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Metabolism of 2,4-Dichlorophenoxyacetic Acid. VII. Comparison of Metabolites from Five Species of Plant Callus Tissue Cultures

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The metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) was investigated in carrot, jackbean, sunflower, tobacco, and corn callus tissue. Amino acid conjugates and hydroxylated metabolites were identified in all plant species although in varying amounts. After 8 days of incubation with 2,4-D water-soluble metabolites were formed in the callus tissue as follows: carrots (13.2%), jackbean (12.8%), sunflower (18.3%), tobacco (42.2%), and corn (64.6%). Five metabolites were identified as aglycones after treatment of these water-soluble fractions with β-glucosidase, three of which were present in all plants, 4-hydroxy-2,3-dichlorophenoxyacetic acid, 4-hydroxy-2,5-dichlorophenoxyacetic acid, and 2,4-D. Two tentatively identified aglycone metabolites, 3-hydroxy-2,4-dichlorophenoxyacetic acid (major) and 4-hydroxy-2-chlorophenoxyacetic acid (minor), were present only in corn. Glutamic acid was identified conjugated with 2,4-D in all tissues examined. The aspartic acid conjugate was present in corn, tobacco, and jackbean. Corn, the only monocot examined, was somewhat unusual since it possessed a high percentage of hydroxylated metabolites, including two not found in the other plant species.

Previous investigations (Feung et al., 1971, 1972, 1973b) have demonstrated that soybean callus tissue cultures readily metabolize 2,4-dichlorophenoxyacetic acid (2,4-D) to at least seven amino acid conjugates (Asp, Glu, Ala, Val, Leu, Phe, and Trp) and two ring hydroxylated metabolites which accounted for 97% of the soluble metabolites. The amino acid conjugates are biologically active and strongly stimulate plant cell elongation and division (Feung et al., 1974). On the other hand the two identified ring hydroxylated metabolites, 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D) and 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D), do not possess any growth stimulatory activity (Feung et al., 1974). 2,4-Dichlorophenoxyacetylaspartic acid (2,4-D-Asp) has also been detected in excised pea roots (Andreae and Good, 1957) and wheat coleoptile sections (Klämbt, 1961)

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and the two ring-hydroxylated metabolites were previously found in bean and other plants by Thomas et al. (1964), Hamilton et al. (1971), Montgomery et al. (1971), and Fleeker and Steen (1971).

Since the amino acid conjugates were major metabolites in soybean callus tissue and possess growth stimulatory activity it is essential to determine the generality of the presence of the amino acid conjugates in other plant species. Therefore, the metabolism of 2,4-D was investigated and compared in five additional callus tissue cultures, four dicots (carrots, jackbean, sunflower, and tobacco) and one monocot (corn).

EXPERIMENTAL SECTION

The plant callus tissues used in these studies were jackbean (Canavalia ensiformis) pod callus, sweet corn (Zea mays) endosperm callus, tobacco (Nicotiana tobacum) pith callus, carrot (Daucus carota var. sativa) pith callus, and sunflower (Helianthus annus) pith callus. All callus stock cultures were grown on a solidified agar medium (Miller, 1963) under continuous fluorescent light at 25° for

Table I. Relative Percentage of Water-Soluble Metabolites of 2,4-D Incubated with Five Species of Plant Callus Tissue for 8 Days[a]

Metabolites		Carrot, % total in		Jackbean, % total in		Sunflower, % total in		Tobacco, % total in		Corn, % total in	
R_f region[b]	Designation	Fraction	Tissue	Fraction	Tissue	Fraction	Tissue	Fraction	Tissue	Fraction	Tissue
0.37–0.41	Ag$_1$(4-OH-2,3-D, 4-OH-2,5-D)	52.5	6.9	53.6	6.9	45.1	8.3	41.3	17.4	48.3	31.7
0.42–0.46	Ag$_2$	24.4	3.2	20.3	2.6	22.4	4.1	19.6	8.3	30.5	19.4
0.47–0.51	Ag$_3$	5.2	0.7	2.8	0.4	4.6	0.8	4.7	2.0	0.8	0.5
0.54–0.58	Ag$_4$	3.2	0.4	2.5	0.3	2.7	0.5	4.5	1.9	1.9	1.2
0.59–0.63	Ag$_5$	0.7	0.1	2.5	0.3	4.1	0.8	5.4	2.3		
0.64–0.68	Ag$_6$									2.4	1.6
0.72–0.76	Ag$_7$	6.5	0.9	8.9	1.1	3.3	0.6	6.2	2.6		
0.84–0.89	Ag$_8$ (2,4-D)	7.5	1.0	9.4	1.2	17.8	3.2	18.3	7.7	16.1	10.2
	Total	100	13.2	100	12.8	100	18.3	100	42.2	100	64.6

[a] Derived from the water-soluble fraction after extraction with diethyl ether at pH 3 followed by (1) extraction into 1-butanol, (2) chromatography on a Bio-Gel P-2 column, (3) treatment with β-glucosidase, and (4) chromatography of the ether-soluble aglycones (Feung et al., 1973b). [b] Descending Whatman No. 1 paper chromatography; solvent system: 1-butanol–95% ethanol–3 N ammonium hydroxide (4:1:5, v/v/v).

Table II. Relative Percentage of Ether-Soluble Metabolites of 2,4-D Incubated with Five Species of Plant Callus Tissue for 8 Days

Metabolites		Carrot, % total in		Jackbean, % total in		Sunflower, % total in		Tobacco, % total in		Corn, % total in	
R_f region[a]	Designation	Fraction	Tissue	Fraction	Tissue	Fraction	Tissue	Fraction	Tissue	Fraction	Tissue
0.22–0.25	Et$_1$									7.6	1.9
0.29–0.31	Et$_2$									6.8	1.7
0.33–0.39	Et$_3$ (2,4-D-Asp)			1.4	1.2			1.5	0.8	6.3	1.6
0.40–0.43	Et$_4$ (2,4-D-Glu)	28.4	23.8	38.1	32.5	6.4	5.0	12.5	6.7	5.3	1.3
0.44–0.48	Et$_5$	0.9	0.8	1.3	1.1	0.9	0.7	3.3	1.8	12.5	3.2
0.51–0.57	Et$_6$	6.9	5.8	6.1	5.2	7.3	5.7	10.2	5.5	3.1	0.8
0.62–0.74	Et$_7$ (2,4-D)	61.8	51.7	53.1	45.4	65.9	51.7	64.1	34.3	48.2	12.5
0.86–0.92	Et$_8$	2.0	1.7			19.5	15.3	8.4	4.5	10.2	2.6
	Total	100	83.8	100	85.4	100	78.4	100	53.6	100	25.6

[a] Descending Whatman No. 1 paper chromatography; solvent system: 1-butanol–95% ethanol–3 N ammonium hydroxide (4:1:5, v/v/v).

6 weeks. Approximately 10 g of each species of callus tissues was aseptically and separately transferred to each 125-ml erlenmeyer flask containing 25 ml of autoclaved liquid nutrient medium (Miller, 1963) minus α-naphthaleneacetic acid (NAA) to which 2 μCi of 2,4-D-1-^{14}C (specific activity 2.44 μCi/μmol) was added. These callus tissues were grown at 25° with gentle shaking for 8 days.

Following 8 days incubation, the treated callus tissues were individually collected on filter paper in a Buchner funnel and briefly rinsed with cold distilled water. The rinsed tissues were stored in separate plastic bags at −20° prior to extraction.

The procedures for extractions, fractionation, isolation, and purification of 2,4-D metabolites were the same as previously described (Feung et al., 1973b).

RESULTS

The relative percentages of the water-soluble and ether-soluble (pH 3) metabolites of 2,4-D in the five plant tissue cultures are given in Tables I and II. The 80% ethanol insoluble residue of all callus tissues contained 3–5% of the applied radioactivity. The relative percentage of metabolites found in the water-soluble fraction is as follows: jackbean (12.8%), carrot (13.2%), and sunflower (18.3%) were relatively small, tobacco gave an intermediate value

(42.2%), and corn possessed a large percentage (64.6%) of water-soluble metabolites. Thus, the plants are species specific in the amounts of metabolites produced. It is interesting to note that the only monocot, corn, which is resistant to the biological effect of 2,4-D, rapidly metabolized 2,4-D to water-soluble metabolites.

The water-soluble metabolites, following treatment with β-glucosidase, and the ether-soluble metabolites were separated by descending paper chromatography on full sheets of Whatman No. 3MM paper in the solvent system 1-butanol–95% ethanol–3 N ammonium hydroxide (4:1:5, v/v/v) (Feung et al., 1973b). The aglycones from the water-soluble fraction were separated into six to eight fractions depending upon the species of callus and were arbitrarily designated the symbols Ag$_1$ through Ag$_8$ corresponding with R_f values of 0.37–0.41, 0.42–0.46, 0.47–0.51, 0.54–0.58, 0.59–0.63, 0.64–0.69, 0.72–0.76, and 0.84–0.89 as shown in Table I. The designated code Ag$_1$–Ag$_8$ does not necessarily correspond with previous designated symbols (Feung et al., 1973b). The fractions Ag$_1$, Ag$_2$, Ag$_3$, Ag$_4$, and Ag$_8$ are common to all callus tissue cultures examined. The metabolite Ag$_6$ was isolated only from corn callus, but metabolites Ag$_5$ and Ag$_7$ are common to the callus of the other four species.

The metabolites eluted from Ag$_1$ were separated into

Table III. Relative Concentrations of Metabolites of 2,4-D

Metabolite		Rel Concn[a]				
R_f region	Designation	Carrot	Jackbean	Sunflower	Tobacco	Corn
0.27	Ag_{1a} (4-OH-2,3-D)	10–20	10–20	10–20	10–20	>90
0.42	Ag_{1b} (4-OH-2,5-D)	80–90	80–90	80–90	80–90	<10
0.14	Ag_{2a} (4-OH-2,3-D)					<10
0.33	Ag_{2b} (4-OH-2,5-D)					<10
0.40	Ag_{2c} (3-OH-2,4-D)					80–90
0.46	Ag_{2d} (4-OH-2-Cl)					<10
0.0–0.002	Et_{6a}	89.6	94.7	90.5	96.2	
0.09–0.11	Et_{6b}	2.1	0	1.9	0	
0.57–0.60	Et_{6c}	8.3	5.3	7.6	3.8	

[a] Relative concentration within Ag_1, Ag_2, and Et_6.

two components, Ag_{1a} (R_f 0.27) and Ag_{1b} (R_f 0.42), on TLC in the solvent system diethyl ether–petroleum ether (60–70°)–formic acid (70:30:2, v/v/v) (solvent system IV) and cochromatographed with 4-OH-2,3-D and 4-OH-2,5-D, respectively, in three solvent systems (I–III, Feung et al., 1973b). The metabolite 4-OH-2,3-D (Ag_{1a}) was found as a minor component in carrot, jackbean, sunflower, and tobacco and as a major component in corn callus (Table III), whereas the metabolite 4-OH-2,5-D (Ag_{1b}) was found as a major component in carrot, jackbean, sunflower, and tobacco, and as a minor component in the corn callus. A single metabolite was obtained from the Ag_8 fraction of all five callus species which cochromatographed with 2,4-D on TLC in three solvent systems (I–III).

The 2,4-D (Ag_8) which is present in significant amounts in all tissues following treatment of the water-soluble fraction with β-glucosidase (Emulsin) may have arisen from hydrolysis of a 2,4-D–glucose–ester which had been postulated by Ojima and Gamborg (1968). In soybean cotyledon callus Ag_8 was present only as a minor component (Feung et al., 1973b). The Ag_2 fraction of corn callus tissue cultures was different from the Ag_2 fraction of the other four species. Ag_2 from corn callus separated into four components on TLC in solvent system IV, Ag_{2a} (R_f 0.14), Ag_{2b} (R_f 0.33), Ag_{2c} (R_f 0.40), and Ag_{2d} (R_f 0.46) (Table III). The latter two components cochromatographed on TLC with 3-hydroxy-2,4-dichlorophenoxyacetic acid (3-OH-2,4-D) and 4-hydroxy-2-chlorophenoxyacetic acid (4-OH-2-Cl), respectively, in four solvent systems (I–IV). The Ag_2 fraction of the other four species of callus did not contain a metabolite that corresponded to either 3-OH-2,4-D or 4-hydroxy-2-chlorophenoxyacetic acid.

A total of five to eight chromatographically distinct fractions were separated in the ether-soluble extracts. The metabolites are arbitrarily referred to as Et_1 through Et_8 having R_f values of 0.22–0.25, 0.29–0.31, 0.33–0.39, 0.40–0.43, 0.44–0.48, 0.51–0.57, 0.62–0.74, and 0.86–0.92, respectively, and the relative amounts of each fraction are listed in Table II. This designated code Et_1–Et_8 does not necessarily correspond with previous designated fractions (Feung et al., 1973b).

Metabolites Et_1 and Et_2 were detected only in the corn callus. Metabolites Et_3, Et_4, and Et_7 cochromatographed (on TLC in solvent systems I–III) and had identical mass spectra with standards 2,4-D-aspartic acid (2,4-D-Asp), 2,4-D-glutamic acid (2,4-D-Glu), and 2,4-D, respectively (Feung et al., 1973b). 2,4-D-Asp (Et_3) was not found in carrot or sunflower but was found as a common small component of the other plant species. Metabolites Et_4, Et_5, Et_6, and Et_7 were found as common components in all callus tissues. Metabolite Et_6 from all five callus species was purified on TLC in solvent system III. Et_6 separated into two to three chromatographically distinct fractions shown in Table III having R_f values of 0.00–0.04

(Et_{6a}), 0.09–0.11 (Et_{6b}), and 0.57–0.60 (Et_{6c}). The major component of Et_6, Et_{6a}, cochromatographed with 2,4-D-lysine (2,4-D-Lys), 2,4-D-histidine (2,4-D-His), and 2,4-D-arginine (2,4-D-Arg), on TLC and paper chromatography (PC) in four solvent systems (I, II, VI, and VII) (Feung et al., 1973b); however no definite identification was made since an insufficient sample was available for spectroscopic identification. These three amino acid conjugates have similar R_f values and cannot be resolved in our chromatographic solvent systems. The metabolites Et_1, Et_2, and Et_5 also remain unknown, presumably amino acid conjugates and await further analyses. Et_8 appears to be a neutral metabolite.

DISCUSSION

2,4-D is metabolized by the five plant callus species to water- and ether-soluble metabolites, and a small portion (2.5%) is found in the insoluble callus residue. The high recovery of labeled metabolites indicates that very little decarboxylation of 2,4-D-1-^{14}C takes place in all callus tissues examined. Five aglycones have been identified following β-glucosidase treatment of the water-soluble extracts of which 2,4-D, 4-OH-2,3-D, and 4-OH-2,5-D were found in all callus tissue. 4-Hydroxy-2-chlorophenoxyacetic acid was found in the corn callus tissue only along with the unusual metabolite 3-OH-2,4-D. The recovery of 2,4-D from the water-soluble extracts may indicate the presence of glucose or sugar esters (Klämbt, 1961). The Ag_1 fraction which constituted a major component ranged from 45.1 to 53.5% in all callus tissue and was found to be a mixture of 4-OH-2,3-D and 4-OH-2,5-D. The hydroxylation pattern seemed unique in corn. The component 4-OH-2,3-D was a major metabolite in corn and was a minor metabolite in the other four species, whereas 4-OH-2,5-D was a minor metabolite in corn and was a major metabolite in the other four species. The metabolite 3-OH-2,4-D which constitutes a relatively major component (80–90% of Ag_2) was found only in the corn tissue. This hydroxylated metabolite (3-OH-2,4-D) was shown to be biologically active in stimulating the elongation of *Avena* coleoptile sections (Feung et al., 1971).

Two ether-soluble metabolites were identified as 2,4-D-Glu and 2,4-D-Asp. The former conjugate was found common to all callus tissue examined. These amino acid conjugates are biologically active and stimulate the growth of soybean cotyledon callus and the elongation of *Avena* coleoptile (Feung et al., 1974).

On the basis of the foregoing results, it seems quite likely that callus tissue, regardless of the species of plant, possesses common metabolic pathways. All callus seemed to be able to metabolize 2,4-D by way of a ring hydroxylation as well as conjugation with amino acids and to possible glucose or other sugars. Although the metabolites of
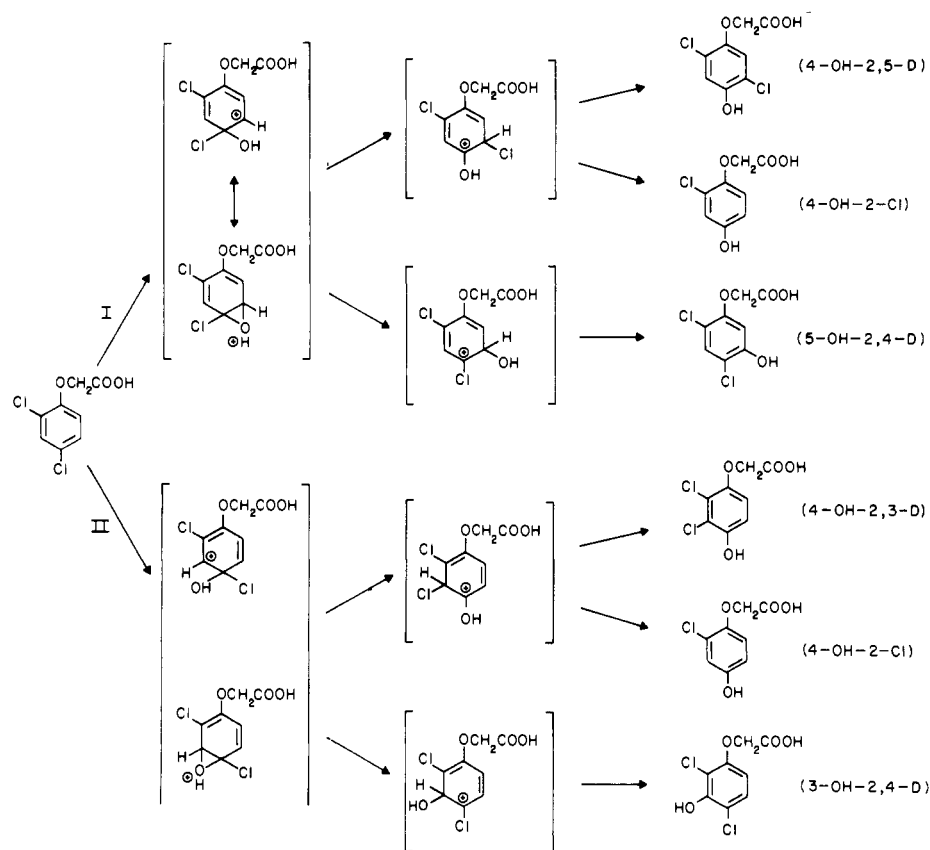
Figure 1. Proposed mechanism for biological hydroxylation of the aromatic ring of 2,4-D.

2,4-D appear to be nearly the same in most plant callus examined the relative percentages of some of these metabolites do vary considerably between plant species. Corn, the only monocot examined, seems to be somewhat different since it possessed an unusually high percentage of aglycones (mainly 4-OH-2,3-D) and contained metabolites not detected in the other plants, e.g. 3-OH-2,4-D and 4-OH-2-Cl.

Since the biological oxidation of 2,4-D in the plants examined involved primarily the C-3, -4, and -5 positions, it suggests initally the enzymatic formation of a C-3,4 or C-4,5 epoxide intermediate followed by a possible NIH shift of the C-4 chloro group (Guroff et al., 1967). Figure 1 shows a proposed theoretical schematic mechanism for the biological hydroxylation of 2,4-D. Two general pathways are conceived. Pathway I, involving a C-4,5 epoxide, could give at least three possible new products, 4-OH-2,5-D, 4-OH-2-Cl, and 5-OH-2,4-D. Pathway II involving a C-3,4 epoxide also could give at least three new products, 4-OH-2,3-D, 4-OH-2-Cl, and 3-OH-2,4-D. Both pathways seem to be operating in all callus tissues; however, pathway I appears to predominate in the four dicot plant tissue cultures examined. On the other hand, pathway II is predominent in corn since 4-OH-2,3-D is the major metabolite and all the theoretical products of pathway II have been found only in corn. This suggests that corn possesses a significant alternative pathway of metabolism and may account for its resistance to treatment with herbicidal quantities of 2,4-D. In contrast, Hagin et al. (1970) have shown that three other grasses, timothy, bromegrass, and orchardgrass, metabolize 2,4-D to 2,4-dichlorophenoxypropionic acid, which is biologically inactive, and is assumed to be a major detoxification mechanism of these grasses.

Additional experiments are needed to determine if different types of calluses from the same plant give similar results and whether the data obtained from callus can be extrapolated to the whole plant.

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