Metabolism of 2,4-Dichlorophenoxyacetic Acid and 2,4,5-Trichlorophenoxyacetic Acid by Bean Plants

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The major metabolite of 2,4-dichlorophenoxyacetic acid in bean plants is 2,5-dichloro-4-hydroxyphenoxyacetic acid, while 2,3-dichloro-4-hydroxyphenoxyacetic acid is a minor metabolite. These metabolites accumulate as glycosides but are also present as the free aglycones. These metabolites were detected in several other plant species. With 2,4,5-T, only elimination of the *p*-chlorine was observed, leading to a minor amount of 2,5-dichloro-4-hydroxyphenoxyacetic acid.

Despite extensive work, the metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) is not completely understood.

Early studies by Weintraub, *et al.* (1952a) on the metabolism of 2,4-D by bean plants indicated side chain metabolism to CO_2, especially from the carboxyl group. Luckwill and Lloyd-Jones (1960a) extended these observations to apple, strawberry, and black current leaves. They also found a metabolite of 2,4-D (Luckwill and Lloyd-Jones, 1960b) which appeared to be a simple phenolic derivative. Fitzgerald, *et al.* (1967) detected 2,4,5-trichlorophenol as a metabolite of 2,4,5-T in woody plants. In contrast to these limited observations in plants, soil bacteria of the genera *Arthrobacter*, *Flavobacterium*, and *Achromobacter* cleave 2,4-D at the ether linkage (Fernley and Evans, 1959; Loos, *et al.*, 1967; Steenson and Walker, 1957), and in addition to 2,4-dichlorophenol, 3,5-dichlorocatechol was found (Bollag, *et al.*, 1968a). These workers have characterized the enzyme from *Arthrobacter* which forms 3,5-dichlorocatechol from 2,4-dichlorophenol. Subsequent metabolism in cell free extracts appeared to give the γ-lactone derivative from a dichloromuconic acid intermediate (Bollag, *et al.*, 1968b). Chloromuconic acids have previously been detected as 2,4-D metabolites from soil bacteria (Fernley and Evans, 1959). This metabolic pathway has been suggested by Tutass (1967) to lead to monochloroacetic acid in higher plants, and this latter substance was suggested to cause the growth regulatory effects.

Extensive studies from Woodcock's laboratory have indicated the major pathway in *Aspergillus niger* is ring hydroxylation. Unsubstituted phenoxyacetic acid was primarily hydroxylated in the para position with small amounts of hydroxylation in the ortho and meta positions (Byrd and Woodcock, 1957). The major metabolite of 2,4-D was 5-hydroxy-2,4-dichlorophenoxyacetic acid (5-OH-2,4-D) with a minor amount of 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D) (Faulkner and Woodcock, 1964, 1965). Holley (1952) found that the major metabolite in bean plants appeared to be a ring hydroxylated glycoside. Thomas, *et al.* (1964b) found glycosides in bean plants which yielded products which cochromatographed with 4-OH-2,4-D and 4-OH-2,3-D (minor), but in oat seedlings only the glucose ester was found (Thomas, *et al.*, 1964a). Bach (1961) found a complex pattern of metabolites in bean petiole. Acid hydrolysis of six water-soluble metabolites yielded either 2,4-D or an unknown. The latter chromatographed like a major ether-soluble unknown. In the early study of Jaworski and Butts (1952), the major 2,4-D metabolite in bean plants released 2,4-D on acid hydrolysis or treatment with amylase. Weintraub, *et al.* (1952b) found almost complete metabolism of 2,4-D in bean plants into an ether-soluble volatile and two ether-insoluble acidic metabolites. Conjugation of 2,4-D to form 2,4-dichlorophenoxyacetylaspartic acid as well as its conjugation to glucose to form the glucose ester has been reported by Klämbt (1961).

An additional pathway of 2,4-D metabolism was recently reported (Linscott, *et al.*, 1968) in alfalfa, where chain extension by two carbon units has been detected.

The objective of this study was to determine what major metabolites of 2,4-D and 2,4,5-T were present in bean plants and their subsequent transformations, if any. These observations were extended to a few other species to determine how general this pathway might be.

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MATERIALS AND METHODS

Some chromatographic properties of hydroxylated 2,4-D derivatives are given in Table I. Synthesis of 4-OH-2,5-D and 4-OH-2,3-D was by the method of Faulkner and Woodcock (1965).

4-Hydroxy-2,5-dichlorophenoxyacetic Acid (4-OH-2,5-D). 2,5-Dichloro-*p*-benzoquinone was reduced by hydrogenation with palladium black at room temperature (mp = 272° C, λ max = 305 mμ). The hydroquinone (700 mg) was dissolved in absolute ethanol (15 ml) and treated with sodium (0.12 g). Ethyl bromoacetate was added slowly (0.85 g) while the solution refluxed 15 min. Two volumes of water were added and the solution adjusted to pH 10. Following ether extraction, the aqueous fraction was refluxed 20 min to hydrolyze the ester. The solution was extracted into ether (3×) following acidification to pH 3 and the combined ether fraction shaken with 5% sodium bicarbonate (2×). The pH of the combined aqueous fraction was adjusted to pH 3 and again extracted with ether (3×). The ether was evaporated and the residue taken up in hot water (150 ml), which yielded a precipitate when cooled. Sublimation (130° to 150° C at 50 μ) yielded about 40 mg of white crystalline product [mp 164°–165.5° C; λ max = 297 mμ at pH 2.0; λ max = 315 mμ at pH 11; also characterized by ir (Faulkner and Woodcock, 1965) and the mass spectrum].

4-Hydroxy-2,3-dichlorophenoxyacetic Acid (4-OH-2,3-D).

Table I. Chromatographic Properties of Hydroxylated 2,4-D and 2,4,5-T Derivatives

	R_f[a]					Color with Diazotized Sulfanilic Acid
	Silica Gel			Paper		
Derivative	1	2	3	4	5	
4-OH-2,5-D	0.44	0.20	0.45	0.25	0.64	Beige
4-OH-2,3-D	0.29	0.15	0.42	0.20	0.59	Reddish
3-OH-2,4-D	0.41	0.15	0.42	0.06	0.40	Yellow
5-OH-2,4-D	0.27	0.10	0.30	0.20	0.48	Dark Pink
6-OH-2,4-D	0.49	0.05	0.28	0.63	0.74	OR-Brown
4-OH-2,3,5-T	0.40	0.17		0.14	0.46	Light Pink
4-OH-2,3,6-T	0.53	...		0.24	0.51	Light Pink
2,4-D	0.63	0.33		0.69	0.72	...
2,4,5-T	0.70	0.33		0.71	0.74	...

[a] The solvents were: (1) Petroleum ether/diethyl ether/formic acid (50:50:2). (2) Benzene/diethyl ether/absolute ethanol/acetic acid (50:40:2:0.2). (3) Benzene/methanol/acetic acid (45:8:4). (4) 2-Propanol/ammonia/water (8:1:1). (5) Ethanol/ammonia/water (80:4:16).

The 2,3-dichloro-hydro-*p*-benzoquinone was prepared by chlorination of the *p*-hydroquinone (4 g) in acetic acid at 50° C (Conant and Fieser, 1923). Chlorine gas was bubbled through the solution until the weight gain was 3 g. The solution was concentrated *in vacuo* (45° C) and the residue taken up in hot water, charcoal added, and the solution filtered. Upon cooling, crystalline material was obtained (330 mg, mp 133–136° C). Upon recrystallization from benzene 206 mg of product (mp 141–142° C) was obtained (E_{max}; $E_{295}^{pH\ 1} = 3.81 \times 10^3$, $E_{316}^{pH\ 12} = 8.60 \times 10^3$).

The synthesis of 4-OH-2,3-D was the same as for 4-OH-2,5-D (180 mg of the 2,3-dichlorohydroquinone). Light tan crystals were obtained from hot water, and after sublimation *in vacuo* (50 μ at 130–142° C) only about 5 mg of 4-OH-2,3-D were obtained (mp 178–180° C; λ_{max} = 298 mμ at pH 1; λ_{max} = 313 mμ at pH 12). Due to the small amount of product, the only other characterization was by paper chromatography and its reaction with diazotized sulfanilic acid.

4-Hydroxy-2,3,5-trichlorophenoxyacetic Acid (4-OH-2,3,5-T). This derivative was prepared only in mixture with 4-OH-2,3,6-trichlorophenoxyacetic acid (4-OH-2,3,6-T). The 2,3,5-trichloro-*p*-benzoquinone was obtained by chlorination of 2,5-dichloro-*p*-benzoquinone (1.77 g) at 55° in 150 ml of glacial acetic acid. The progress was monitored each hour by tlc (Silica gel G with ultraviolet indicator; Benzene). When traces of tetrachloro-*p*-benzoquinone were detected the reaction was stopped by evaporation of the reaction mixture *in vacuo* (rotary evaporator, 40° C). The residue was taken up in benzene (100 ml) and hydrogenated in the presence of palladium black (30 mg). After uptake of one equivalent of hydrogen gas, the solution was filtered and crystallized from the cold benzene (mp 134–135° C); tlc (Silica gel G; benzene: ethyl acetate, 95 to 5) indicated mostly one component (R_f 0.2) with traces of the tetrachlorohydroquinone (R_f 0.26) and dichlorohydroquinone (R_f 0.14). Following the reaction of 2,3,5-trichloro-*p*-benzohydroquinone with ethyl bromoacetate, the product contained two isomers (4-OH-2,3,5-T, and 4-OH-2,5,6-T) which could be separated by tlc (Silica gel G; petroleum ether, diethyl ether, ethyl acetate, trichloroacetic acid, 75:75:7.5:3 g) R_f 0.38 and and 0.49 (also see Table I).

For most studies 2,4-D-1-^{14}C was used (2.44–21.1 mCi per mmole) but a few experiments with 2,4-D-2-^{14}C indicated similar metabolites were present. Pinto bean plants were usually 14 to 20 days old when treated. In initial experiments 10 μl of an ethanolic solution of 2,4-D containing 0.5% Tween-20 was spotted on the midvein of each primary leaf. The treated area was confined with vaseline and discarded prior to extraction of the leaf and stem tissue with 5 volumes of boiling ethanol. In later experiments stems were injected at the primary leaf axis by the use of small glass capillary tubes. Prior to injection the capillary was filled with water and 10 μl of 2,4-D-1-^{14}C in ethanol (0.075 μCi per plant; 2.44 mCi per mmole) was added only to those capillaries in which uptake of water was observed. In other experiments excised roots of plants were surface sterilized with 5% hydrogen peroxide and shaken with 2,4-D-1-^{14}C for 6 to 8 hr. The roots were washed several times with water prior to extraction. Tissue not immediately extracted was stored in plastic bags at −20° C.

The extraction and fractionation procedure is outlined in Figure 1. In some experiments the ether fraction was shaken twice with 5% $NaHCO_3$. Following acidification with phosphoric acid (pH 3), the aqueous layer was extracted three times with equal volumes of diethyl ether. The combined ether extracts contained 90 to 95% of the ^{14}C initially present in the ethanol fraction. This bicarbonate partition step improved tlc and paper chromatography for detection of 2,4-D and the aglycones.

The column concentrate (about 25 ml) was adjusted to pH 6.7 and treated with 0.7 mg per ml of β-glucosidase (emulsin, Nutritional Biochemical Co.) for 5 hr at 37° C. All thin-layer chromatography was on silica gel G plates (0.25 mm) which were activated by heating at least 2 hr at 140° C. Ascending paper chromatography was on Whatman 3MM paper. In most cases synthetic standards were mixed with the labeled metabolites prior to chromatography. The known compounds were located with diazotized sulfanilic acid (first sprayed with fresh diazotized sulfanilic acid in 1 *N* HCl then with 10% sodium carbonate), and the labeled metabolites by radioautography or scanning. Counting was by either gas flow GM (plate and strip scanning) or by liquid scintillation (PPO 5 g, POPOP 0.1 g, Triton X-100 333 ml, toluene 667 ml). The insoluble residue and pigmented samples were combusted by the oxygen flask method (Kalberer and Rutschman, 1961) prior to liquid scintillation counting in Brays solution (Bray, 1960). Counts were corrected for background and quenching (external standard).

RESULTS AND DISCUSSION

In preliminary experiments primary leaves of 2-week-old bean plants were treated with 2,4-D-1-^{14}C (0.01 μM, 0.024 μCi) and the leaf tissue collected after 6 hr in order to evaluate initial metabolites. The amount of butanol-soluble metabolites formed varied from 7 to 13% of the total ethanol soluble radioactivity. Following Sephadex G-10 chromatography, two incompletely separated radioactive peaks were observed but recovery of ^{14}C was only about 50%. The major labeled product after treatment with β-glucosidase cochromatographed with synthetic 4-OH-2,5-D in solvents 1, 2, 3, 4, and 5 (Table I). In subsequent experiments a minor component running below 4-OH-2,5-D was confirmed to be 4-OH-2,3-D by cochromatography in the same solvents. In a preparative experiment enough major aglycone was obtained for a mass spectrum. Although the isolated metabolite is impure, the mass ion peaks at 236, 238, and 240 correspond to the expected pattern for a compound with two chlorine groups and agree with the synthetic material. The phenol fragments at mass 177, 179 are also obvious in both.

Stem injection was used to introduce a known amount of 2,4-D-1-^{14}C into bean plants in a short interval. The leaves

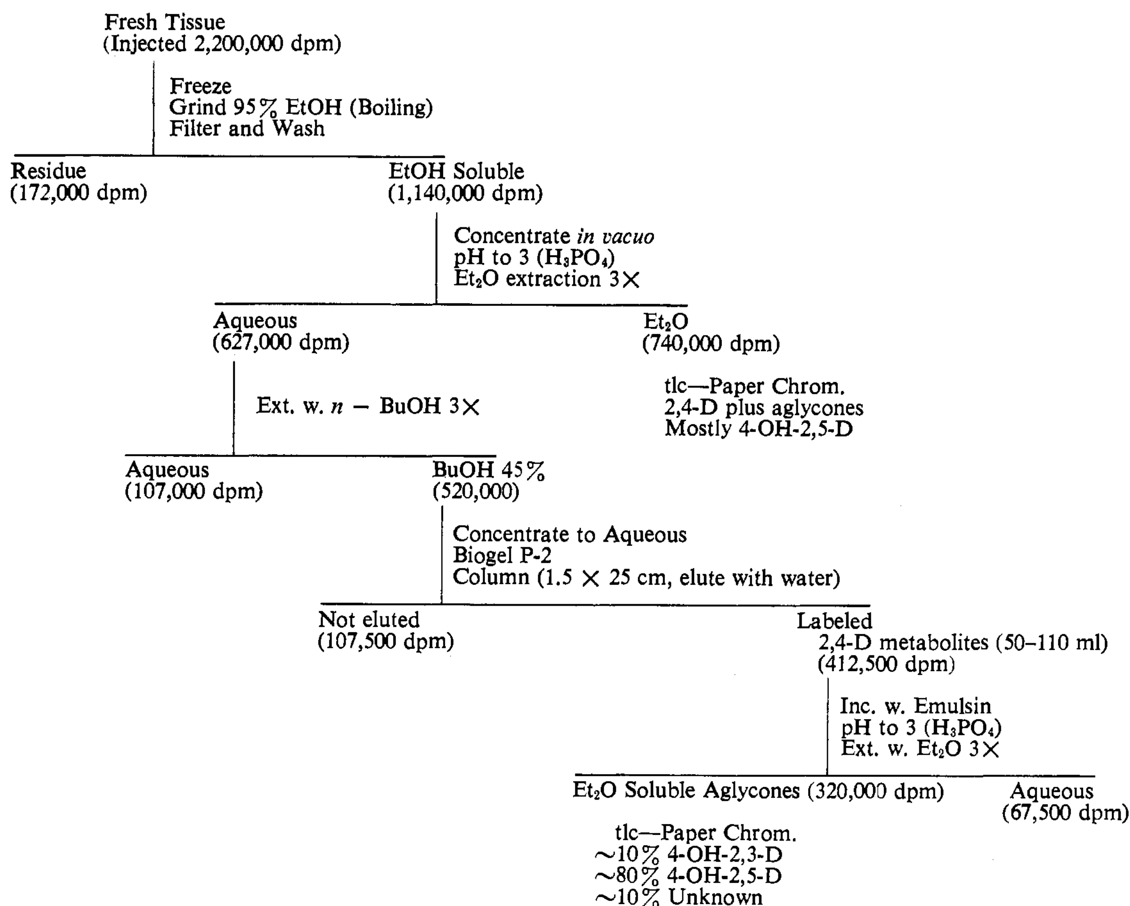

Figure 1. Flow diagram for the standard extraction procedure and radioactivity found in various fractions from 48 hr 2,4-D-1-^{14}C stem injected bean plants

and stems were then sampled at intervals following treatment, and the distribution of radioactivity into various fractions or substances was determined. Duplicate samples were counted and corrected for quenching and efficiency. The total radioactivity in the residue plus the soluble fractions was considered to be 100%. The actual distribution of ^{14}C after 48 hr is shown in Figure 1, while the changes in the percent of the total radioactivity in some of the fractions or substances isolated are shown in Figure 2. The rapid decrease in the percentage of 2,4-D is correlated with the appearance of these metabolites. At first there are more aglycones (almost entirely 4-OH-2,5-D) but later the glycosides are the major metabolites. The glycosides would seem to be relatively stable in this tissue, since they still predominate 22 days following treatment. About 70% of the radioactivity injected was accounted for, and there was little decrease in the total radioactivity found in the tissues even after 22 days. Therefore, one can conclude that decarboxylation or cleavage of 2,4-D at the ether linkage cannot be a major pathway in this tissue. In addition to the fractions shown (Figure 2), there was some (2.75 to 6.75%) residual water-soluble radioactivity following butanol extraction which increased to 23 and 31% of the total ^{14}C after 15 and 22 days, respectively. Also, 15 to 30% of the radioactivity from the Biogel P-2 column was not hydrolyzed by β-glucosidase. Of the ether soluble products resulting from β-glucosidase treatment, 10 to 15% of the ^{14}C was 4-OH-2,3-D 70 to 80% was 4-OH-2,5-D, and a minor unknown with an R_f (solvent 1) just higher than 2,4-D was also detected. Prior to hydrolysis, paper chromatography in 2-propanol, acetic acid, water (8:1:1) indicated an R_f of about 0.4 (diffuse spot) for the major glycoside(s) with a minor component at R_f 0.8.

Survey experiments with corn, buckwheat, oats, wheat

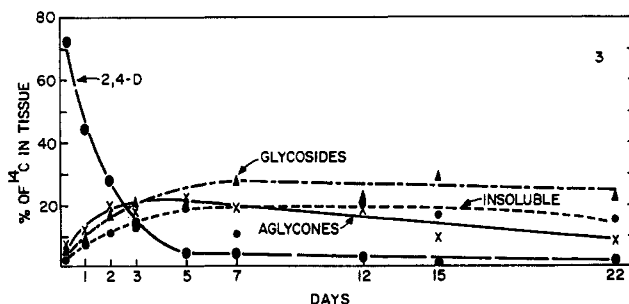

Figure 2. Changes in the percent of total ^{14}C found in various substances or fractions following stem injection of 14-day-old bean plants with 2,4-D-1-^{14}C. Fifteen plants were extracted and fractionated as indicated in Figure 1 after each time interval

Table II. Metabolic Products Found in 2-Week-Old Pinto Bean Plants 24 Hr After Stem Injection of 2,4,5-T-1-^{14}C

Fraction or Substance	Total dpm[a]	% Ethanol Soluble
(Total injected)	3,330,000	
Ethanol soluble	2,445,000	(100)
Ether soluble	1,920,000	79%
Butanol soluble	75,550	3.1%
Water residual	10,000	0.4%
Ether Soluble Tlc[b]		
Cochromat. w. 2,4,5-T	1,840,000	76%
Cochromat. w. 4-OH-2,5-D	47,500	1.9%
Unknown	44,550	1.8%
Butanol Soluble		
Ether soluble after		
β-glucosidase treatment	38,400	1.6%
Cochromat. w. 2,4,5-T[b]	16,000	0.7%
Cochromat. w. 4-OH-2,5-D	11,950	0.5%
Unknown	10,450	0.4%

[a] Average of two experiments of 20 plants each. [b] Tlc solvent was petroleum ether, diethyl ether, trichloroacetic acid, ethyl acetate (75:75:3 gm:7.5 ml).

soybeans, and barley were undertaken to determine if the ring hydroxylated glycoside pathway was present in these species. Preliminary experiments indicated that surface-sterilized bean roots accumulated significant quantities of the ring-hydroxylated glycosides in 6 hr. Therefore, 2,4-D-1-^{14}C was either applied to the foliage of intact plants (48 hr) or surface sterilized excised roots (6 hr) were used. The aglycones released from the β-glucosidase treatment were examined by cochromatography with standards in solvents 1, 2, and 3. The results indicate that these metabolites are formed in wheat, barley, soybeans, and oats, but not in buckwheat or corn. Other major unknowns are present in oats and soybean. One of these appears to be the glucose ester of 2,4-D, as has been reported (Klämbt, 1961). Both excised roots and leaves appear to form these metabolites.

The detection of 4-OH-2,5-D and 4-OH-2,3-D in bean plants appears to confirm that the NIH shift reaction (Guroff, *et al.*, 1967) takes place in this tissue. Since this reaction has been detected in liver microsomes, an attempt was made to demonstrate the hydroxylation with plant preparations. The enzymatic hydroxylation of 2,4-D *in vitro* could not be demonstrated with bean crude homogenates or microsomal preparations from bean seedlings, bean roots, or wheat roots. Microsomal preparations from pea seedlings (Russell and Conn, 1967) which convert *trans*-cinnamic acid to *p*-coumaric acid did not convert 2,4-D-1-^{14}C to hydroxylated derivatives, nor was phenylalanine converted to tyrosine.

The metabolism of 2,4,5-T in bean plants was examined by stem injection of 2,4,5-T-1-^{14}C (0.07 μCi; 2.21 mCi/mM). Two major findings (Table II) were that 2,4,5-T is much more stable in bean plants than 2,4-D, and that in addition to unknowns, 4-OH-2,5-D was present while no 4-OH-2,3,5-T was detected. This indicates that elimination of the chlorine group occurs in this case rather than the expected NIH shift to the 3 position.

The synthetic aglycones 4-OH-2,5-D and 4-OH-2,3-D were bioassayed in the *Avena* section test (Wang, *et al.*, 1968) for their ability to induce cell elongation, and in the bean bud test (Brown and Weintraub, 1950) for formative effects on leaves. In both tests no activity was observed, so these metabolites may be considered to be detoxification products. The fact that both grass and broad-leaf species form these metabolites indicates that tolerance of grasses to 2,4-D is probably unrelated to this detoxification pathway.

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