Metabolism of 2,4-Dichlorophenoxyacetic Acid by Soybean Cotyledon Callus Tissue Cultures

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The major water soluble metabolites in soybean callus tissue after 2,4-dichlorophenoxyacetic acid (2,4-D) treatment are ring hydroxylated glycosides of 2,4-D. 4-Hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D), the major aglycone, 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D), an intermediate aglycone, and two minor unknown aglycones were detected after emulsin treatment. The conjugation of labeled 2,4-D with glutamic acid to form a major ether soluble metabolite was observed. The ether soluble fraction also contained two other unidentified metabolites of 2,4-D and free 2,4-D. Further, *Avena* section test bioassays of the ring hydroxylated 2,4-D derivatives and 2,4-D glutamic acid indicated that only 3-hydroxy-2,4-dichlorophenoxyacetic acid (3-OH-2,4-D) and the 2,4-D glutamic acid metabolite were active.

The metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) by plants has been studied extensively and has been reviewed by Crosby (1964), Thomas and Loughman (1964), Thomas, et al. (1963, 1964), and Tutass (1967). Conversely, its metabolism in plant tissue cultures has received little attention. Ojima and Gamborg (1968) reported that the highest yields of cell suspension cultures derived from soybean roots were obtained when 2,4-D was incorporated into the base medium consisting of mineral salts, B vitamins, and sucrose. Glutamic acid in the culture medium also enhanced 2,4-D uptake. In addition, these workers demonstrated that 2,4-D was the major ether soluble constituent extracted from the cultures, while the glucose ester of 2,4-D was suggested to be present in the water soluble fraction.

This paper describes investigations concerning the metabolism of 2,4-D in callus tissue from soybean cotyledons. Although this specific callus tissue normally requires a cytokinin (kinetin or analog) and an auxin (indole-3-acetic acid or α-naphthalene acetic acid) for growth and maintenance in culture, 2,4-D will stimulate cell division and enlargement in the absence and presence of a conventional cytokinin (Witham, 1968). It was of interest, therefore, to investigate the metabolism of 2,4-D after its application to the callus tissue.

MATERIALS AND METHODS

The tissue used was originally derived from cotyledons of soybean [*Glycine max* (L.) Merrill, var. Acme]. Stock cultures have been maintained on an agar-sucrose-salt-vitamin basal medium (Miller, 1963) containing 0.5 mg/l. of kinetin and 2.0 mg/l. of α-naphthalene acetic acid. Further, the stock cultures have been routinely subcultured aseptically on the same medium once a month without observable changes in growth patterns or physical properties.

Four-week-old callus clumps were transferred to a sterile Petri dish and sliced with a sterile scalpel. Approximately 10 g of the callus pieces were placed into each 125-ml flask containing autoclaved nutrient medium (Miller, 1963) and one microcurie (1 μCi) of 2,4-D-1-^{14}C (specific activity 2.44 mCi/mM) in place of NAA. The labeled 2,4-D was dissolved in a few microliters of 95% ethanol and added to the auto-claved liquid nutrient medium with the aid of a microliter syringe.

After 2 days incubation at room temperature, the labeled 2,4-D treated callus tissues were surface-rinsed rapidly with cold distilled water on filter paper in a Buchner funnel. The rinsed solution removed by suction was saved for determination of the labeled 2,4-D remaining in the medium. The surface-rinsed tissues were weighed and stored in plastic bags at −20° C.

The frozen callus was thoroughly ground with a mortar and pestle to which equal volumes of 95% ethanol had been added. The homogenate was filtered in a Buchner funnel with suction, and the residue was rinsed repeatedly with 80% ethanol until it became grayish in color. The remaining procedures of extraction and fractionation were the same as those previously described (Hamilton, et al., 1971). The ethanol fraction was concentrated and the remaining aqueous concentrate (pH 3.0) was extracted three times with diethyl ether. The ether soluble acids (98% of ^{14}C) were partitioned into 5% NaHCO₃ and following acidification (pH 3.0) were reextracted into ether. The aqueous fraction remaining after the initial ether extraction was washed with *n*-butanol three times and the residue (in water) was run on a Biogel P-2 column. The water-soluble radioactive fractions were incubated with emulsin, and the aglycones extracted into diethyl ether following acidification.

The radioactivity in each fraction was measured by liquid scintillation counting (PPO, 5 g; POPOP, 0.1 g; Triton X-100, 333 ml; toluene, 667 ml). The residue was combusted by the oxygen flask method of Kalberer and Rutschman (1961) prior to liquid scintillation counting in Brays solution (Bray, 1960). All counts were corrected for quenching (external standard) and background.

The final extracts contained aglycones derived from the water-soluble fraction by emulsin treatment, and from the ether-soluble fraction containing free labeled 2,4-D and three labeled 2,4-D metabolites. The extracts and synthetic standards were spotted on Whatman No. 1 paper and developed by ascending chromatography in: isopropyl alcohol/ammonia/water (8:1:1, solvent 4), ethanol/ammonia/H₂O (80:4:16, solvent 5), or *n*-butanol/ethanol/3 N ammonia (4:1:5, solvent 6). Metabolites were also detected by thin-layer chromatography (tlc) on activated 0.25 mm silica gel (Adsorbosil-2; Applied Science Laboratories) plates which were developed with anhydrous diethyl ether/petroleum ether (Benzine, boiling range 37.0 ∼ 46.2° C)/formic acid (50:50:2,

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Soybean Callus Tissue
(Incubated in 2,4-D-1-^{14}C, 2 μCi = 4,400,000 dpm)

Freeze
Grind in 95% Ethanol
Filter and wash with 80% ethanol

Medium remaining   Residue   Ethanol fraction
(455,000 dpm)   (252,232 dpm)   (3,492,600 dpm)

Concentrate
Adjust pH to 3.0 with
3 N H$_3$PO$_4$
Extract four times with diethylether

Water fraction   Ether fraction
(685,450 dpm)   (2,797,000 dpm)

Extract four times with   Extract four times with
n-butanol   5% NaHCO$_3$

Aqueous   Butanol fraction   Ether fraction   NaHCO$_3$ fraction
(32,600 dpm)   (658,800 dpm)   (24,700 dpm)   (2,697,310 dpm)

Concentrate   Adjust pH to 3 with
Biogel P-2 column   3 N H$_3$PO$_4$
(1.5 × 24 cm)   Extract four times with
Elute with distilled   diethylether
water

Not Eluted   2,4-D metabolites   Ether fraction   NaHCO$_3$ fraction
                 (670,670 dpm)   (2,620,400 dpm/5.7 ml)   (41,000 dpm)

Incubate in emulsin
Adjust pH to 3 with 3 N H$_3$PO$_4$
Extract four times with diethylether

Aqueous   Aglycones
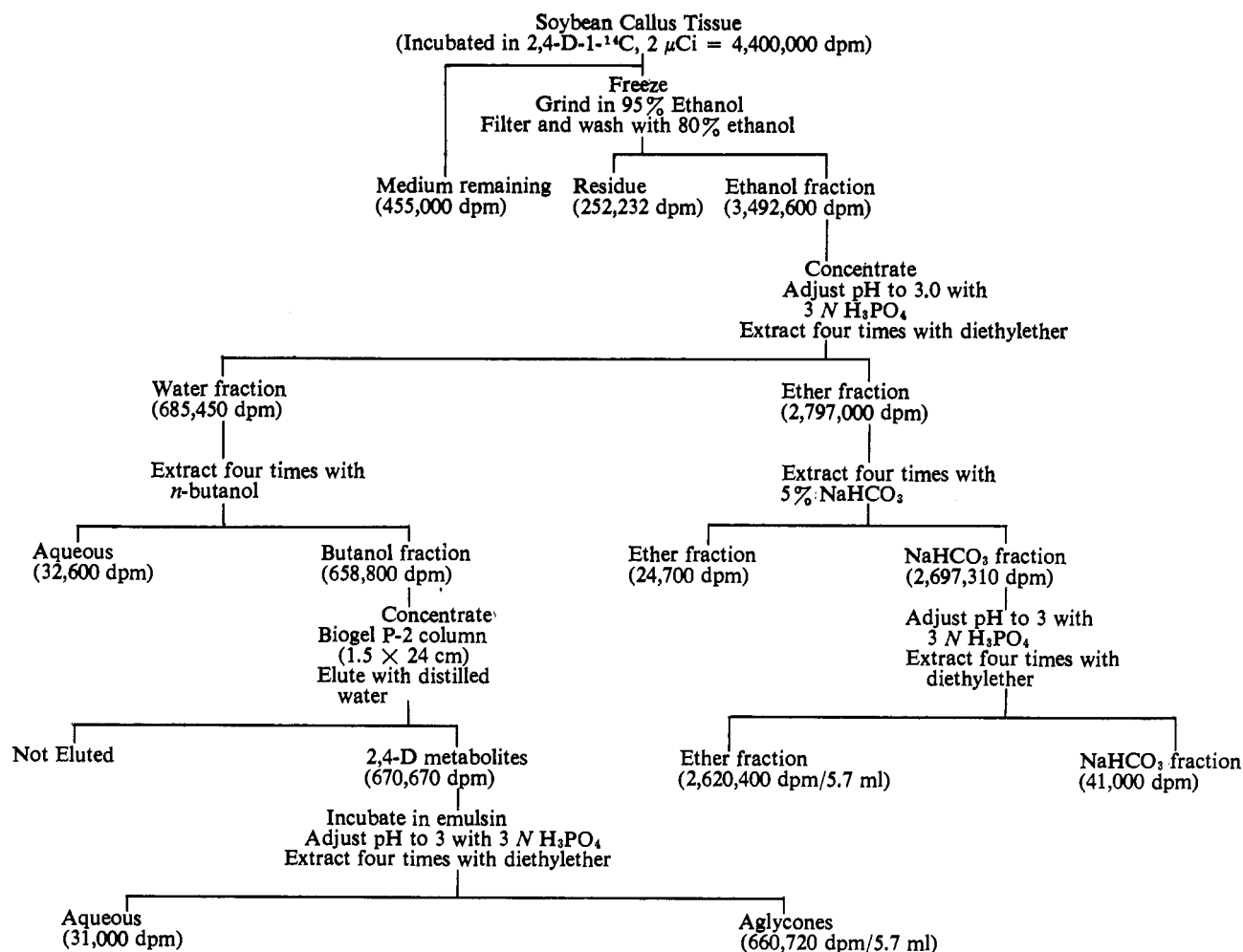(31,000 dpm)   (660,720 dpm/5.7 ml)

Figure 1. The extraction procedure and radioactivity in various fractions from soybean cotyledon callus after 48 hr incubation in 2,-4-D-1-^{14}C

solvent 1), benzene/dioxane/acetic acid (90:25:4, solvent 7), and solvent 4. The aglycones and labeled 2,4-D metabolites were located either by scanning with a radiochromatogram scanner or by autoradiography.

The hydroxylated 2,4-derivatives used in the cochromatography studies were either synthesized in this laboratory (Hamilton, et al., 1971) (4-OH-2,5-D and 4-OH-2,3-D) or were obtained from D. Woodcock, University of Bristol (3-OH-2,4-D, 5-OH-2,4-D, and 6-OH-2,4-D). The synthetic standards were identified and located by spraying with fresh diazotized sulfanilic acid (Hamilton, et al., 1971).

Purification of the aglycones was achieved by preparative descending paper chromatography on full sheets of Whatman 3 MM paper developed in solvent 4. Those aglycones located by autoradiography were eluted with 50% ethanol. The eluted aglycone concentrates were again streaked across a full sheet of Whatman No. 1 paper (45.5 cm) and developed by descending chromatography in solvent 6. The labeled aglycones were again eluted with 50% ethanol, concentrated, and taken up with 95% ethanol.

The identification of purified aglycones was accomplished by two-dimensional cochromatography. The purified aglycones were spotted along with the synthetic standards on tlc plates and developed either in solvent 1 or solvent 7 in the first dimension and in solvent 4 in the second dimension. The labeled aglycones were located by autoradiography and the standards were located by spraying with fresh diazotized sulfanilic acid.

Initial purification of labeled 2,4-D metabolites (fractionated from the ether soluble fraction) was performed in the same manner as for the aglycones. The eluted labeled 2,4-D metabolites were further purified by descending two-dimensional chromatography on Whatman No. 1 paper using 80% phenol in the first dimension and solvent 6 in the second. The labeled 2,4-D metabolites were eluted with 50% ethanol, concentrated, and taken up with 95% ethanol.

The nature of the major ether soluble labeled 2,4-D metabolites was first determined by chromatography of acid hydrolyzates (6 N HCl at 65° C for 24 hr) using 2,4-D-1-^{14}C standards and amino acid standards. The chromatograms were examined by autoradiography and then sprayed with ninhydrin reagent. The amino acids before and after acid hydrolysis were also determined using an amino acid analyzer (Technicon).

The synthetic 2,4-dichlorophenoxyacetyl glutamic acid (2,4-D glutamate) used as a standard was made from 2,4-dichlorophenoxyacetyl chloride (2,4-D-Cl) and glutamic acid by the method of Wood and Fontaine (1952). 2,4-D-Cl was prepared in a manner analogous to that described by Freed (1946) by reacting 5 g 2,4-D with 2.5 mg thionyl chloride. The 2,4-D-Cl (3.5 g) was crystallized as white needle-like crystals. 2,4-D glutamate was synthesized by reacting 0.014 mole 2,4-D-Cl with 0.014 mole L-glutamic acid in 1 N NaOH (43 ml) and 75 ml water at 5 to 10° C. The 2,4-D glutamate was crystallized from water as white crystals with a yield of 1.68 g from 3.5 g of 2,4-D-Cl and a melting

Table I. The R_f Values of the Isolated Aglycone Metabolites of 2,4-D

	$R_f{}^a$				
	Silica Gel		Paper		
Derivative	1	7	4	5	6
Ag₁	0.33	0.18	0.15	0.33	0.28
Ag₂	0.38	0.25	0.17	0.35	0.30
Ag₃	0.43	0.34	0.25	0.40	0.31
Ag₄	0.49	0.40	0.39	0.50	0.62

[a] The solvents were: (1) Anhydrous diethyl ether/petroleum ether/ formic acid (50:50:2). (7) Benzene/dioxane/acetic acid (90:25:4). (4) Isopropyl alcohol/ammonia/water (8:1:1). (5) Ethanol/ammonia/ water (80:4:16). (6) n-Butanol/ethanol/ammonia (4:1:5).

point of 178-179.5° C. The uv absorption spectrum exhibiting absorption maxima at 292, 284, 228, and 210 nm were identical with those of 2,4-D.

The 2,4-D glutamate and the isolated metabolite which was extracted and purified from the ether-soluble fraction were esterified with diazomethane (Schlenk and Gellerman, 1960). 2,4-D methyl glutamate was prepared by reacting 350 mg of 2,4-D glutamate with diazomethane (from 645 mg of diazald, N-methyl-N-nitroso-p-toluenesulfonamide). The 2,4-dichlorophenoxyacetylmethyl glutamate was crystallized from water as white cotton fiberlike crystals, with a melting point of 69.5-70° C. The uv absorption spectrum was identical to that of 2,4-D and 2,4-D glutamate. The structure of 2,4-D-methyl glutamate and the esterified metabolites was characterized in an LKB model 9000 mass spectrometer using the direct sample inlet.

The synthetic standards 2,4-D, 3-hydroxy-2,4-dichlorophenoxyacetic acid (3-OH-2,4-D), 5-OH-2,4-D, 6-OH-2,4-D, and the labeled (isolated) major metabolite from the ether soluble fraction were bioassayed in the Avena coleoptile section test (Wang, et al., 1968). The Avena coleoptile sections were incubated in 2 ml of 2% sucrose and 0.01 M KH₂PO₄ buffer solution in 3 cm Petri dishes to which test compound had been added. After 24 hr incubation, the length of the sections was measured with a millimeter ruler.

RESULTS

The results of numerous experiments indicated that after 48 hr incubation, 89-96% of the applied labeled 2,4-D was taken up by the callus tissue. The radioactivity in the residue, water-soluble and ether-soluble extracts of the 48 hr 2,4-D treated soybean callus were measured. The residue radioactivity ranged from 3-6%, the water-soluble radioactivity ranged from 17-23%, and the ether-soluble radioactivity ranged from 70-76% of the total in the tissue. The data presented in Figure 1 represent those of one such experiment. Aglycones were obtained from the water-soluble fraction

following n-butanol extraction, Biogel P-2 elution, and emulsin (β-glucosidase, Nutritional Biochemical Co.) treatment. A major unknown and two minor unknown 2,4-D metabolites and free 2,4-D were detected in the ether-soluble fraction.

Aglycones were spotted along with the synthetic standards on thin-layer chromatography plates and Whatman No. 1 paper, and developed in solvents 1, 7, and 4 for tlc and solvents 4, 5, and 6 for paper chromatography. The aglycone (Ag) fraction separated into at least four spots (Ag₁, Ag₂, Ag₃, and Ag₄) on both tlc and paper chromatograms. The R_f values of Ag₁, Ag₂, Ag₃, and Ag₄ on tlc and paper chromatograms are listed in Table I. The R_f values of the synthetic standards on paper chromatograms developed in solvent 6 were: 0.30 (3-OH-2,4-D), 0.29 (5-OH-2,4-D), 0.30 (4-OH-2,5-D), 0.60 (6-OH-2,4-D), and on tlc with solvent 7 were: 0.26 (3-OH-2,4-D), 0.28 (4-OH-2,3-D), 0.33 (4-OH-2,5-D), 0.18 (5-OH-2,4-D), and 0.15 (6-OH-2,4-D), respectively. For the other solvents used, the R_f values of the standards were the same as those described previously (Hamilton, et al., 1971).

The relative proportions of the aglycones separated by paper chromatography (Table II) were: Ag₁ (minor, 7.8%), Ag₂ (intermediate, 33.4%), Ag₃ (major, 49.6%), and Ag₄ (minor, 9.2%). The four labeled aglycones listed above were identified by cochromatography with the known standards on two-dimensional tlc. Chromatograms were examined by autoradiography and sprayed with diazotized sulfanilic acid. The metabolites were found to be 4-OH-2,5-D (Ag₃ major), 4-OH-2,3-D (Ag₂ intermediate), possibly 5-OH-2,4-D, and an unknown (Ag₄ minor). Traces of labeled 2,4-D were found after hydrolysis of the water soluble extracts. This may indicate that the glucose ester of 2,4-D was also present in callus tissue in small amounts (Audus, 1964; Hilton, 1966; Ojima and Gamborg, 1968).

The labeled ether soluble 2,4-D metabolites were spotted, along with the synthetic standards on tlc plates and Whatman No. 1 paper and developed in solvent 4. The labeled metabolites could be separated into Et₁, Et₂, Et₃, and Et₄ having R_f values of 0.19, 0.41, 0.49, and 0.65, respectively. The R_f values on tlc plates developed in solvent 1 were: 0.13, 0.28, 0.40, and 0.44, respectively. The compound in Et₄ was identified as 2,4-D, Et₂ and Et₃ are still unknown, while Et₁ (major) was identified as the 2,4-D glutamic acid conjugate.

The relative proportions of the radioactive 2,4-D-metabolites in the ether soluble fraction as detected by paper chromatography (Table III) were Et₁ (major, 81.6%), Et₂ (9.6%), Et₃ (1.5%), and Et₄ (2,4-D, 7.3%). The major unknown labeled 2,4-D metabolite (Et₁) purified by two-dimensional chromatography was cochromatographed with the synthetic 2,4-dichlorophenoxyacetyl glutamic acid on paper and tlc in solvents 4, 5, 6, 1, and 7. The ¹⁴C-labeled metabolite (Et₁) exactly matched the synthetic 2,4-D glutamic acid (located by uv absorbance, 254 nm). The R_f values of the major un-

Table II. Relative Amounts of the Aglycone Metabolites of 2,4-D on the Chromatogram[a]

Metabolites	Radioactivity in Each Spot dpm	Percent Metabolite in 80 μl %	Calculated Radioactivity of Metabolite in 5.7 ml Fraction dpm	Percent Metabolite from Total 2,4-D Applied to Tissue %
Ag₁	756	7.8	53,865	1.2
Ag₂	3223	33.4	229,639	5.2
Ag₃	4789	49.6	341,216	7.8
Ag₄	886	9.2	63,129	1.4

[a] 80 μl of the aglycone fraction (660,720 dpm/5.7 ml) were spotted on paper which was developed by ascending chromatography in n-butanol/ethanol/ 3 N ammonia (4:1:5).

Table III. Relative Amounts of the Ether Soluble Metabolites of 2,4-D on the Chromatogram[a]

Metabolites	Radioactivity in Each Spot dpm	Percent Metabolite in 50 μl %	Calculated Radioactivity of Metabolite in 5.7 ml Fraction dpm	Percent Metabolite from Total 2,4-D Applied to Tissue %
Et₁	19,714	81.6	2,247,386	51.1
Et₂	2,329	9.6	265,506	6.0
Et₃	368	1.5	41,952	0.9
Et₄	1,762	7.3	200,868	4.6

[a] 50 μl of the final ether soluble fraction (2,620,400 dpm/5.7 ml) were spotted on paper which was developed by ascending chromatography in n-butanol/ethanol/3 N ammonia (4:1:5).

known 2,4-D metabolite (Et₁) on paper chromatograms developed in solvents 4, 5, and 6 were 0.25, 0.45, and 0.43, respectively. The R_f values in solvent 1 and 7 were 0.15 and 0.20, respectively.

The purified 2,4-D metabolite (Et₁) was completely hydrolyzed in 6 N HCl for 24 hr at 65° C, but incomplete hydrolysis was obtained at room temperature. The resulting hydrolyzates contained free labeled 2,4-D and three amino acids which were identified with the amino acid analyzer as glutamic acid (0.091 μM), aspartic acid (0.038 μM), and phenylalanine (0.039 μM). The 2,4-D content of these samples was 0.114 μM, as calculated on the basis of specific activity. Also, when the purified metabolite (Et₁) was examined prior to hydrolysis, no amino acids were detected. These data indicated that 2,4-D was conjugated with glutamic acid. However, the possibility that 2,4-D was conjugated with a glutamylphenylalanine and a glutamylaspartate cannot be excluded, since on a molar basis the free aspartic acid and phenylalanine added up to almost as much as glutamic acid. Conversely, the nearly equal amounts of phenylalanine and aspartic acid indicate that these two amino acids may have been derived from a contaminating phenylalanyl aspartic peptide.

The labeled 2,4-dichlorophenoxyacetylglutamic acid was also characterized in the mass spectrometer after it was esterified with diazomethane. The characteristic mass ion peaks of the synthetic 2,4-dichlorophenoxyacetylmethylglutamic acid are 377, 342, 318, 286, 282, 258, and 184, as shown in Figure 2. In addition to those peaks, there are peaks at m/e 356 and 239, and these peaks are presumed to be due to contamination of the ethyl ester. The base peak is a m/e 342, and a loss of one chlorine is indicated. Loss of

—C(=O)—OCH₃ gives rise to the ion fragment at m/e 318. Loss of two chlorines gives rise to a mass ion fragment at m/e 286. Loss of two —C(=O)—O—CH₃ gives rise to the peak at m/e 258.

The characteristic mass ion peaks of the isolated 2,4-D metabolite (Et₁) are also at 342 (base), 318, 258, and 184, as shown in Figure 2. Although the weak mass ion peak was not detected in the isolated metabolite, the fragmentation pattern was very similar to synthetic 2,4-D glutamic acid.

Bioassay experiments with Avena coleoptiles were performed to determine the biological activity of the synthetic compounds and the isolated 2,4-D-glutamic acid conjugate in inducing cell elongation. The experimental results summarized in Figure 3 demonstrate that 3-OH-2,4-D was the most active in inducing Avena coleoptile cell elongation. The optimal concentration of 3-OH-2,4-D, the labeled 2,4-D glutamate conjugate, and synthetic 2,4-D in inducing Avena coleoptile cell elongation is 10⁻⁵ M, 10⁻⁵ M, and 10⁻⁶ M, respectively. 3-OH-2,4-D in all concentrations tested in these experiments was highly effective in inducing Avena coleoptile cell elongation. Avena coleoptile epinastic curvatures were observed in the medium containing either synthetic
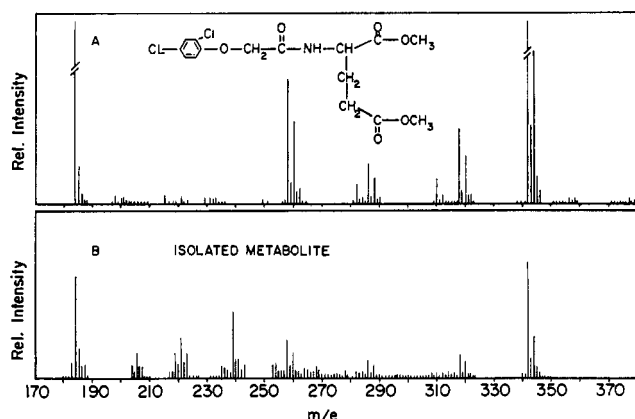

Figure 2. (A) Mass spectrum of the methyl ester of 2,4-dichlorophenoxyacetylglutamic acid. (B) Mass spectrum of the major ether soluble metabolite isolated from soybean callus. Mass spectra were obtained with a LKB Model 9000 mass spectrometer using the direct sample inlet
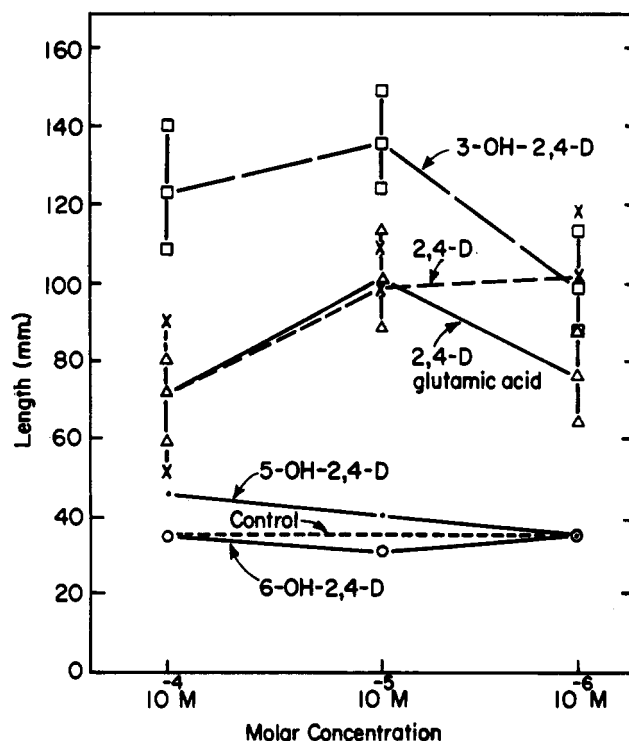

Figure 3. Effect of molar concentration on cell elongation of Avena coleoptile. Each point represents the mean of eight samples and the standard error is indicated

2,4-D or isolated 2,4-D glutamic acid conjugate at a concentration of 10^{-4} M, but were not observed when 3-OH-2,4-D was incorporated into the medium. Both 5-OH-2,4-D and 6-OH-2,4-D were inactive in these experiments. Previous results with 4-OH-2,5-D and 4-OH-2,3-D indicated they are inactive when bioassayed in the *Avena* coleoptile section test (Wang, *et al.*, 1968).

DISCUSSION

On the basis of the foregoing data, it is reasonable to conclude that over 50% of the applied labeled 2,4-D conjugated with glutamic acid in the soybean callus tissue (Table III). This conjugated compound was as active as 2,4-D in the *Avena* section test. Ojima and Gamborg's observation (1968) that glutamic acid in the medium enhanced 2,4-D uptake appears to be related to our observations. These workers probably did not detect this conjugate due to their use of an acidic solvent for paper chromatography.

In pea epicotyls, only small amounts of the applied 2,4-D were reported to be conjugated with aspartic acid (Andreae and Good, 1957). Several investigators (Andreae and Good, 1957; Klämbt, 1961; van Overbeek, 1964) have suggested that the process of conjugation of 2,4-D with an amino acid or protein provides the means for 2,4-D detoxification. However, extensive data on synthetic 2,4-D-L-amino acid conjugates by Krewson, *et al.* (1954) have shown such derivatives to be as active as auxins.

The nuclear hydroxylation of 2,4-D appears to be one of the essential processes involved in the metabolism of 2,4-D by the soybean callus. The soybean callus tissues converted over 20% of the applied labeled 2,4-D into acidic, water-soluble compounds. Four ether-soluble metabolites released from the water-soluble product after enzymatic hydrolysis were identified as 4-OH-2,5-D (major), 4-OH-2,3-D (intermediate), possibly 5-OH-2,4-D (minor), and one minor unknown, as indicated from the two-dimensional cochromatography with the synthetic standards. Further, the presence of at least six unknown metabolites in trace amounts were shown by autoradiography. Although 3-OH-2,4-D was very active in the *Avena* coleoptile section test, all the other hydroxylated 2,4-D derivatives were inactive. It is interesting to note that Tutass (1967), working with tobacco stem pith callus, reported that 3-OH-2,4-D, 5-OH-2,4-D, and 4-OH-2,5-D had growth stimulating properties. The growth promoting properties of the isolated metabolites on the soybean callus is under current investigation.

We have found the major water-soluble metabolites in the tissues 48 hr after 2,4-D treatment to be similar to the ring hydroxylated glycosides of 2,4-D previously reported (Hamilton, *et al.*, 1971) in bean tissues. A major 2,4-D ether-soluble metabolite has been identified as the 2,4-D glutamic acid conjugate.

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