Metabolism of 2,4-Dichlorophenoxyacetic Acid by Wheat Cell Suspension Cultures

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Wheat cells in suspension culture absorbed [acetic-2-^{14}C]2,4-dichlorophenoxyacetic acid ([acetic-2-^{14}C]2,4-D) rapidly from B5 nutrient medium. After 4 days of incubation, the distribution of radiolabel in the culture system reached a steady state. After 6 to 8 days, the major metabolism pathway for 2,4-D involved ring hydroxylation followed by conjugation with sugars since over 37% of the applied radiolabel was present in the cells as water-soluble/ether-insoluble metabolites. Following hydrolysis, extraction into ether, and separation by TLC, 4-hydroxy-2,5-dichlorophenoxyacetic acid was identified as the major aglycon present. Lesser amounts of 4-hydroxy-2,3-dichlorophenoxyacetic acid, 4-hydroxy-2-chlorophenoxyacetic acid, and 2,4-D were detected. Ether-soluble amino acid conjugates and free 2,4-D present in the cells after 6 to 8 days represented only 17 and 13%, respectively, of the applied radiolabel. A considerable amount (12%) of the applied radiolabel was bound to insoluble cellular tissue while 10% was present in the extracellular medium. By difference, apparently 9.4% was lost from the system as a volatile metabolite. The results of this model study are compared with those reported for other plants in tissue culture and support the hypothesis that the resistance of some plants to the herbicidal action of 2,4-D is related to their species specific ability to accomplish detoxification by conversion to water-soluble metabolites.

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has consistently been one of the most widely used pesticides in modern agriculture. Despite numerous investigations, the metabolism and fate of 2,4-D in plants is not understood very well (Loos, 1975; Robertson and Kirkwood, 1970; Mumma and Hamilton, 1976). Early studies were directed toward elucidation of the mode of action of this plant growth regulator and were conducted largely on susceptible plant species. They revealed the occurrence of specific metabolic pathways. However, these limited studies resulted in the development of only a partial picture and sometimes confusing results (e.g., Steen et al., 1974; Hagin et al., 1970).

More recent studies, in which careful account is made for the fate of all of the 2,4-D applied to the system under study, have provided a more complete picture for the metabolism of 2,4-D in plants (Arjmand and Mumma, 1976; Mumma and Hamilton, 1976; Feung et al., 1971, 1972, 1973a,b, 1974, 1975, 1976; Chkanikov et al., 1976; Montgomery et al., 1971; Hamilton, et al., 1971; Ojima and Gamborg, 1968). Much of this work has been conducted in callus tissue culture systems, models that are ideally suited for total fate studies and detailed analysis of metabolism products. As a result, several distinct metabolic pathways for 2,4-D in plant tissue culture have been recognized. They involve side-chain degradation, conjugation with amino acids, conjugation with sugars (carboxylic glycosides), ring hydroxylation followed by conjugation with sugars, and conjugation of 2,4-D, 2,4-D metabolites, or 2,4-D side-chain fragments with insoluble cellular tissue (bound residue).

Our interest in 2,4-D metabolism is focused on understanding its fate in resistant plants that are treated commercially for weed control. Such information is needed before adequate residue methods can be developed and questions regarding the fate of 2,4-D in commercial foodstuffs can be resolved. Because 2,4-D is used extensively on small grain crops, the objective of this study was to determine the detailed metabolism and total fate of this herbicide in a representative model. The system chosen was wheat cell suspension culture derived from a well-characterized cell line. Several experiments which delineate the behavior of 2,4-D and some of its metabolites under the conditions used to extract, separate, and quantify them in wheat tissue culture are also reported.

EXPERIMENTAL SECTION

Materials. [acetic-2-^{14}C]2,4-D (4.2 mCi/mmol, of >98% radiochemical purity) was purchased from Mallinckrodt, St. Louis, Mo. Nonradioactive 2,4-D purchased from Eastman Chemical Co., Rochester, N.Y., was recrystallized from benzene. Authentic samples of 2-hydroxy-4-chlorophenoxyacetic acid (2-HO-4-CPA), 4-hydroxy-2-chlorophenoxyacetic acid (4-HO-2-CPA), 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-HO-2,3-D), 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-HO-2,5-D), 5-hydroxy-2,4-dichlorophenoxyacetic acid (5-HO-2,4-D), and 6-hydroxy-2,4-dichlorophenoxyacetic acid (6-HO-2,4-D) were obtained through the courtesy of Dr. J. R. Fleeker, Department of Biochemistry, North Dakota State University, and Dr. R. C. Steen, Chemagro Agricultural Division, Mobay Chemical Corp., Fort Collins, Col. All solvents were ACS reagent grade, distilled in a glass apparatus before use. Wheat callus tissue derived from *Triticum monococcum* L. was originally obtained from Dr. O. L. Gamborg, Prairie Regional Laboratory, Saskatoon Sask., Canada.

Treatment of Suspension Cell Cultures with [acetic-2-^{14}C]2,4-D. Wheat callus tissue was cultured as individual cells or small aggregates of cells in B5 medium containing nonradioactive 2,4-D (2.25 μM) according to procedures outlined by Gamborg and Eveleigh (1968) and Oleson et al. (1974a,b). After about 13 days of growth, the medium from an individual culture, 250 mL in a 1-L De long flask, was removed by aspiration through a sterile, sintered glass bubbler tube. Sterile B5 medium (250 mL) containing 5.57 × 10^{-7} mol of [acetic-2-^{14}C]2,4-D (1.38 mCi/mmol) was added to the cells (about 5 g wt weight) remaining in the flask. A small portion of the B5 medium was removed for assay of the total radiolabel applied to the cells before each radioactive culture was incubated in the dark at 25 °C on an orbital shaker (110 rpm) for the desired period of time.

General Methods and Procedures. Following incubation, the radiolabel present in each culture was separated into several characteristic fractions by the procedure outlined in Figure 1, a modification of those developed by Feung et al. (1971, 1972, 1973b) and Hamilton et al. (1971).

The volume of each liquid fraction was measured, duplicate 1.0-mL samples were withdrawn, and the total radiolabel present was measured by liquid scintillation counting in Insta-gel (Packard Instrument Co., Downers Grove, Ill.). Counting efficiency for each sample was determined by the automatic external standard channels ratio method. All samples possessing activity above 1000 cpm/mL were measured at 1% and those below at 3% standard error (95% confidence limit). The radiolabel present in lyophilized samples of either the intact filtered cells or the tissue residue remaining after extraction was determined by liquid scintillation counting after combustion to $^{14}CO_2$ in a Packard Model 306 Tri-Carb sample oxidizer.

Cells were harvested by vacuum filtration on a coarse sintered disc funnel and surface rinsed with a total of 50 mL of cold water in three portions. They were transferred quantitatively to a blender cup using 60 mL of 95% ethanol and homogenized in a high-speed blender. The ethanol extracts were decanted through a coarse sintered disc funnel. The remaining insoluble tissue was homogenized in a mixture of 55 mL of 95% ethanol plus 5 mL of 0.10 N $NaHCO_3$ and filtered. The blender cup and the insoluble tissue residue were rinsed three times with 5-mL portions of 95% ethanol to complete the transfer. The combined extracts were concentrated to 1–3 mL of an aqueous liquor on a rotary vacuum evaporator. This was transferred to a 50-mL graduated cylinder, modified to accept a Teflon lined screw cap, using five rinsings of 0.10 N $NaHCO_3$ totalling 10 mL. The alkaline solution was acidified to pH ≤ 1 with 1.0 N H_2SO_4, diluted to 25.0 mL with distilled water, and treated with 7.0 g of NaCl. The resulting solution was extracted three times with 15-mL portions of ethyl ether without delay. The combined ethereal extracts were dried over anhydrous Na_2SO_4, evaporated just to dryness under a stream of dry nitrogen, and taken up in 1.0 mL of methanol for TLC analysis. The aqueous fraction was refluxed for 15–20 h in either 0.10 N H_2SO_4 or NaOH. The hydrolyzate was extracted three times with 15-mL portions of ethyl ether (alkaline hydrolyzate acidified to pH ≤ 1 with concentrated H_2SO_4 before extraction). The combined ethereal extracts were dried, evaporated, and taken up in methanol for two-dimensional TLC analysis.

For each radioactive culture, the extracellular medium and rinsings obtained by filtration of the cells were combined. After the total volume was determined, a 50-mL portion was acidified to pH ≤ 1 with concentrated H_2SO_4 and extracted three times with 15-mL portions of ethyl ether. Each extraction was followed by centrifugation to break the emulsion that formed.

In both 2- and 8-day incubation experiments, harvested cells were split into two equal parts by weight. One half was treated as shown in Figure 1; the other was lyophilized, weighed, and assayed for radiolabel content.

The stability of the water-soluble, radiolabeled compounds present in the concentrated ethanol extract toward aqueous acid (pH ≤ 1) was examined by repetitive extraction of a portion of the combined ethanol extracts from a 4-day incubation. The time required from acidification through completion of the first extraction (three times with 15-mL portions of ethyl ether) was 5 min. Extraction of the aqueous fraction was repeated at 0.5, 1, 2, 4, and 8 h after acidification. The total radiolabel present in each phase after each extraction was assayed by liquid scintillation counting.

Incubation of [*acetic*-2-^{14}C]2,4-D in Extracellular Medium. The extracellular B5 medium of a 14-day wheat
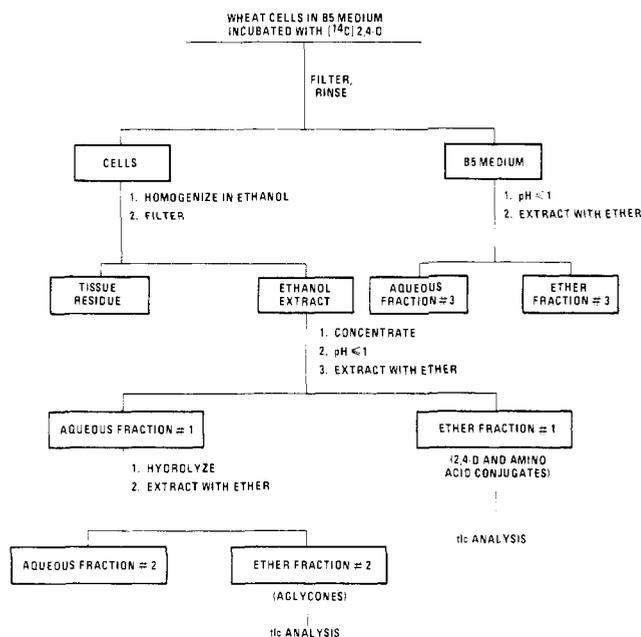

Figure 1. Flow diagram of the separation procedure used to isolate [^{14}C]2,4-D and its radiolabeled metabolites from wheat cell suspension culture.

cell suspension culture that had been grown with non-radioactive 2,4-D was separated from cells by filtration through Miracloth. The filtrate was clarified by centrifugation at 10 000g for 15 min before duplicate portions (100 mL) of the supernatant solution were treated with 0.20 mL of a 1.11 mM solution of [^{14}C]2,4-D (sp act. 1.38 mCi/mmol) in ethanol. One portion was used as an unincubated control and the other was sterilized by filtration through a membrane filter (0.20 μm porosity) into a sterile De long flask. Aliquots were withdrawn from both mixtures so that the total radiolabel applied could be assayed. The sterile mixture was incubated for 4 days under conditions identical with those used for cell cultures. Triplicate 25-mL portions of both the 4-day incubated medium and the unincubated control medium were extracted and assayed for radiolabel by the procedure used for the B5 medium (Figure 1).

Thin-Layer Chromatography. Quantum Industries LQDF (20 cm × 5 cm) and LQF (20 cm × 20 cm) silica gel thin-layer plates were used for one- and two-dimensional analyses, respectively. Plates were spotted with 10–40 μL of the radioactive solutions to be analyzed, overspotted with either 10 μg of 2,4-D in solution or 10 μL of a solution containing 1 μg/μL of each of the available authentic aglycon standards and developed to 15 cm. For two-dimensional analysis, plates developed in the first dimension were removed from the tank, dried under a gentle flow of warm air, rotated 90°, and developed in a second solvent system. Solvent systems used routinely were: (I) ethyl ether–hexane–formic acid (70:30:2, v/v/v), (II) ethyl ether–hexane–formic acid (70:30:6, v/v/v), (III) benzene–dioxane–acetic acid (90:25:4, v/v/v), and (IV) benzene–dioxane–formic acid (90:25:4, v/v/v). Spots located by fluorescence quenching (254 nm), exposure to iodine vapor, or radioautography were scraped from the plates into counting vials and assayed for radiolabel content.

RESULTS AND DISCUSSION

The behavior of free (unmetabolized) 2,4-D in the separation scheme utilized in this work was determined in two separate experiments. In a triplicate, reagent spike

TABLE I. RADIOLABEL PRESENT IN WHEAT CELLS AS DETERMINED BY ALTERNATIVE PROCEDURES

| | RADIOLABEL FOUND AS A PERCENTAGE OF TOTAL APPLIED | | | |
| | EXTRACTION OF CELLS WITH ETHANOL | | | DIRECT COMBUSTION OF LYOPHILIZED CELLS[a] |
INCUBATION PERIOD (DAYS)	ETHANOL EXTRACT	TISSUE RESIDUE	TOTAL IN CELLS[b]	TOTAL IN CELLS
2	29.0	4.0	33.0	29.9
8[b]	66.1 ± 1.7	12.8 ± 3.8	78.9 ± 5.5	81.0 ± 2.0

[a] ALL COMBUSTION ANALYSES WERE PERFORMED IN DUPLICATE.

[b] DUPLICATE EXPERIMENT REPORTED AS MEAN ± AVERAGE DEVIATION.

experiment, solutions of [^{14}C]2,4-D in ethanol, corresponding to the ethanol extract of Figure 1, were concentrated, acidified and extracted with ethyl ether. Of the total radiolabel present, 97 ± 3.2% (mean ± standard deviation) partitioned into the ether fraction while 0.87 ± 0.10% remained in the aqueous fraction. If NaHCO$_3$ solution was not used to complete the transfer step after concentration, recoveries were lower and quite variable. In a zero-time control experiment, where wheat cells were treated with [^{14}C]2,4-D and then harvested after less than 5 min of incubation, 94% of the applied radiolabel was recovered in the B5 medium and rinsings. Essentially all of the radiolabel associated with the cells (5.3%) was ethanol soluble, of which 96 and 0.81% were recovered in ether and aqueous fractions no. 1, respectively, of Figure 1.

To insure the efficient extraction of free 2,4-D (pK_a of 2.73; Hunter and Faust, 1969) and the monobasic amino acid conjugates of 2,4-D (Mumma and Hamilton, 1976) into ether, the pH of each aqueous fraction in Figure 1 was adjusted to 1 or less. Under these conditions, some of the water-soluble metabolites of aqueous fraction no. 1 underwent slow hydrolysis. More specifically, 7.1, 5.1, 4.8, 3.6, and 3.1% of the total radiolabel originally present in the ethanol extract of a 4-day culture was removed by repetitive extraction with ethyl ether at 0.5, 1, 2, 4, and 8 h after acidification. Because the initial extraction could be completed in less than 5 min, the distribution of radiolabel between the aqueous and ether fractions in Figure 1 would not be affected significantly by such slow hydrolysis.

Figure 2 shows that the wheat cell suspension culture system is characterized by rapid absorption of radiolabeled 2,4-D from the B5 medium. After 6 to 8 days of incubation, further changes in the distribution of the radiolabel within the system were not observed. Over 80% of the applied radiolabel was present in the cells, while 10.4% was present in the extracellular medium. The percentages of the applied radiolabel present in the cells of 2- and 8-day incubations, determined either as the sum of the radiolabel present in the ethanol and tissue residue fractions or by combustion analysis of intact cells, were in very close agreement (Table I). The determination of the level of cellular radiolabel by alternative procedures verified the gradual decrease in material balance observed with increasing incubation period (Figure 2). During 8 days of incubation, 9.4% of the total radiolabel applied was lost from the system, apparently as a volatile metabolite. Although trapping experiments were not conducted with wheat cultures, numerous studies have demonstrated that degradation of side-chain labeled 2,4-D with evolution of $^{14}CO_2$ occurs in plants (Loos, 1975). Feung et al. (1975, 1976) have shown that decarboxylation is a significant metabolic pathway in rice root but not in other species of callus tissue culture. By comparison, wheat tissue culture appears to be intermediate in its ability to decarboxylate 2,4-D.
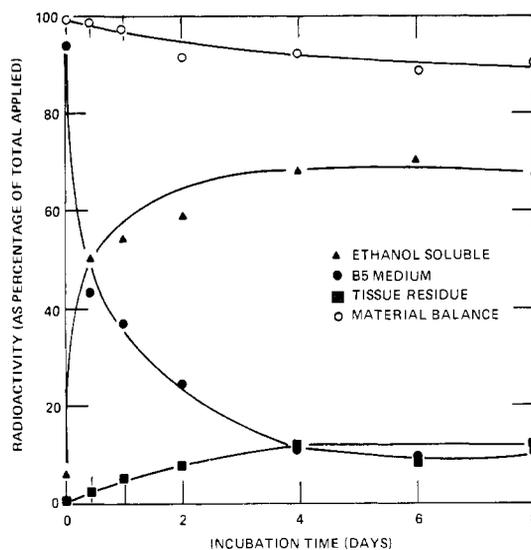

Figure 2. Distribution of the radiolabel applied as [^{14}C]2,4-D to wheat cell suspension cultures as a function of incubation time. The data at 1, 2, 4, and 8 days represent the averages of three, four, five, and four replications, respectively. Those at 0, 0.4, and 6 days represent single incubations.

The rapid decrease in the amount of radiolabel remaining in the wheat culture extracellular medium was accompanied by a rapid increase in the amount of water-soluble radiolabel present (from 10% after 1 day of incubation to 38% after 6 to 8 days). The presence of water-soluble radiolabel indicates that small but significant quantities of polar 2,4-D metabolites had accumulated in the extracellular medium. Extracellular enzymes are known to be produced during the growth of cultured plant cells (Gamborg and Eveleigh, 1968; Srivastava and van Huystee, 1973; Oleson et al., 1974a,b). The possibility that extracellular enzymes from wheat cells might decarboxylate or convert 2,4-D to polar metabolites was excluded by a control experiment in which [^{14}C]2,4-D was incubated with a sterile extracellular medium obtained from a 14-day culture. After 4 days, extraction of the acidified medium with ether resulted in the recovery of 100% of the radiolabel added. Because 2,4-D was stable in the extracellular medium, the observed buildup of water soluble metabolites must have been the result of either the excretion of intracellularly formed polar metabolites or slight lysis of cells during incubation. In either case, from 94 to 100% of the [^{14}C]2,4-D added to wheat cell suspension cultures was absorbed by the cells rather than the apparent 90% indicated by the amount of radiolabel remaining in the extracellular medium (Figure 2).

Frear and Swanson (1975) found significant amounts of a polar metabolite of cisanilide in the extracellular media of carrot and cotton suspension cell cultures, but did not determine the reason for such accumulation. Feung et al. (1976) found that the extracellular medium of rice root callus tissue contained a relatively large amount (25.2%)

TABLE II. RECOVERY OF LABELED AGLYCONES FOLLOWING HYDROLYSIS IN EITHER 0.10 N SULFURIC ACID OR SODIUM HYDROXIDE SOLUTION[a]

HYDROLYSIS CONDITIONS	PERCENT[b] RECOVERED AS		
	ETHER FRACTION NO. 2	AQUEOUS FRACTION NO. 2	TOTAL RECOVERY
0.10 N H2SO4	83.0 ± 2.47	8.96 ± 0.32	92.0 ± 2.5
0.10 N NaOH	81.2 ± 9.49	10.49 ± 0.93	91.7 ± 10.3

[a]DATA WAS OBTAINED FROM THE AQUEOUS FRACTION NO. 1 OF FOUR DIFFERENT 8-DAY CULTURES WHICH WERE DIVIDED INTO EQUAL PARTS BY VOLUME. ONE HALF WAS HYDROLYZED IN ACID, THE OTHER IN BASE.

[b]AVERAGE ± STANDARD DEVIATION.

of the applied radiolabel as 2,4-D after 7 days of incubation. They interpreted this as an indication that rice root callus tissue did not readily absorb and metabolize or store 2,4-D but did not exclude the possibility that excretion could have occurred. On the other hand, other species of plant callus tissue studied by Feung et al. (1975) rapidly absorbed all of the 2,4-D applied to the system. The possibility that wheat and rice, both resistant monocots, might reduce their cellular burden of 2,4-D and/or 2,4-D metabolites by excretion needs further evaluation.

Figure 3 presents the distribution of the radiolabel present in wheat cells at harvest as a function of incubation period. After 6 to 8 days of incubation, 15% is bound to the macromolecular tissue residue (i.e., insoluble in 95% ethanol and in ethanolic sodium bicarbonate solution). The soluble radiolabel was separated into water- and ether-soluble fractions as shown in Figure 1. The concentration of free [14C]2,4-D, separated from the ether-soluble metabolites of 2,4-D by TLC, decreased rapidly to only 17% of the radiolabel present in the cells after 6 to 8 days of incubation. The remaining radiolabel, assumed to represent 2,4-D amino acid conjugates, increased to 20% of that present in the cells and showed several spots of lower R_f values than 2,4-D by TLC analysis. Feung et al. (1973b, 1975) have shown that callus tissue cultures of six different plants produce a complex mixture of ether-soluble 2,4-D amino acid conjugates, the exact composition of which is species specific. Because wheat cell culture
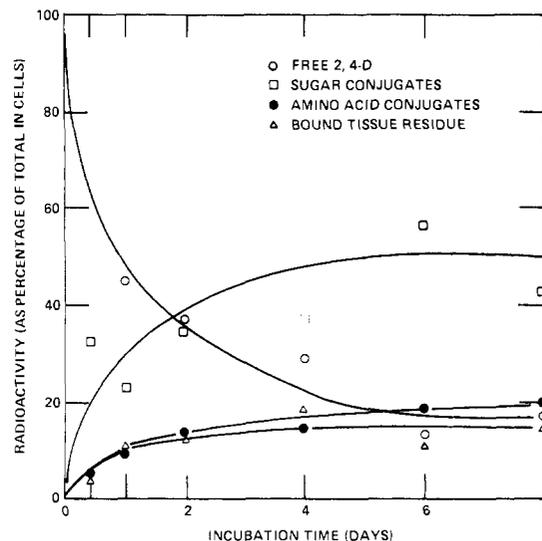

Figure 3. Distribution of cellular radioactivity after different periods of incubation.

produced more than twice the amount of water-soluble than ether-soluble metabolites of 2,4-D, the individual 2,4-D amino acid conjugates were not identified but were measured together to give the curve shown in Figure 3.

The water-soluble metabolites, which increased to over 46% of the radiolabel present in the cells after 6 to 8 days of incubation, were converted to aglycons by hydrolysis in either acid or alkali (Table II). Hydrolysis in acid was found to be more convenient and reproducible and was preferred. The small amount (10%) of radiolabel that remains water soluble after hydrolysis may correspond to $^{14}C_2$ side-chain degradation products such as glyoxylic acid (Fleeker, 1973). The 92% recovery of the radiolabel subjected to hydrolysis may indicate that a small amount of water-soluble metabolite undergoes decarboxylation under these conditions.

The ether-soluble aglycons were separated by two-dimensional TLC (Figure 4) and quantitated by liquid

TABLE III. RELATIVE AMOUNTS[a] OF WATER SOLUBLE METABOLITES OF [14C] 2,4-D PRODUCED BY WHEAT CELL SUSPENSION CULTURE AS A FUNCTION OF INCUBATION PERIOD[b]

R_f VALUES[c]	DESIGNATION	INCUBATION PERIOD, DAYS					
		1	2	4	6	8	$\overline{X} \pm s$
0.82 (0.44)	Ag1 2,4-D	4.0	2.8	3.7	8.0	2.6	4.2 ± 2.2
0.81 (0.25)	Ag2 6-HO-2,4-D	1.7	1.4	4.3	3.4	2.4	2.6 ± 1.2
0.78 (0.42)	Ag3 4-HO-2,5-D	44.1	46.4	40.4	41.5	47.7	44.0 ± 3.1
0.72 (0.16)	Ag4 5-HO-2,4-D ⎫						
0.68 (0.21)	Ag5 4-HO-2,3-D ⎭	17.2	18.6	13.0	10.8	19.3	15.8 ± 3.7
0.65 (0.15)	Ag6 4-HO-2-CPA	6.7	6.6	10.0	11.6	5.1	8.0 ± 2.7
0.41 (0.06)	Ag7 2-HO-4-CPA	0.8	0.4	0.5	0.7	0.4	0.6 ± 0.2
SUBTOTAL		74.5	76.2	71.9	76.0	77.5	75.2 ± 2.1
0.00-0.35(0.00-0.35)	ZONE 1	1.7	1.2	1.2	1.3	2.0	1.5 ± 0.4
0.35-0.76(0.00-0.35)	ZONE 2	4.8	7.1	1.8	3.7	1.7	3.8 ± 2.2
0.76-1.00(0.00-0.35)	ZONE 3	11.2	5.2	13.9	10.1	2.8	8.7 ± 4.5
0.38-0.70(0.35-0.75)	ZONE 4	1.2	1.8	0.6	0.7	2.9	1.4 ± 0.9
0.70-1.00(0.35-0.75)	ZONE 5	6.0	3.0	1.0	0.7	6.0	3.3 ± 2.6
0.0(0.0)	ORIGIN	1.9	1.0	0.8	1.6	0.7	1.2 ± 0.5
TOTAL RECOVERY		101.3	95.5	91.2	94.1	93.6	95.1 ± 3.7

[a]REPORTED AS A PERCENTAGE OF THE TOTAL 14C SUBJECTED TO tlc ANALYSIS.

[b]EACH VALUE REPRESENTS THE AVERAGE OF AT LEAST THREE DETERMINATIONS ON EACH REPLICATE INCUBATION.

[c]THE FIRST VALUE IS THAT OBTAINED BY DEVELOPMENT IN SOLVENT SYSTEM II, THAT IN PARENTHESES CORRESPONDS TO THE R_f VALUE FOUND FOR DEVELOPMENT IN THE SECOND DIMENSION IN SOLVENT SYSTEM III.

TABLE IV. DISTRIBUTION OF 2,4-D METABOLITES PRODUCED BY DIFFERENT SPECIES OF PLANT CALLUS TISSUE CULTURE

METABOLITE	% OF TOTAL RADIOACTIVITY IN TISSUE[a]							
	WHEAT[b]	RICE[c]	CORN[d]	CARROT[d]	JACKBEAN[d]	SUNFLOWER[d]	TOBACCO[d]	SOYBEAN[e]
ETHER SOLUBLE FRACTION	36.7	26.2	25.6	83.8	85.4	78.4	53.6	62.2
AMINO ACID CONJUGATES	21.0	2.0	13.1	32.1	40.0	26.7	19.3	18.5
FREE 2,4-D	15.7	24.2	12.5	51.7	45.4	51.7	34.3	33.7
WATER SOLUBLE FRACTION	46.4	65.3	64.6	13.2	12.8	18.3	42.2	33.8
AGLYCONES	43.9	3.2	54.4	12.2	11.6	15.1	34.5	33.0
2,4-D SUGAR ESTER	2.5	45.7	10.2	1.0	1.2	3.2	7.7	0.8
TISSUE RESIDUE	14.6	8.6	4.5	4.5	4.5	4.5	4.5	2.8

[a]ALL TISSUE CULTURES WERE SUSPENSION CELL CULTURES EXCEPT SOYBEAN WHICH WAS GROWN ON AGAR.

[b]DATA TAKEN FROM THIS WORK, AVERAGE OF RESULTS FOR 6 AND 8 DAY CULTURES.

[c]DATA TAKEN FROM FEUNG ET AL. (1976), 7 DAY CULTURES.

[d]DATA TAKEN FROM FEUNG ET AL. (1975), 8 DAY CULTURES.

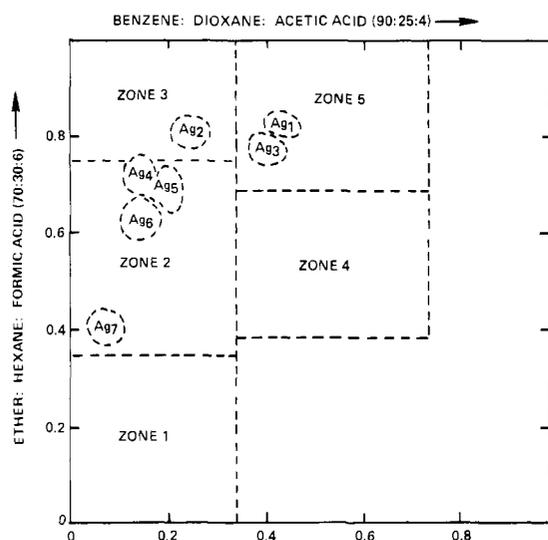[e]DATA TAKEN FROM FEUNG ET AL. (1973a), 13 DAY CULTURES.

Figure 4. Two-dimensional thin-layer chromatograms of a mixture of authentic aglycons.

scintillation counting. The R_f values, identity, and relative amounts of the seven spots (arbitrarily designated Ag_1–Ag_7) characterized are presented in Table III. Separate spots corresponding to 4-HO-2,3-D and 5-HO-2,4-D were discernible but not completely resolved and were scraped and quantitated together. However, radioautography showed that very little 5-HO-2,4-D was present. No changes in the relative amounts of ring hydroxylated metabolites present after different periods of incubation were observed in contrast with those reported for the formation of 2,4-D amino acid conjugates by soybean callus tissue culture (Feung et al., 1972).

The area of the TLC plate that remained after spots Ag_1–Ag_7 were removed was divided into zones as shown in Figure 4 and analyzed for radiolabel (Table III) to detect any aglycons for which authentic standards were not available. By analogy to literature R_f values for 3-HO-2,4-D (Feung et al., 1975; Montgomery et al., 1971; Hamilton et al., 1971), this compound would have occurred in our system in zone 3 below 4-HO-2,5-D or possibly in zone 2 above 4-HO-2,3-D. While zone 3 did contain a significant amount of residual radiolabel, it is clear that no major metabolites other than Ag_3, Ag_5, and Ag_6 were present. A likely explanation for the presence of radiolabel in zones 1–5 and at the origin involves partial air oxidation of the phenolic metabolites during application and drying of the plates between developments. Separated spots for the mixture of authentic standard Ag_2–Ag_7 were air oxidized

to yellow, brown, or red spots on standing for 12 or more hours after development and streaking was apparent only in the first direction of development using the two-dimensional technique. The low recovery of radiolabel as Ag_1–Ag_7 (75%) is attributed to air oxidation since inclusion of that detected in zones 1–5 and at the origin gives an average recovery of over 95%. Accordingly, the amounts of Ag_1–Ag_7 reported in Table III may be underestimated by as much as 25%. The small amounts of radiolabel corresponding to Ag_2 and Ag_7 may also be due to air oxidation of Ag_3–Ag_6 rather than the occurrence of 6-HO-2,4-D and 2-HO-4-CPA as metabolites.

The small amount of 2,4-D found in the aglycon fraction (4% of the ^{14}C present) may indicate the formation of either sugar esters (Feung et al., 1975, 1976; Loos, 1975) or dibasic amino acid conjugates not soluble in ether (Mumma and Hamilton, 1976). The stabilities of such metabolites toward the isolation conditions utilized in this and other studies are not known. However, if such carboxylic conjugates of 2,4-D were formed in wheat cells, and if they are extremely labile (hydrolyzed in 5 min at pH ≤1 or in about 20 min in 0.1 N NaHCO$_3$ at room temperature), then they would have been isolated and analyzed as ether-soluble, free 2,4-D in this work (cf. Figure 1). Because the amount of free 2,4-D found was small (13% of that applied), hydrolysis of carboxylic conjugates could represent only a small error in our results.

The foregoing results demonstrate that wheat cells in suspension culture absorb and metabolize [^{14}C]2,4-D rapidly by several different pathways. Loss of a volatile degradation product, probably ^{14}CO$_2$, may proceed through metabolism of the side-chain to form ^{14}C$_2$ fragments as intermediates (Fleeker, 1973). Bound residue may possibly arise by incorporation of ^{14}C$_2$ fragments and/or ^{14}CO$_2$ into insoluble cellular tissue. Thus, on the basis of the total radiolabel added to the cell culture system, side-chain degradation may account for 21% or more of the metabolism of 2,4-D. A small amount (4 to 10%) of cellular 2,4-D and/or polar 2,4-D metabolites are returned to the medium either by excretion or cell lysis. Less than 13% of the 2,4-D added to the system remains free (unmetabolized) in the cells while 17% is found conjugated with amino acids. The major metabolism pathway in wheat involves ring hydroxylation followed by conjugation with sugars to form water-soluble glycosides (38% of the radiolabel applied). The amount of 4-HO-2,5-D, the major metabolite found in wheat cells at long incubation periods, actually exceeds that of free 2,4-D by a factor of 1.3 or more. A small amount of the applied 2,4-D (at least 1.6%) is apparently converted to either a sugar ester (carboxylic

glycoside) or a dibasic amino acid conjugate.

The metabolism of 2,4-D by wheat cell culture can be compared with its metabolism by cell cultures derived from several other plant species (Feung et al., 1973a, 1975, 1976; Mumma and Hamilton, 1976). The observed distributions of metabolites found in the different tissues are presented in Table IV. Wheat, rice, and corn are resistant monocots while the other plants studied are all dicots that are susceptible to the herbicidal action of 2,4-D. The most striking feature of the data in Table IV is the low concentration of 2,4-D present in the tissues of the resistant plants wheat, rice, and corn relative to that in the susceptible plants. Montgomery et al. (1971) reported a similar finding in their study using corn, bluegrass, and bean plants. The combined amounts of 2,4-D and 2,4-D amino acid conjugates in wheat, rice, and corn are also lower than in any of the susceptible plant cell cultures studied. Conversely, wheat, rice, and corn culture tissues contain higher concentrations of water-soluble metabolites than the other tissues.

The amino acid conjugates of 2,4-D all possess stimulating plant growth regulator properties, some in excess of 2,4-D itself, and their formation during 2,4-D metabolism cannot be considered to be a detoxification mechanism, per se (Feung, 1973b, 1974). The formation of ring hydroxylated 2,4-D metabolites, however, may represent detoxification since they exhibit no auxin properties in the *Avena* coleoptile section bioassay test with the exception of 3-HO-2,4-D (Feung, 1971; Wang et al., 1968). The fact that the general pattern for metabolism of 2,4-D by wheat resembles that by rice and corn confirms the suggestion by Montgomery et al. (1971), Mumma and Hamilton (1976), and Feung et al. (1976) that the resistance of plants to the herbicidal action of this plant growth regulator may stem from their ability to detoxify it by ring hydroxylation. While ring hydroxylated metabolites and 2,4-D amino acid conjugates have been identified as being formed in a variety of studies utilizing whole plants (Fleeker and Steen, 1971; Montgomery et al., 1971; Hamilton et al., 1971; Loos, 1975), their relative amounts and the amount of free 2,4-D present were not determined quantitatively. Accordingly the significance of the occurrence of these metabolism pathways could not be fully assessed. However, the widespread occurrence of both ring hydroxylated metabolites and amino acid conjugates of 2,4-D indicates that these pathways may well represent major routes for 2,4-D metabolism in most plants. On the other hand, the major pathway for 2,4-D metabolism in rice root callus tissue (Feung et al., 1976) and possibly other plants appears to involve the formation of carboxylic glycosides of 2,4-D and appears to be more significant in the resistant monocots than the susceptible dicots studied in tissue culture. Therefore, the ability of some plants to lower their cellular burden of 2,4-D and biologically active amino acid conjugates by the formation of carboxylic glycosides and the degradation of the side chain of 2,4-D may also contribute to their overall resistance. Accordingly, the hypothesis explaining the resistance of plants to the herbicidal action of 2,4-D can be restated in terms of their ability to detoxify it, not only by ring hydroxylation, but by any or all of the several metabolic pathways leading to the formation of water-soluble metabolites. The relative significance of the competing pathways may vary from one plant species to another but they all serve to lower the cellular burden of ether-soluble 2,4-D and its biologically active amino acid conjugates by conversion to water-soluble metabolites.

Closer examination of the identity and relative amounts of ring hydroxylated metabolites produced by wheat and corn cell cultures reveals some differences that are not explained readily. Both tissues contained 4-HO-2,5-D, 4-HO-2,3-D, and 4-HO-2-CPA. However, while 4-HO-2,5-D is the major hydroxylated metabolite in wheat, Feung et al. (1975) reported that 4-HO-2,3-D was the major hydroxylated metabolite and that 3-HO-2,4-D was also present in corn. In rice (Feung et al., 1976), 4-HO-2,3-D appeared to predominate but both 4-HO-2,3-D and 4-HO-2,5-D were present only in trace amounts. The results with corn and rice cell cultures are surprising, since virtually all other studies have reported that 4-HO-2,5-D is the major ring hydroxylated metabolite of 2,4-D in a variety of plants (Loos, 1975). The metabolite 3-HO-2,4-D was not detected in whole corn plants by Montgomery et al. (1971) and had not been reported to be a metabolite of 2,4-D in other studies. Hamilton et al. (1971) reported that no quantities of any ring hydroxylated metabolites were formed in corn plants. These discrepancies may arise either from the much greater difficulty encountered in working with intact plants than with tissue cultures or from an inability of the tissue culture model systems used to date to accurately mimic the metabolic pathways existent in the differentiated tissue of intact plants. They clearly point out the need for the development of better separation methods and characterization procedures that can be applied to the detailed study of the metabolic pathways for 2,4-D in both model systems and intact plants.

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Occurrence of Aflatoxin B_{2a} in Cottonseed Meal

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Aflatoxin B_{2a}, 17 ppb, was found in cottonseed meal contaminated with aflatoxin B_1 and B_2. Aflatoxin B_{2a} was not detected in feed-grade meal or in meal which had been decontaminated by the ammonia process. Other fungal metabolites such as zearalenone, sterigmatocystin, ochratoxin A, and aflatoxins M_1 and M_2 were not detected.

Since the discovery that a peanut meal containing aflatoxin was responsible for many deaths among turkey poults and ducklings (Allcroft et al., 1961), a massive literature has appeared describing the efforts devoted to aflatoxin and other mycotoxins that affect agricultural products. Aflatoxins, which are secondary fungal metabolites, include at least 16 closely related chemicals with highly substituted furofuran rings of which aflatoxin B_1 is the most important (Rodricks, 1969). The aflatoxins, which are acutely toxic and oncogenic mold products, are elaborated by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Sargeant et al., 1961). These molds can grow readily on feedstuff if favorable conditions of temperature and humidity prevail (Goldblatt, 1969; Borker et al., 1966; Ciegler and Lillehoj, 1968). These compounds fluoresce brightly under long-wave UV light. This important property has been utilized as the basis for an extremely sensitive method for their detection, isolation, and analytical quantitation (Goldblatt, 1969).

Chemical characteristics of the aflatoxins have been determined by various workers and the structures of aflatoxins B_1, B_2, G_1, and G_2 have been elucidated (Asao et al., 1963, 1965; Hartley et al., 1963; van der Merwe et al., 1963). The biotransformation of aflatoxin B_1 results in hydroxylated derivatives, namely aflatoxins M_1 and M_2; these were first isolated from cow's milk (de Iongh et al., 1964). The structures of both of these compounds have been elucidated (Holzapfel et al., 1966). Two new hydroxylated aflatoxins produced by mold cultures of *A. flavus* have been described and designated aflatoxins B_{2a} and G_{2a} (Dutton and Heathcote, 1966, 1968). It has been shown that these two compounds can be formed from aflatoxins B_1 and G_1, respectively, by treatment with strong acid and heat (Pons et al., 1972; Pohland et al., 1968). The occurrence of aflatoxins B_{2a} and G_{2a} in cottonseed meal has not been reported.

Aflatoxigenic *Aspergilli* are ubiquitous in nature (Hesseltine et al., 1966; Wogan, 1966) and contamination of cottonseed by aflatoxin occurs in the field (Ashworth, 1972). In addition to aflatoxins, the *Aspergilli* can produce other toxic substances on feed materials (Wilson and Wilson, 1964). For this reason we screened aflatoxin-contaminated cottonseed meals for fungal metabolites other than aflatoxins B_1 and B_2. Naturally infected cottonseed meals usually contain only aflatoxins B_1 and B_2 (McMeans et al., 1968). In controlled laboratory conditions, however, a strain of *A. flavus* grown on media containing cottonseed products elaborated all four aflatoxins, B_1, B_2, G_1, and G_2 (Mayne et al., 1966).

This report describes the isolation and quantitation of aflatoxin B_{2a} found in contaminated cottonseed meal. This procedure did not detect zearalenone, sterigmatocystin, ochratoxin A, and aflatoxins M_1 and M_2.

EXPERIMENTAL PROCEDURES

Materials. Three different cottonseed meals were used. One was a meal free of aflatoxin B_1 and B_2 contamination. A second contained 948 μg/kg (ppb) aflatoxin B_1 and 170 ppb aflatoxin B_2. The third was the same contaminated meal, which had been detoxified in the pilot plant by the ammonia process (Mann et al., 1970). All chemicals and solvents were reagent grade. Silicic acid for column chromatography, 100 mesh powder, analytical reagent, was from Mallinckrodt Chemical Works, St. Louis, Mo. Adsorbosil-1 (Applied Science Laboratories, State College, Pa.) was used to coat thin-layer plates 0.5 mm prepared according to methods already described in the literature (AOAC, 1975). Aflatoxin B_{2a} (kept under N_2 at 4 °C) and ochratoxin A were obtained from W. A. Pons, Jr., SRRC, and zearalenone was supplied by Commercial Solvents, Terre Haute, Ind. Sterigmatocystin was purchased from Aldrich Chemical Co. Aflatoxins M_1 (10186-65-A) and M_2 (8578-95-A) were obtained from R. D. Stubblefield, NRRC, Peoria, Ill. Resolution of B_{2a} was evaluated visually in a View Box Spectroline, Black Light, Model 3F, Eastern Corp., Westbury, L.I., New York. Quantitative estimation was made with a Photovolt Model 530A densitometer,,

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