Metabolism of 2,4-Dichlorophenoxyacetic Acid. 10. Identification of Metabolites in Rice Root Callus Tissue Cultures

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The metabolism of 1-^{14}C-labeled 2,4-dichlorophenoxyacetic acid (2,4-D) was investigated in rice root callus tissue and found to be quite different from other callus tissue. After 7 days incubation, 39.7% of the applied dose was found in the water-soluble and 15.9% in the ether-soluble tissue fractions. The residue and media contained 5.2 and 25.2%, respectively. Almost all of the ether-soluble label was unmetabolized 2,4-D. The major metabolites in the water-soluble fraction of rice root callus tissue appear to be sugar esters of 2,4-D (carboxylic glycosides) since β-glycosidase releases mainly 2,4-D. Amino acid conjugates of 2,4-D, which were major metabolites in other callus tissues (especially dicots), were not detected. The two ring hydroxylated aglycones, 4-hydroxy-2,3-dichlorophenoxyacetic acid and 4-hydroxy-2,5-dichlorophenoxyacetic acid represented only 0.4% of the applied dose in rice root callus tissue, but in other callus tissues (especially the monocot corn) these ring hydroxylated metabolites were major metabolites of 2,4-D.

Previously it was reported that plant callus tissue cultures from several plant species converted 2,4-dichlorophenoxyacetic acid (2,4-D-*1*-^{14}C) to biologically active amino acid conjugates (ether soluble) and to biologically inactive ring-hydroxylated glycosides (water soluble) as well as, to a small extent, to glucose or sugar esters (Feung et al., 1971, 1972, 1973, 1974, 1975). Enzymatic hydrolysis of water-soluble metabolites gives rise to free 2,4-D (Klämbt, 1961; Hilton, 1966; Ojima and Gamborg, 1968; Feung et al., 1971, 1973, 1974) and ring-hydroxylated 2,4-D metabolites (Thomas et al., 1964; Fleeker and Steen, 1971; Hamilton et al., 1971; Feung et al., 1971, 1973, 1974). On the other hand, Hagin et al. (1970) demonstrated 2,4-D was chain elongated to 2,4-dichlorophenoxypropionic acid by several grasses (timothy, bromegrass, and orchardgrass) and that a major portion of this metabolism took place on the surface of the grasses. In a study on the metabolism of 2,4-D in several callus tissues it was shown that corn-endosperm callus tissue more readily metabolized 2,4-D to biologically inactive ring-hydroxylated glycosides and to sugar ester glycosides (carboxylic glycosides) compared to other dicot callus tissue examined (carrot, sunflower, tobacco, jackbean, and soybean) (Feung et al., 1975). This article reports an extension of this investigation of the metabolism of 2,4-D by another monocot, rice root callus tissue.

MATERIALS AND METHODS

The callus used was derived from roots of rice plants (*Oryza sativa* var. Starbonnet) and was obtained from Dr. C. C. Still, Department of Biochemistry and Microbiology, Rutgers University. Stock cultures have been incubated at 30 °C in the dark on 0.6% agar containing a basal medium of Heller's salts, yeast extract, vitamins, sucrose, and 2 mg/l. 2,4-D (Yatazawa et al., 1967).

Approximately 10 g of 3-week-old root callus tissues was aseptically transferred into each 125-ml flask containing 50 ml of autoclaved liquid basal nutrient medium (Yatazawa et al., 1967) and 5 μCi (35.7 μg) of 2,4-D-*1*-^{14}C (specific activity 140 μCi/mg). The treated root callus tissues were incubated at 30 °C with gentle shaking in the dark for 7 days. The tissue was collected on a Buchner funnel (Whatman No. 1 paper) and surface rinsed with cold distilled water. The filtrate and the tissue residue were stored at −18 °C prior to extraction.

The procedures for extraction, fractionation, separation, and purification were similar to those previously reported (Feung et al., 1973). The frozen tissues were thoroughly macerated in a Waring Blendor with 95% ethanol (5 ml/g). The homogenate was filtered with suction. The residue was boiled in 80% ethanol for 5 min and filtered, and the residue washed six times with 80% ethanol. The combined ethanol filtrates were concentrated, acidified (pH 2), and extracted four times with diethyl ether. The aqueous phase was subsequently extracted twice with 1-butanol, which was then evaporated to dryness. The residue was dissolved in 10 ml of distilled water and adjusted to a pH of 4.5 with 1% $NaHCO_3$. Emulsin (25 mg, Nutritional Biochemical Co.) was added and the solution incubated for 72 h at room temperature. The aglycones were extracted into diethyl ether (4×) after the hydrolysate was acidified to pH 2. The original ether-soluble and the aglycone fractions were evaporated to dryness, and the residue was dissolved in 90% ethanol (10 ml), placed in 15-ml centrifuge tubes, and stored at −18 °C overnight. The chilled aglycones and ether-soluble fractions were centrifuged at 300*g* for 5 min at 0 °C and the precipitate discarded (no radioactivity). The supernatant was concentrated to ca. 6 ml and the procedure was again repeated. The supernatant was stored at −18 °C prior to separation.

The aglycone fraction was fractionated by thin-layer chromatography (TLC) and the original ether-soluble fraction (pH 2) was separated by paper chromatography (PC). All metabolites were subsequently purified by TLC. The labeled 2,4-D metabolites were located with autoradiography and the synthetic standards were located by viewing with short-wave uv or by spraying with bromocresol green (Bryant and Overell, 1953) or with diazotized sulfanilic acid. The isolated and purified 2,4-D-*1*-^{14}C metabolites were characterized by paper or thin-layer cochromatography with synthetic standards and also analyzed by mass spectrometry using a direct sample inlet (Model 1290 G, Nuclide Corp.).

The radioactivity in each fraction was measured by liquid scintillation counting (Aquasol). The ethanol insoluble tissue residue was combusted by the oxygen flask method (Kalberer and Rutschman, 1961) prior to liquid scintillation counting in Bray's solution (Bray, 1960). All counts were corrected for quenching (external standards) and background.

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Table I. Relative Percentage of Metabolites of 2,4-D Incubated with Rice Root Callus Tissue for 7 Days[a]

Water-soluble metabolites[b]				Ether-soluble metabolites			
Metabolites				Metabolites			
R_f region[c]	Designation	% total in fraction	% total in tissue	R_f region[d]	Designation	% total in fraction	% total in tissue
0.39	Ag_1 (4-OH-2,3-D)	1.0	0.3	0.65	Et_1	1.2	0.2
0.56	Ag_2	50.3	15.0	0.75	Et_2	1.7	0.3
	Ag_{2a}[e] (4-OH-2,5-D)	0.2	0.1	0.81	Et_3 (2,4-D)	92.6	14.7
	Ag_{2b}[e] (2,4-D)	50.1	14.9	0.88	Et_4	4.5	0.7
0.68	Ag_3	2.8	0.8				
0.76	Ag_4[f] (Et-2,4-D)	43.4	12.9				
0.84	Ag_5	1.8	0.5				
0.89	Ag_6	0.7	0.2				
		Total 100	29.7			Total 100	15.9

[a] The residue and media contained 5.2 and 25.2% of the applied radioactivity, respectively. [b] Only 75% of the total 39.7% water-soluble metabolites were hydrolyzed with Emulsin yielding 29.7% of the total applied radioactivity which was further fractionated. [c] Thin-layer chromatography, solvent system: diethyl ether–petroleum ether–formic acid (70:30:2, v/v/v). [d] Descending paper chromatography, solvent system: 1-butanol–95% ethanol–3 N ammonia hydroxide (4:1.36:5, v/v/v). [e] Following rechromatography Ag_2 separated into two distinct regions, Ag_{2a} and Ag_{2b} of R_f 0.53 and 0.58, respectively. [f] Ag_4 is an artifact of the isolation procedure and presumably derived from the large amount of 2,4-D (Ag_{2b}) which resulted from Emulsin treatment of the sugar esters.

Four TLC solvents were employed: (I) diethyl ether–petroleum ether (bp 35–60 °C)–formic acid (70:30:2, v/v/v); (II) chloroform–methanol–concentrated ammonia hydroxide (70:35:2, v/v/v); (III) chloroform–ethyl acetate–formic acid (35:55:10, v/v/v); (IV) diethyl ether–benzene–ethanol–acetic acid (40:50:20:0.5, v/v/v/v). Descending PC (Whatman No. 1 paper) employed the solvent 1-butanol–ethanol–3 N ammonium hydroxide (4:1.36:5, v/v/v) (solvent V). Supelcosil 12A (Supelco, Inc.) was used as the absorbent for TLC and a zinc phosphor was used for detection.

RESULTS

The total recovery of the applied 2,4-D-*1*-^{14}C from rice root callus tissue cultures and liquid media after 7 days incubation was 86.0%, with 25.2% of the applied 2,4-D remaining (as 2,4-D) in the liquid medium. The water-soluble tissue extracts (before Emulsin treatment), the ether-soluble tissue extracts, and the ethanol-insoluble residue consisted of 39.7, 15.9, and 5.2% of the applied dose, respectively. Approximately 75% of the water-soluble extract, which represented 29.5% of the applied dose, was hydrolyzed by β-glucosidase (Emulsin).

The relative proportions of the labeled 2,4-D metabolites in the aglycone fraction, as separated by thin-layer chromatography (TLC), are given in Table I. Six distinct areas obtained from TLC employing solvent I were arbitrarily referred to as Ag_1 through Ag_6 (Table I), and these designations do not necessarily correspond with previous symbols (Feung et al., 1973).

The metabolite Ag_1 was identified as 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D). Ag_2 separated into two components on TLC when it was rechromatographed in solvent system I, having R_f 0.53 (Ag_{2a}) and 0.58 (Ag_{2b}) and subsequenty identified as 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D, 0.1%) and free 2,4-D (14.9%), respectively. The free 2,4-D presumably is derived from a sugar ester (carboxylic glycoside) (Ojima and Gamborg, 1968). Ag_4 was further purified on TLC in solvent system III and acid hydrolysis of Ag_4 (6 N HCl at 75 °C for 24 h) yielded only one detectable compound, 2,4-D. Ag_4 did not correspond chromatographically or mass spectrometrically with 2,4-dichlorophenoxypropionic acid or with 2,4-dichlorophenoxybutyric acid. Mass spectrometric analysis (70 eV, 60 °C) of Ag_4 (Figure 1B) showed it to be the ethyl ester of 2,4-D (Figure 1A) having characteristic m/e ions of 248 (M), 213, 185, 175 (base), 162, 147, 145, 135, 133, 111, and 109. The metabolites Ag_3, Ag_5, and Ag_6 consisted of only 1.5% of the total radioactivity and still remain unknown.

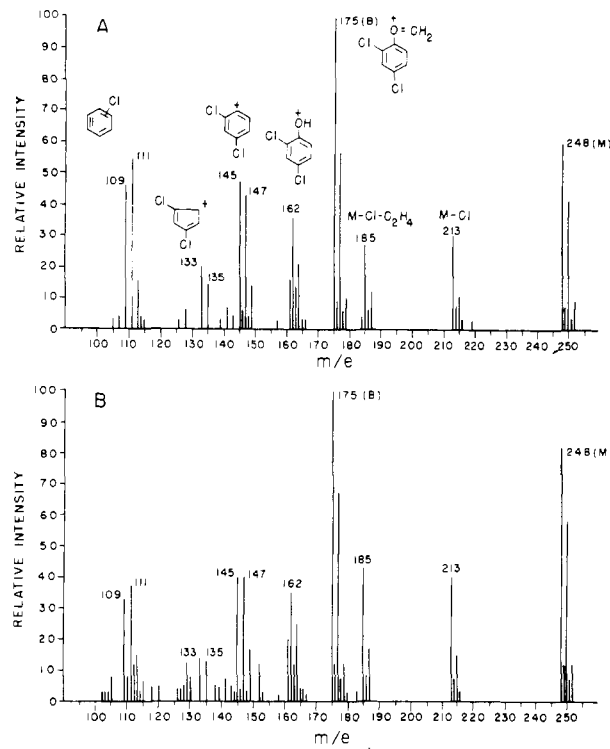

Figure 1. Mass spectra of (A) ethyl ester of 2,4-D and (B) isolated metabolite Ag_4.

The ether-soluble extract migrated into four distinctive fractions having R_f values of 0.65, 0.75, 0.81, and 0.88, respectively, in PC solvent system V (Table I). These components are arbitrarily designated Et_1 through Et_4 in order of increasing R_f values.

Et_3, which was the major metabolite (14.7% of the applied dose) in the ether-soluble extract, was identified as free 2,4-D. The remaining metabolites Et_1, Et_2, and Et_4 constituted only 1.2% of the applied dose and have not been identified.

DISCUSSION

After 7 days incubation with rice root callus tissue the liquid nutrient media still contained 25.2% of the applied dose as free 2,4-D. In previous experiments with other tissue cultures the media contained essentially no ra-

diolabeled products (Feung et al., 1971, 1973, 1974). These data indicate that rice root callus tissue evidently does not readily metabolize or absorb and store free 2,4-D.

Since 14.9% of the radioactivity of the applied 2,4-D-*1*-^{14}C is not accounted for, it suggests that perhaps decarboxylation is of some significance. In previous studies with soybean callus tissue, decarboxylation was shown to be a minor pathway (Feung et al., 1972).

The diethyl ether extract contained 15.9% of the applied dose from which four metabolites were separated. Free 2,4-D consisted of 92.6% of this fraction and no amino acid conjugates were identified. Unfortunately Et_1, Et_2, and Et_4 were present in such small quantities (1.2%) that identification was not possible. In contrast, all dicot callus tissue examined previously contained large quantities of the amino acid conjugates of 2,4-D (Feung et al., 1971, 1973, 1975). On the other hand, the callus tissue of the monocot corn possessed very low levels of amino acid conjugates which suggests that perhaps monocots do not accumulate amino acid conjugates or that the amino acid conjugate pathway is not significant in these plants.

The tissue converted 39.7% of the applied 2,4-D dose into water-soluble (ether insoluble but 1-butanol soluble) conjugated metabolites. Following Emulsin treatment six bands or metabolites were isolated and characterized. Two of the metabolites, Ag_{2_b} and Ag_4, consisted of 93.5% of this fraction. Ag_{2_b}, the major metabolite, was identified as free 2,4-D and thus must have arisen from some conjugate (water soluble) precursor, presumably the glucose ester (carboxylic glucoside). Free 2,4-D has also been found in other callus tissues, following Emulsin treatment of the water-soluble extract; however, the 50.1% found in the rice root callus tissue far exceeds any percentage previously reported (Feung et al., 1975). Interestingly, corn, the other monocot callus tissue examined, also had a high relative proportion of 2,4-D following Emulsin treatment. These data suggest that the glucose ester pathway of metabolism of 2,4-D is very significant in rice root callus tissue and may be more important in monocots.

Ag_4 was identified as the ethyl ester of 2,4-D. This compound was not present in the original extract of the tissue; however, it was an artifact of the isolation procedure. The aqueous fraction was dissolved in ethanol several times and in all probability the ethyl ester was derived from the large amount of 2,4-D that was present in this fraction after Emulsin treatment of the glycones. Since Hagin et al. (1970) reported the presence of chain elongation metabolism of 2,4-D in grasses, careful consideration was given to these potential metabolites. No evidence could be found for the presence of either 2,4-dichlorophenoxypropionic acid or 2,4-dichlorophenoxybutric acid. Et_4 was not identified because of the difficulty presented by its low percentage relative composition (0.7%). Hagin et al. (1970) reported the chain elongation pathway to be primarily a surface phenomena; thus these metabolites may not be found in tissue culture techniques.

Two other aglycones, Ag_1 and Ag_{2_a}, have been identified as 4-OH-2,3-D and 4-OH-2,5-D but these are present in only minor amounts relative to the applied doses (0.3 and 0.1%, respectively). In all other callus tissue examined (Feung et al., 1973, 1975) these hydroxylated aglycones were very significant, especially in the corn callus tissue (31.7%). In an earlier investigation (Feung et al., 1975) it was reported that the 3,4-epoxy pathway (pathway II) predominated in corn relative to the 4,5-epoxy pathway (pathway I) which predominated in other dicots. Correspondingly, rice root callus tissue accumulated more 4-OH-2,3-D (pathway II) than 4-OH-2,5-D (pathway I) and thus is similar to the results obtained with corn, although it differs greatly in the amount of these metabolites observed.

In conclusion, the metabolism of 2,4-D by rice root callus tissue is considerably different from the metabolism of 2,4-D by other callus tissues examined. The major pathway of metabolism appears to be the formation of sugar esters of 2,4-D (carboxylic glucosides). Ring hydroxylation and amino acid conjugation, which were major metabolic intermediates in other callus tissues examined, were not significant metabolites in rice root callus tissue.

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