible for rapid ^{14}C incorporation into these C_2 compounds. Since carbon dioxide is a product in the metabolism of the 2,4-D side-chain,[8] photosynthetic incorporation of $^{14}CO_2$ into glycine and glyoxylate could occur via the glycolate pathway,[12] irrespective of whether side-chain carbons were removed as C_1 or C_2 units. To test this possibility, glycine and glyoxylic acid were isolated from excised *Ribes sativum* leaves 6 hr after uptake of 2,4-D-1-^{14}C and 2,4-D-2-^{14}C, and the distribution of radioactivity in the carbon-chains determined. The results are shown in Table 2. If ^{14}C had been incorporated into glycine and glyoxylic acid only by the glycolate pathway, the expected distribution of the isotope would have been similar for either

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carboxyl- or methylene-labeled herbicide. The data, however, are consistent with a mechanism in which the acetate-group of 2,4-D was removed as a C_2 unit.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN GLYCINE AND GLYOXYLIC ACID ISOLATED FROM EXCISED *Ribes sativum* LEAVES 6 hr AFTER UPTAKE OF 2,4-D-1-^{14}C AND 2,4-D-2-^{14}C

Precursor	Compound degraded	C_1 and C_2		C_1		C_2*	
		dpm/mmol	%†	dpm/mmol	%	dpm/mmol	%
2,4-D-1-^{14}C	Glycine	1450	100	1420	97·9	0	0
2,4-D-1-^{14}C	Glyoxylic acid	5320	100	4660	87·6	380	7·1
2,4-D-2-^{14}C	Glycine	3860	100	1250	32·4	2500	64·8
2,4-D-2-^{14}C	Glyoxylic acid	2300	100	730	31·7	1380	60·0

* C_2 of glycine isolated and counted as the dimedone derivative of formaldehyde-^{14}C. All other carbons were collected and counted as $Ba^{14}CO_3$.

† % of specific activity obtained from total oxidation of glycine or glyxoylic acid.

Table 3 summarizes the results of an experiment in which the metabolism of 2,4-D-1-^{14}C and glyoxylic acid-1-^{14}C are compared after a 6-hr period in excised leaves. Almost all of the glyoxylic acid-1-^{14}C taken up by the leaves was metabolized to other substances, while nearly two-thirds of the herbicide was recovered unchanged. A significant amount of radioactivity from both compounds was incorporated in the ethanol-insoluble plant residue and carbon dioxide.

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN MATERIAL OBTAINED FROM EXCISED *Ribes sativum* LEAVES 6 hr AFTER UPTAKE OF 2,4-D-1-^{14}C AND GLYOXYLIC ACID-1-^{14}C

Material	2,4-D-1-^{14}C (mμCi)	Glyoxylic acid-1-^{14}C (mμCi)
Radioactivity absorbed	2750	1850
EtOH-insoluble residue	270	572
Recovered 2,4-D	1790	—
Glycine	9·5	27·0
Glyoxylic acid	11·7	30·6
Unidentified water-soluble ^{14}C	399	888
$^{14}CO_2$	189	293
Radioactivity recovered	2670	1810

In earlier work with *Ribes sativum*, radioactivity from 2,4-D-1-^{14}C and 2,4-D-2-^{14}C was reported to be incorporated into leaf tissue in a form not extractable with organic or aqueous solvents.[8] The chemical form in which the label was incorporated was not known. Table 4 shows the results of an experiment in which *Ribes sativum* shoots were allowed to metabolize 2,4-D-2-^{14}C-4-^{36}Cl over a 24-hr period. The side-chain carbons were incorporated more efficiently into the ethanol-insoluble residue than the ring-label. Radioactive glycine, resulting from the metabolism of the side-chain carbons of 2,4-D, and incorporated into

protein, could account for a portion of the incorporated ^{14}C. The ratio of ^{14}C to ^{36}Cl in the residue suggests that up to two-thirds of the radioactivity incorporated could have come from the intact herbicide molecule. Hydrolysis of the residue with 2 N HCl for 3 hr solubilized 11·4% of the residue-bound ^{14}C. The solubilized, radioactive material behaved as 2,4-D (PC).

TABLE 4. RATIO OF ^{14}C TO ^{36}Cl IN FRACTIONS OBTAINED FROM *Ribes sativum* SHOOTS 24 hr AFTER UPTAKE OF 2,4-D-2-^{14}C-4-^{36}Cl

Material	$^{14}C/^{36}Cl$ ratio
2,4-D-2-^{14}C-^{36}Cl fed to plants	1·96
EtOH-extract	1·93
EtOH-insoluble residue	2·91

DISCUSSION

A mechanism by which the acetate-moiety of 2,4-D is removed as glycine, glycolate, or glyoxylate is consistent with the data presented here. These compounds are metabolically related.[12] An attractive possibility is an oxidation on the methylene-carbon of the herbicide yielding glyoxylate and dichlorophenol. The presence of dichlorophenol in plants fed 2,4-D has been reported for many species.[13–15]

Studies concerning the removal of the side-chain carbons of 2,4-D and MCPA by microbes suggest an oxidative process. Tiedje and Alexander[16] obtained a soluble enzyme preparation from an *Arthrobacter* species which gave 2,4-dichlorophenol and alanine on incubation with 2,4-D. They presented evidence that suggested glyoxylate was the initial product of the side-chain cleavage and was subsequently converted to alanine. An extract of a *Pseudomonas* species was found to convert MCPA to 2-methyl-4-chlorophenol.[17] The side-chain carbons were recovered in 82% yield as the 2,4-dinitrophenylhydrazone of glyoxylic acid.

EXPERIMENTAL

Radioactive compounds. Glyoxylic acid-1-^{14}C, 2,4-D-1-^{14}C, and 2,4-D-2-^{14}C were purchased from Amersham/Searle Corporation. $H^{36}Cl$ was purchased from International Chemical and Nuclear Corp. The method of Mikulski and Eckstein[18] was used to prepare 2,4-D-4-^{36}Cl. All radioactive compounds were checked for radiochemical purity after PC with a Packard Radiochromatogram scanner.

Plant growth conditions. Ribes sativum plants were grown in the greenhouse in soil. The plants were watered once a week with half-strength Hoagland's solution,[19] at other times with tap-water. Leaves used in the experiments were 20–30 days old.

Measurement of radioactivity. Radioactivity was determined in a liquid scintillation spectrometer. Plant residue (10–20 mg) was partially solubilized by standing overnight in 1·0 ml Soluene TM-100; before count-

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ing, 0·5 ml H_2O, 0·25 ml acetic acid and 15 ml scintillation solvent were added. $Ba^{14}CO_3$ was powdered, weighed and suspended in 15 ml scintillation solvent containing 5% Cab-O-Sil Before counting. $^{14}CO_2$ from plant tissue was trapped and assayed by the method of Jeffay and Alvarez.[20] The procedures of Hetenyi and Reynolds[21] were adopted for counting samples containing both ^{14}C and ^{36}Cl. The scintillation solvent used contained 5 g PPO, 0·5 g POPOP in 500 ml toluene and 500 ml absolute EtOH.

Feeding of 2,4-D-2-^{14}C-4-^{36}Cl. Excised shoots (50 g) were allowed to take up 2·0 mg 2,4-D-2-^{14}C-4-^{36}Cl (5·00 μCi ^{14}C, 2·55 μCi ^{36}Cl) in 2·0 ml potassium phosphate buffer (0·02 M, pH 7·5). The precursor solution was taken up in 35 min and the shoots allowed to metabolize the herbicide for 24 hr. The photoperiod was 16 hr, the temp. 28°, and light intensity 15 000 lx. The shoots were frozen in liquid N_2 and stored at −30° until extraction.

Feeding of 2,4-D-1-^{14}C *and* 2,4-D-2-^{14}C. Excised *Ribes sativum* leaves were weighed (5–6 g) and the petioles quickly immersed in a 5 ml solution of 2,4-D-1-^{14}C or 2,4-D-2-^{14}C (0·17 μmol, 5·0 μCi). After 4 min, the leaves were transferred to a beaker and the petioles immersed in 20 ml H_2O. The temp. was 28° and light intensity 15 000 lx at the leaf surface. After a suitable incubation period, the leaves were frozen in liquid N_2 and stored at −30° until extracted.

Feeding of 2,4-D-1-^{14}C *and glyoxylic acid*-1-^{14}C. Excised *Ribes sativum* leaves were weighed (5–6 g) and the petioles immersed in a 5·0 ml solution containing 2,4-D-1-^{14}C (5·0 μCi, 0·17 μmol) or glyoxylic acid-1-^{14}C (5·0 μCi, 0·64 μmol). After 4 min, the leaves were transferred to a 3·7 l. bell-jar, and the petioles immersed in 20 ml H_2O. Air was introduced into the jar through a 4 mm opening on the side, 3 cm from the bottom, and pumped out of the jar through a 4 mm opening at the top. Air passed from the jar through a drying tube of 100–200 mesh silica gel to 2 traps, each containing 25 ml $^{14}CO_2$-trapping solution,[20] and to a pump. The flow-rate of air was 250 ml/min. The $^{14}CO_2$-traps were changed at 2-hr intervals. After 6 hr the leaves were removed from the jar, frozen in liquid N_2, and stored at −30° until extraction.

Extraction of plant tissue. Plant tissue was extracted with 80% EtOH by homogenization in a blender. Carrier glycine and glyoxylic acid were added, the homogenate filtered, and the residue washed 3 × with 25 ml portions of Me_2CO. The residue was dried 3 hr at 35°, then stored at −30°. The combined EtOH–Me_2CO extract was concentrated to an aqueous solution by warming at 40° under reduced-pressure. The aqueous solution was adjusted to pH 2 with 6 N HCl, extracted 3 × with equal vols. of CH_2Cl_2 and retained for isolation of glycine and glyoxylic acid. Essentially all of the radioactivity in the CH_2Cl_2 phase cochromatographed with authentic 2,4-D.

Isolation of glycine-^{14}C. The aqueous solution obtained from extraction of plant tissue was passed through a column of Dowex 50 × 8 (H^+) resin (17·5 × 0·8 cm), followed by 20 ml H_2O. The effluent was saved for isolation of glyoxylic acid. The column was washed with 60 ml 0·4 N NaOH. The second effluent was adjusted to pH 6·5 with 0·5 N HCl, and evaporated to dryness. The residue was taken up in 3 ml water, diluted with 650 mg copper–picrate in 5 ml H_2O, and after standing overnight at 4°, the copper–picrate derivative of glycine collected, washed twice with 3 ml portions of cold H_2O, and air-dried.[22] After recovery from the derivative, glycine was determined colorimetrically[23] and the total ^{14}C in glycine determined by reverse isotope dilution. Radiochemical purity was checked by PC.

Isolation of glyoxylic acid-^{14}C. The first effluent from the Dowex 50 column was adjusted to pH 7 with KOH and evaporated to 10 ml. The solution was applied to a column of Dowex 1 × 8 (acetate) resin (17·5 × 0·8 cm). The column was eluted successively with 100 ml H_2O, 200 ml 0·5 N HOAc, and 400 ml 1·5 N HOAc acid. Fractions (15 ml) from the 1·5 N HOAc effluent were collected and assayed for glyoxylic acid.[24] Fractions containing glyoxylic acid were pooled, evaporated to dryness, and taken up in 10 ml H_2O. Glyoxylic acid was determined colorimetrically[25] and the total ^{14}C-content in the acid determined by reverse isotope dilution. Radiochemical purity was checked by PC.

Degradation of glycine-^{14}C *and glyoxylic acid*-^{14}C. Radioactive glycine was degraded with ninhydrin.[26] The carboxyl-carbon was collected as $Ba^{14}CO_3$. The methylene-carbon was released as formaldehyde and trapped as the dimedone derivative. Glyoxylic acid-^{14}C was reduced to glycolic acid,[24] then degraded according to the method of Willard and Young.[27] The carboxyl- and α-carbons were collected separately as $Ba^{14}CO_3$.

Hydrolysis of plant residue. EtOH-insoluble plant residue (100 mg) was suspended in 20 ml 2 N HCl and heated on the steam bath 3 hr. After cooling, the hydrolysate was extracted twice with 10 ml portions of CH_2Cl_2. An aliquot of the CH_2Cl_2-extract was assayed for ^{14}C and the rest chromatographed on paper with authentic 2,4-D-^{14}C. Essentially all of the radioactivity obtained cochromatographed with authentic 2,4-D.

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PC. Glycine, glyoxylic acid, and 2,4-D were separated by PC with *n*-BuOH–HOAc–H_2O (12:3:5). In addition, PC of glyoxylic was carried out with Et_2O–HOAc–H_2O (4:1:1); 2,4-D with *n*-PrOH–conc.NH_3 (7:3); and glycine with *t*-BuOH–H_2O–MeEtCO–Et_2NH (80:80:40:8). Chromatograms with low radioactivity were cut into 1·5 cm sections, placed in counting-vials, and assayed in a liquid scintillation spectrometer.

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