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Comparative Metabolic Fate of 2,4-Dichlorophenoxyacetic Acid in Plants and Plant Tissue Culture

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The metabolic fate of 2,4-dichlorophenoxyacetic acid (2,4-D) in soybean cotyledon callus tissue culture was compared with the metabolism in soybean and corn plants to determine the usefulness of plant tissue culture as a research tool for pesticide analysis. Both plants and callus tissue were able to metabolize 2,4-D by common metabolic pathways, by way of ring hydroxylation as well as conjugation of the carboxyl with amino acids and with sugars. Qualitatively the metabolites of 2,4-D were the same in callus tissue and in plants but they varied quantitatively. Soybean callus tissue formed amino acid conjugates and hydroxylated derivatives more extensively than soybean or corn plants. The plants contained a higher relative proportion of hydroxylated derivatives than amino acid conjugates. Therefore, 2,4-D metabolism in callus tissue is a good indication of the operating metabolic pathways.

The metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) has been studied extensively in plants (Butts and Fang, 1956; Andreae and Good, 1957; Klämbt, 1961; Thomas et al., 1964; Faulkner and Woodcock, 1964; Audus, 1964; Hilton, 1966; Ojima and Gamborg, 1968; Fleeker and Steen, 1971; Hagin et al., 1970; Hamilton et al., 1971; Chkanikov et al, 1976) and in plant tissue cultures (Bristol et al., 1977; Feung et al., 1971, 1972, 1973, 1975, 1976). Soybean cotyledon callus tissue cultures rapidly convert externally applied 2,4-D-*1*-^{14}C into at least seven biologically active amino acid conjugates, at least two biologically inactive ring-hydroxylated metabolites, and, to a smaller extent, into glucose or sugar esters (Feung et al., 1973, 1974).

Experimentally it is easier and quicker to identify metabolites from plant callus tissue than from whole plants because of the absence of interfering plant pigments and starches (Mumma and Hamilton, 1976). Until experiments are conducted comparing pesticide metabolism in both callus tissue and in the whole plant, the potential advantages of using plant callus tissue as a research tool will remain in doubt. Therefore, we now report the comparative metabolism of 2,4-D by soybean and corn plants and by soybean callus tissue. Results of 2,4-D metabolism by corn endosperm tissue cultures under somewhat different conditions were previously reported (Feung et al., 1975).

MATERIALS AND METHODS

Soybean (*Glycine max* (L.) Merrill var. *Acme*) and corn (*Zea mays* (L.) [Su-1]) were grown in soil in the greenhouse for 2 weeks prior to treatment with 2,4-D-*1*-^{14}C (sp act. 52 mCi/mmol or 236 μCi/mg). With the aid of a syringe, 5 μCi 2,4-D-*1*-^{14}C (21 μg), dissolved in 10 μL of ethanol–acetone solution (1:1), was directly injected into the stem of each plant. Soybean and corn plants were separately harvested 14 days following treatment by cutting at the soil surface, and the tissue was stored in plastic bags at −18 °C for 24 h.

Callus tissue, derived from cotyledon of soybean (*Glycine max* (L.) Merrill var. *Acme*), was grown on autoclaved agar nutrient medium (Miller, 1963) plus α-naphthaleneacetic acid (NAA) in a 125-mL flask for 4 weeks. Then, 5 μCi 2,4-D-*1*-^{14}C (21 μg) in 10 μL of ethanol–acetone (1:1) was aseptically injected into three callus clumps (8–10 g total) which were then allowed to grow on the agar in the growth chamber for 7 days. The callus clumps were harvested and stored in plastic bags at −18 °C for 24 h prior to extraction.

The procedures used for extraction and fractionation of 2,4-D-*1*-^{14}C metabolites of callus tissues and plants were the same as those previously described (Feung et al., 1976). The frozen 2,4-D-*1*-^{14}C treated soybean and corn plants were separately cut into pieces 1 cm in length and subsequently homogenized in a Waring Blendor with 95% ethanol. The frozen callus tissue was also directly ground in a Waring Blendor with 95% ethanol. The homogenates were filtered with suction. The residue was boiled in 80% ethanol for 5 min, filtered with suction, and washed six times with 80% ethanol. The ethanol filtrate was combined, concentrated, adjusted to pH 2 with 3 N H_3PO_4, and then extracted four times with diethyl ether. The aqueous phase was subsequently extracted twice with 1-butanol and the 1-butanol layer was evaporated to dryness. This residue was dissolved in 15 mL of distilled water and adjusted to a pH of 4.5 with 1% $NaHCO_3$, Emulsin was added (Nutritional Biochemical Corporation), and the solution was incubated at room temperature for 72 h. The aglycons were obtained by acidification and extraction with diethyl ether. This aglycon solution and the original ether extract were evaporated to dryness, 90% ethanol (1 mL) was added to these residues, and the resulting solutions were stored at −18 °C for 12 h. The cold alcoholic solution of the original ether extract was then centrifuged at 300*g* for 5 min and the precipitate was discarded (precipitate

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Table I. Relative Percentage of Water-Soluble 2,4-D-*1*-^{14}C Metabolites Isolated from Soybean Callus, Soybean Plant, and Corn Plant as the Aglycons[a]

metabolites[a]		soybean callus % total in		soybean plant % total in		corn plant % total in	
R_f region[b]	designation	fraction	tissue	fraction	tissue	fraction	tissue
origin	Ag_0	13.57	5.18	2.18	0.39	2.38	0.94
0.16–0.21	Ag_1	4.38	1.67				
0.23–0.25	Ag_2	4.69	1.79	0.83	0.15	1.86	0.73
0.38–0.43	Ag_3 4-OH-2,3-D 5-OH-2,4-D	21.68	8.27	8.12	1.47	6.23	2.45
0.50–0.55	Ag_4 4-OH-2,5-D	42.67	16.28	17.78	3.20	4.27	1.67
0.57–0.61	Ag_5 2,4-D	10.54	4.02	50.56	9.05	61.49	24.15
0.67–0.70	Ag_6	2.47	0.94	13.72	2.46	4.18	1.64
0.74–0.78	Ag_7 ethyl-2,4-D[c]			6.81	1.22	19.59	7.70
total		100.	38.15	100.	17.94	100.	39.28

[a] Ether-soluble metabolites obtained after treatment of water-soluble metabolites with Emulsin. [b] Thin-layer chromatography employing solvent systems: diethyl ether–petroleum ether–formic acid (70:30:2, v/v/v). [c] An artifact of the isolation procedure and probably is derived from the carboxylic glycoside and should be considered with Ag_5.

did not contain ^{14}C). The supernatant was concentrated (0.5 mL) and this procedure was repeated. Following the centrifugation the supernatant was stored at −18 °C prior to separation.

The radioactivity in each fraction was measured by liquid scintillation counting (Aquasol). The dried tissue residue was combusted by the oxygen flask method (Kalberer and Rutschmann, 1961) prior to liquid scintillation counting in Brays solution (Bray, 1960). All counts were corrected for quenching (external standard) and background.

The procedures for separation and purification of the 2,4-D metabolites were the same as those previously reported (Feung et al., 1973). The aglycons were separated by thin-layer chromatography (TLC) and the ether-soluble metabolites were first separated by paper chromatography (PC). All metabolites were subsequently purified by TLC. The 2,4-D-*1*-^{14}C metabolites were located with autoradiography and the synthetic standards were located by viewing with short-wave UV light or by spraying with bromocresol green (Bryant and Overell, 1953) or diazotized sulfanilic acid. The isolated and purified 2,4-D-*1*-^{14}C metabolites were further characterized by PC and TLC by comparison with synthetic standards.

Seven TLC solvents were employed: I, benzene–dioxane–formic acid (90:25:2, v/v/v); II, chloroform–methanol–concentrated ammonium hydroxide (70:35:2, v/v/v); III, diethyl ether–petroleum ether–formic acid (70:30:2, v/v/v); IV, chloroform–ethyl acetate–formic acid (35:55:10, v/v/v); V, diethyl ether–benzene–ethanol–acetic acid (40:50:20:0.5, v/v/v); VI, 1-butanol–acetic acid–water (4:1:5, v/v/v); VII, phenol–water (80%). Descending PC was used (Whatman No. 1 paper) employing the solvent 1-butanol–ethanol–3 N ammonium hydroxide (4:1.36:5, v/v/v) (solvent VIII). Supelcosil 12A (Supelco, Inc., Bellefonte, Pa.) was used as the absorbent for TLC and a zinc phosphor was used for detection.

RESULTS AND DISCUSSION

The total recovery of the applied radioactivity from plants (stems and leaves) and plant callus tissues after 14 days of incubation (injection) was 90–95%. The radioactivity remaining in the residue constituted 4.50% of the administered dose in soybean callus tissue, 4.20% in soybean plants, and 4.90% in corn plants. The ether-soluble metabolites (pH 2) found in soybean callus, soybean, and corn plants were 47.75, 73.31, and 46.00% of the applied dose, respectively. The relative amounts of the water-soluble fraction found in soybean callus, soybean plant, and corn plant were 38.15, 17.94, and 39.28% of the applied dose, respectively. About 20% of the radiolabel in the water-soluble fraction from all tissues studied was not converted to ether-soluble material following hydrolysis by Emulsin.

The ether-soluble 2,4-D-*1*-^{14}C metabolites were separated by descending PC in solvent system VIII and the water-soluble metabolites, following treatment with Emulsin, were separated by TLC in solvent system III. The compositions of the ether-soluble and the water-soluble fractions are presented in Tables I and II.

The aglycons from the water-soluble extracts were separated into seven fractions and were arbitrarily referred to as Ag_0 through Ag_7 (Table I). These designated symbols do not necessarily correspond with previous designated symbols (Feung et al., 1973). The fractions Ag_0, Ag_2, Ag_3, Ag_4, Ag_5, and Ag_6 are common to plants and callus tissue examined. The fraction Ag_1 was found only in soybean callus tissues and fraction Ag_7 was found only in soybean and corn plants.

The metabolites eluted from Ag_3 were identified by cochromatography with synthetic standards on two-dimensional TLC as 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D) and 5-hydroxy-2,4-dichlorophenoxyacetic acid (5-OH-2,4-D) in four solvent systems, I, III, IV, and V. The metabolites of Ag_4 and Ag_5 cochromatographed with synthetic standards 4-hydroxy-2,5 dichlorophenoxyacetic acid (4-OH-2,5-D) and 2,4-D, respectively, in two-dimensional TLC in the same four solvent systems. The metabolite Ag_7 cochromatographed with the ethyl ester of 2,4-D-*1*-^{14}C, which was recently identified from rice root callus tissue (Feung et al., 1976) and is probably an artifact of isolation. The remaining metabolites Ag_0, Ag_1, Ag_2, and Ag_6 are still unknown.

The recovery of free 2,4-D after enzymatic hydrolysis of the water-soluble extracts indicates that the carboxylic glycosides of 2,4-D are present not only in a tolerant corn plant but also in a susceptible soybean plant and callus (Andus, 1964; Klämbt, 1961; Hilton, 1966; Ojima and Gamborg, 1968; Feung et al., 1971, 1975, 1976). The carboxylic glycosides of 2,4-D (Ag_5) are the major metabolites in corn and soybean plants and the relative amounts of these ester glycosides are considerably greater than in soybean callus. The carboxylic glycosides were also the principal 2,4-D metabolites in corn and rice callus (Feung et al., 1975, 1976).

This suggests that these water-soluble conjugates of 2,4-D in a tolerant species may be important for the detoxification of the herbicide. It is possible that a small portion of the ether-soluble 2,4-D (Et_{11}) represents hydrolysis of the carboxylic glycoside of 2,4-D during work-up

Table II. Relative Percentage of the Ether-Soluble (at pH 2) 2,4-D-*1*-^{14}C Metabolites Isolated from Soybean Callus, Soybean Plant, and Corn Plant

metabolites		soybean callus % total in		soybean plant % total in		corn plant % total in	
R_f region[a]	designation	fraction	tissue	fraction	tissue	fraction	tissue
0.29–0.35	Et_1	1.18	0.50	4.28	3.14	trace	trace
0.36–0.38	Et_2 5-OH-2,4-D 4-OH-2,3-D 4-OH-2,5-D	3.10	1.48	4.77	3.50	15.25	7.08
0.42–0.46	Et_3 [b]	20.07	9.59	12.08	8.87	1.48	0.69
0.47–0.51	Et_4 [b]	3.12	1.49	2.64	1.94	0.54	0.25
0.52–0.54	Et_5 Asp-2,4-D	1.29	0.62	trace	trace	0.35	0.16
0.56–0.50	Et_6 Asp-2,4-D Glu-2,4-D[c]	23.34	11.15	5.47	4.02	0.61	0.28
0.61–0.63	Et_7	5.58	2.67	3.03	2.22	1.07	0.50
0.65–0.68	Et_8	4.19	2.00	2.17	1.59	1.34	0.62
0.69–0.73	Et_9	3.63	1.73	2.01	1.48	0.78	0.36
0.74–0.76	Et_{10} Ala-2,4-D[c] Val-2,4-D	8.51	4.07	1.86	1.37	1.86	0.86
0.79–0.85	Et_{11} Val-2,4-D 2,4-D[c]	24.93	11.91	59.47	43.55	63.80	29.60
0.88–0.92	Et_{12} Phe-2,4-D[c] Trp-2,4-D[c] Leu-2,4-D	1.06	0.51	2.22	1.63	12.92	5.60
total[d]		100.	47.72	100.	73.31	100.	46.00

[a] Descending Whatman No. 1 paper chromatography employing solvent system: 1-butanol–95% ethanol–3 N ammonium hydroxide (4:1.36:5, v/v/v). [b] Evidence indicates these compounds are amino acid conjugates of hydroxylated 2,4-D metabolites primarily of glutamic acid and 4-OH-2,5-D. [c] Major product. [d] The residue contained 4.50, 4.20, and 4.90% of the applied ^{14}C dose in the soybean callus tissue, soybean plant, and corn plant, respectively.

procedures, but this is kept to a minimum by immediate ether extraction following acidification. Also the isolated ethyl ester of 2,4-D (Ag_7) presumably is derived from the carboxylic glycoside. It is possible that the true magnitude of the carboxylic pathway is best represented by a summation of Ag_5 and Ag_7. The corn plant contains a greater percentage 4-OH-2,3-D than the soybean plant or callus. A similar observation was made with corn endosperm callus (Feung, et al., 1975).

The aglycon metabolites of 2,4-D in soybean callus tissue are qualitatively similar to the metabolites found in the whole plants but their relative percentage composition varies. No metabolites were found in the whole plants that were not present in the callus except for Ag_7 which is probably an artifact of the workup rather than a true metabolite (Feung et al., 1976). One metabolite (Ag_1) was found in the callus and was not present in the plants.

A total of 12 chromatographically distinct fractions of the ether-soluble extracts (pH 2) were separated by descending paper chromatography (Figure 1). The metabolites are arbitrarily designated the symbol Et_1 through Et_{12} corresponding with increasing R_f values as shown in Table II. These 12 fractions were found common to all tissues examined.

Six ether-soluble metabolites found in all tissues studied had been previously identified in this laboratory in soybean callus tissue (Feung et al., 1973). They were again confirmed by two dimensional TLC to be 2,4-D-aspartic acid, 2,4-D-glutamic acid, 2,4-D-alanine, 2,4-D-valine, 2,4-D-phenylanine, and 2,4-D-tryptophan. The eluted unknown metabolites Et_2 were separated by TLC employing solvent system III into three components having R_f values of 0.38 (Et_{2_a}), 0.42 (Et_{2_b}), and 0.52 (Et_{2_c}). These three metabolites (Et_{2_a}, Et_{2_b}, Et_{2_c}) cochromatographed with synthetic standards on two-dimensional TLC in all solvent systems and thus are identified as free 4-OH-2,3-D, 4-OH-2,5-D and 5-OH-2,4-D, respectively. The metabolites Et_3 and Et_4 were completely hydrolyzed in 6 N HCl for 24 h at 85 °C, but were incompletely hydrolyzed at room temperature for a similar period to time. The resulting hydrolyzates were individually analyzed and compared with synthetic

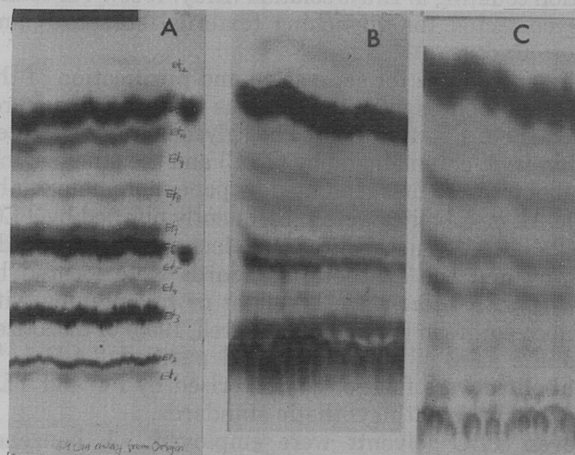

Figure 1. Radioautogram of paper chromatograms of ethyl ether extract of: (A) soybean cotyledon callus tissue; (B) soybean plant; and (C) corn plant, incubated with 2,4-D-*1*-^{14}C.

standards on two-dimensional TLC. The hydrolyzate of Et_3 from soybean callus consisted of a mixture of amino acids, primarily glutamic and aspartic acids, and hydroxylated metabolites of 2,4-D (4-OH-2,5-D, 95%; 4-OH-2,3-D, 3%; and 5-OH-2,4-D, 2%). The acid hydrolyzates of Et_4 contained glutamic acid and 4-OH-2,5-D. The metabolites of Et_3 and Et_4 were individually examined prior to hydrolysis, and no amino acids were detected. The remaining four unknown metabolites (Et_1, Et_7, Et_8, and Et_9) in soybean callus, soybean plant, and corn plant constitute 6.9, 8.4, and 1.5% of the total radioactivity in these tissues, respectively.

No qualitative differences in the ether-soluble extracts were found between callus and plant species studied, but considerable differences in the relative quantities of some of these metabolites were found as can be seen in Table II. These results indicated that 2,4-D is converted to ether-soluble products at a differential rate: soybean callus > corn plant > soybean plant. The formation of amino acid conjugates by soybean callus was much more extensive than in soybean or corn plants and may reflect the acti-

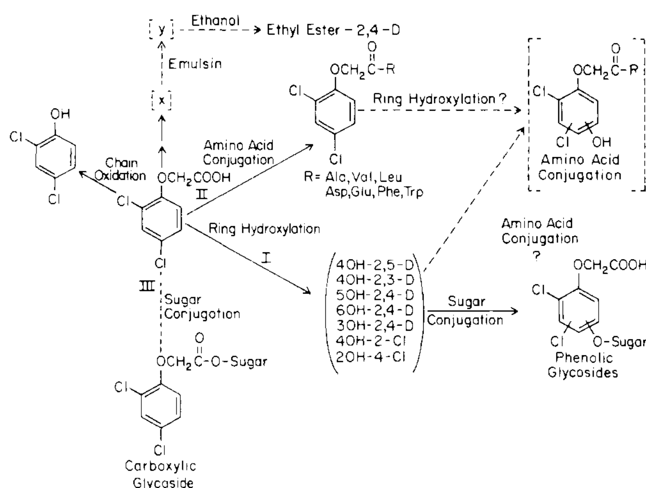

Figure 2. Scheme of metabolism of 2,4-D by plants.

vation of the inducible enzyme system involved in formation of amino acid conjugates. Chkanikov et al. (1976) reported amino acid conjugates as significant metabolites in beans.

Of considerable interest is the identification of free ring hydroxylated metabolites (Et_2) in all tissues and their higher relative composition in the whole plants. In our earlier callus tissue culture studies these metabolites (Et_2) were usually not observed though they were present in bean plants (Hamilton et al., 1971). Chkanikov et al. (1976) also reported free ring hydroxylated metabolites in wheat and bean. Their accumulation mainly as glucosides in the callus tissue cultures in fresh medium may be related to sugar pools in the tissue and possibly age of the tissue. There is increasing evidence that some plant tissue cultures do not form glycosides as readily as whole plants. The hydrolytic data suggest that Et_3 and Et_4 are amino acid conjugates of ring hydroxylated 2,4-D and are common to all plant tissues examined. The relatively high concentration of metabolites Et_1–Et_4 compared to previous studies (Feung et al., 1973, 1975, 1976) reflects different experimental conditions. In these experiments the 2,4-D was directly injected into the tissue growing on agar, creating a micro-environment in the tissue of a high dose of 2,4-D (5 μCi or 21.2 μg) relative to previous experiments where the 2,4-D was incubated with the callus tissue in solution shake cultures. Also, in this experiment incubations were conducted for 7 days which is longer than most previous experiments (2 days). These data suggest that the conditions of administration of the pesticide, the length as well as the method of incubation, age of tissue, and nutritional status may be quantitatively (if not qualitatively) significant factors in pesticide metabolism and these parameters need to be studied further. The method of injecting the 2,4-D-*1*-^{14}C directly into 4-week-old callus growing on agar used in these experiments is different than methods previously used, but was chosen to correlate with the techniques employed with the whole plants.

On the basis of the foregoing data, it seems quite likely that plant tissues, regardless of whether it is a plant callus or a whole plant, possess common metabolic pathways. Both plants and plant callus were able to metabolize 2,4-D-*1*-^{14}C by way of ring hydroxylation as well as conjugation of the carboxyl with amino acids and with sugars. Under these experimental conditions soybean callus tissues metabolize 2,4-D more readily than the plant. Qualitatively, the metabolites of 2,4-D appear to be nearly the same in callus and in the plants examined but the relative percentages of the metabolites do vary. Therefore, it is reasonable to use plant callus tissues as a tool for the identification of metabolites and for the determination of metabolic pathways.

Three general pathways of metabolism of 2,4-D-*1*-^{14}C are conceived (Figure 2). These three pathways seem to be operating in the examined plants and plant callus tissue. Pathway III predominates in corn plants, while pathways I and II predominate in soybean callus and soybean plants.

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