Table III gives the R_f values of the 20 amino acid conjugates of 2,4-D in six tlc solvent systems and in one paper chromatographic solvent system. Using this technique, most of the 20 conjugates can be recognized. The Leu and Ile conjugates and the Met and Val conjugates have similar chromatographic properties and cannot be identified easily in this manner; however, they can be readily identified by mass spectrometry.

Collectively these mass spectral and chromatographic data provide additional information for the identification of hitherto structurally unrecognized amino acid conjugates of 2,4-D. In addition, these data should be useful for the identification of amino acid conjugates of other plant growth regulators. These studies suggest that the 2,4-dichlorophenoxyacetyl group may be a desirable derivative of peptides for mass spectral investigations, similar to the long-chain fatty acyl derivatives of peptides (Barber, 1965a,b). The fragmentations of high m/e are few in number, usually intense, predictable, and characterized by the chlorine isotope peaks.

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Metabolism of 2,4-Dichlorophenoxyacetic Acid. V. Identification of Metabolites in Soybean Callus Tissue Cultures

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The metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenoxyacetylglutamic acid (2,4-D-Glu) was investigated in soybean callus tissue cultures and the various metabolites were quantified. In addition to the major amino acid conjugates previously reported, 2,4-D-Glu and 2,4-dichlorophenoxyacetylaspartic acid (2,4-D-Asp), five new 2,4-D conjugates have been isolated and identified: alanine, valine, leucine, phenylalanine, and tryptophan. Callus tissue converted 2,4-D-Glu to a number of compounds, of which two ether-soluble metabolites have been identified (2,4-D-Asp and 2,4-D) and three aglycone metabolites have been identified (2,4-D, 4-hydroxy-2,5-dichlorophenoxyacetic acid, and 4-hydroxy-2,3-dichlorophenoxyacetic acid). These metabolites account for 83.5% of the radioactivity found in the tissue. A greater proportion of the 2,4-D-Glu was metabolized and converted to the ring-hydroxylated metabolites and to 2,4-D-Asp than was 2,4-D.

2,4-Dichlorophenoxyacetic acid (2,4-D) is rapidly metabolized by soybean cotyledon callus tissues into amino acid conjugates and two ring-hydroxylated derivatives (Feung *et al.,* 1971, 1972; Hamilton *et al.,* 1971). Two amino acid conjugates, which retain growth stimulatory activity, were identified as 2,4-dichlorophenoxyacetylglutamic acid (2,4-D-Glu) and 2,4-dichlorophenoxyacetylaspartic acid (2,4-D-Asp). The latter has also been detected in excised pea roots (Andreae and Good, 1957) and wheat coleoptile sections (Klämbt, 1961). The ring-hydroxylated metabolites, which do not possess growth stimulatory activity, were identified as 4-hydroxy-2,5-dichlorophenoxyacetic acid (4-OH-2,5-D) and 4-hydroxy-2,3-dichlorophenoxyacetic acid (4-OH-2,3-D). These two metabolites were previously found in bean and other plants by Thomas *et al.* (1964), Hamilton *et al.* (1971), and Fleeker and Steen (1971). Faulkner and Woodcock (1964) identified 4-OH-2,5-D as a metabolite in *Aspergillus niger.*

We now report the identification of additional amino acid conjugates of 2,4-D in soybean cotyledon callus tissue and we have shown *in vivo* conversion of 2,4-D-Glu to ring-hydroxylated metabolites, 2,4-D, and other amino acid conjugates.

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EXPERIMENTAL SECTION

Soybean (*Glycine max* L. Merrill var. Acme) cotyledon callus stock cultures were grown on an agar solidified medium (Miller, 1963) under continuous fluorescent light at 25° for 5 weeks. Approximately 10 g of this tissue was aseptically transferred to each 125-ml Erlenmeyer flask containing 50 ml of sterile liquid medium (Miller, 1963), minus α-naphthalene acetic acid (NAA) to which 4 μCi (1.63×10^{-6} M)

of either 2,4-D-*1*-^{14}C (specific activity 2.44 mCi/m*M*) or 4 μCi of isolated and purified 2,4-D-*1*-^{14}C-Glu was added. These callus tissues were grown at 25° with gentle shaking for either 8 days (2,4-D-*1*-^{14}C treatment cultures) or for 12 days (2,4-D-*1*-^{14}C-Glu treatment cultures).

Following incubations, the treated callus tissues were separately collected on filter paper in a Buchner funnel and surface-rinsed with cold distilled water. The tissues were stored in separate plastic bags at −20° prior to analysis.

The techniques used for extraction and fractionation of 2,4-D and 2,4-D-Glu metabolites were the same as those previously reported (Feung *et al.*, 1971, 1972). The frozen callus tissues of individual treatments were thoroughly ground in a Waring Blendor with 95% ethanol. The homogenate was filtered with suction and the residue was repeatedly washed with 80% ethanol. The filtrate was concentrated and adjusted to pH 3, and was then extracted three times with diethyl ether. The aqueous phase was subsequently extracted three times with 1-butanol, which was then evaporated to dryness. The 1-butanol soluble residue was dissolved in 5 ml of distilled water and placed on a Biogel P-2 column (Hamilton *et al.*, 1971) and eluted. The fractions containing the metabolites were incubated with Emulsin (Nutritional Biochemical Corporation) and the aglycones were obtained by subsequent acidification and extraction with diethyl ether. The aglycones and the ether-soluble fractions (pH 3) both contained metabolites moving to eight separate zones on thin-layer chromatography (tlc).

The procedures for separation and purification of the metabolites were the same as those previously reported (Feung *et al.*, 1971, 1972). Both aglycones and ether-soluble 2,4-D metabolites were isolated in micro- or submicrogram quantities by solvent extraction, followed by paper chromatography (pc) and tlc. These metabolites were located with autoradiography. A diazotized sulfanilic acid spray was employed to visualize the aglycones or standards and bromocresol green (Bryant and Overell, 1953) to visualize the ether-soluble metabolites when present in microgram quantities. The isolated and purified compounds were characterized by paper or thin-layer cochromatography with synthetic compounds (Feung *et al.*, 1973a) and were also analyzed by mass spectrometry (AEI Model MS902 mass spectrometer) using a direct sample inlet.

Five 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 (60–70°)–formic acid (70:30:2, v/v/v); IV, benzene–triethylamine–methanol–concentrated ammonium hydroxide (85:15:20:2, by vol); and V, benzene–methanol–cyclohexane–formic acid (80:10:20:2, by vol). Descending pc was used (Whatman No. 1 paper) employing the solvent 1-butanol–ethanol–3 *N* ammonium hydroxide (4:1:5, v/v/v) (solvent VII).

Soybean cotyledon callus tissue incubated with 2,4-D-*1*-^{14}C for 8 days was selectively extracted. The tissue radioactivity was found in three fractions: ethanol-insoluble residue (1.5–4.0%), water-soluble (30–35%), and ether-soluble (61–66%). After 8 days the medium contained about 4–6% of the total radioactivity put into the flasks.

The ether-soluble metabolites and the water-soluble metabolites, following treatment with Emulsin (β-glucosidase), were separated by descending paper chromatography employing Solvent VII. The quantitative results of a typical experiment are given in Table I. The ether-soluble metabolites were separated into eight zones having R_f values of 0.18–0.26, 0.26–0.30, 0.30–0.40, 0.40–0.43, 0.45–0.56, 0.58–0.66, 0.68–0.74, and 0.76–0.84. These zones of the paper chromatograms were arbitrarily referred to as Et_1 through Et_8 in order of increasing R_f values, as in our previous investigation (Feung *et al.*, 1972).

Three metabolites were previously identified as 2,4-D-Asp (Et_2), 2,4-D-Glu (Et_4), and 2,4-D (Et_7) (Feung *et al.*, 1971, 1972). The material isolated from Et_6 (*ca.* 9% of total ether-soluble fraction) was separated on tlc into two components having R_f values of 0.48 (Et_{6a}) and 0.52 (Et_{6b}) employing solvent III. These metabolites cochromatographed on tlc with synthetic 2,4-D-alanine (Et_{6a}) and 2,4-D-valine (Et_{6b}) in five solvents (I–V) and they possessed mass spectra identical to these synthetic compounds (Feung *et al.*, 1973a). The metabolites isolated from region Et_8 (*ca.* 6% of the total ether-soluble fraction) were separated on tlc into three components having R_f values of 0.27 (Et_{8a}), 0.49 (Et_{8b}), and 0.56 (Et_{8c}) using solvent III. These metabolites cochromatographed on tlc in five solvents (I–V) with synthetic 2,4-D-tryptophan (Et_{8a}, 2,4-D-Try), 2,4-D-phenylalanine (Et_{8b}, 2,4-D-Phe), and 2,4-D-leucine (Et_{8c}, 2,4-D-Leu), respectively. The mass spectra of these isolated metabolites were also identical to the corresponding spectra of the synthetic conjugates (Feung *et al.*, 1973a). Metabolites Et_1 and Et_5 have not been identified; however, they only constitute *ca.* 4.5% of the total ether-soluble metabolites and are not any of the 20 previously synthesized amino acid conjugates of 2,4-D, based upon chromatographic evidence.

The water-soluble metabolites of 2,4-D yielded at least eight aglycone metabolites, possessing ether solubility following Emulsin treatment. These components, which are arbitrarily referred to as Ag_1–Ag_8, migrated upon paper chromatography in solvent system VII to R_f regions 0.24–0.31, 0.32–0.34, 0.34–0.38, 0.38–0.44, 0.44–0.47, 0.49–0.54, 0.59–0.65, and 0.72–0.78. The radioactive material isolated from region Ag_1 separated into two components on tlc in solvent system III: a major component Ag_{1a} (R_f 0.37) and a minor component Ag_{1b} (R_f 0.42). Ag_{1a}, Ag_{1b}, and Ag_8 cochromatographed on tlc with 4-OH-2,3-D, 4-OH-2,5-D, and 2,4-D, respectively, in five solvent systems (I–V), and possessed mass spectra identical with those of the synthetic compounds. These results are in agreement with our previous findings (Feung *et al.*, 1971, 1972). The remaining six unknown metabolites constitute 22% of the radioactivity in this fraction.

Soybean cotyledon callus tissue was incubated with isolated and purified 2,4-D-*1*-^{14}C-Glu for 12 days. The callus tissue was extracted as previously described and the residue, water, and ether extracts possessed 3.9, 79.6, and 16.5% radioactivity, respectively. The total radioactivity left in the medium was about 4–6% of that supplied. Upon paper chromatography in solvent system VII, the ether-soluble metabolites were separated into four distinct regions and the water-soluble aglycones separated into five distinct regions (Table II). The ether-soluble metabolites were arbitrarily designed Etg_1 through Etg_4 in order of increasing R_f values. Etg_1, Etg_3, and Etg_4 were identified by tlc and mass spectrometry as 2,4-D-Asp, 2,4-D-Glu, and 2,4-D, respectively. The aglycones were separated into five regions on paper chromatography in solvent system VII and arbitrarily designated Agg_1 through Agg_5. The major component (Agg_2) was identified as a mixture of 4-OH-2,5-D (major) and 4-OH-2,3-D (minor), and Agg_5 corresponds to 2,4-D.

DISCUSSION

2,4-D is metabolized by soybean callus tissue to ether- and water-soluble metabolites, and a small portion is found in the callus tissue residue (1.5–4.0%). Therefore, relatively little 2,4-D is associated with macromolecular fractions. Also, high recovery of label precludes appreciable decarboxylation in this tissue. Seven of the ether-soluble metabolites have been identified and in the illustrated experiment they constitute 95.8% of the ether fraction and 59.6% of the total radioactivity found in the tissue. The identified amino acid conjugates of 2,4-D are glutamic acid, aspartic acid, alanine, valine, leucine, phenylalanine, and tryptophan. 2,4-D-Glu is the major metabolite in the 8-day

Table I. Relative Percentage Metabolites of 2,4-D Incubated with Soybean Callus Tissue for 8 Days[a]

Ether-soluble metabolites				Water-soluble metabolites			
Metabolites		% total in fraction	% total in tissue	Metabolites		% total in fraction	% total in tissue
R_f region[b]	Designation			R_f region[b]	Designation		
0.18–0.26	Et_1	1.9	1.2	0.24–0.31	Ag_1 (4-OH-2,5-D) (4-OH-2,3-D)	75.8	26.3
0.26–0.30	Et_2 (2,4-D-Asp)[c]	5.9	3.7	0.32–0.34	Ag_2	7.2	2.5
0.30–0.40	Et_3[d]	0.0	0.0	0.34–0.38	Ag_3	3.1	1.1
0.40–0.43	Et_4 (2,4-D-Glu)	20.8	12.9	0.37–0.44	Ag_4	2.8	1.0
0.45–0.56	Et_5	2.3	1.4	0.44–0.47	Ag_5	2.2	0.8
0.58–0.66	Et_6 (2,4-D-Ala, Val)	8.6	5.3	0.49–0.54	Ag_6	2.6	0.9
0.68–0.74	Et_7 (2,4-D)	54.0	33.7	0.59–0.65	Ag_7 (2,4-D)	2.3	0.8
0.76–0.84	Et_8 (2,4-D-Leu, Phe, Try)	6.5	4.0	0.72–0.78	Ag_8	4.0	0.4
	Total	100%	62.2%			100%	33.8%

[a] Residue contained 4.0% of the total radioactivity. [b] Descending Whatman No. 1 paper chromatography, solvent system; 1-butanol–95% ethanol–3 *N* ammonium hydroxide (4:1:5, v/v/v). [c] Following purification the R_f is 0.37–0.40. [d] Et_3 was detected only after longer incubation with 2,4-D.

Table II. Relative Percentage Metabolites of 2,4-D-Glutamic Acid (Et_4) Incubated with Soybean Callus Tissue for 12 Days[a]

Ether-soluble metabolites				Water-soluble metabolites			
Metabolites		% total in fraction	% total in tissue	Metabolites		% total in fraction	% total in tissue
R_f region	Designation			R_f region	Designation		
0.20–0.25	Etg_1 (2,4-D-Asp)	44.3	11.7	0.25–0.30	Agg_1	5.2	3.7
0.38–0.42	Etg_2 (2,4-D-Glu)	25.6	6.7	0.30–0.32	Agg_2 (4-OH-2,5-D) (4-OH-2,3-D)	77.5	54.9
0.42–0.47	Etg_3	7.3	1.9	0.38–0.42	Agg_3	8.0	5.7
0.62–0.73	Etg_4 (2,4-D)	22.8	6.0	0.47–0.55	Agg_4	3.3	2.3
				0.65–0.73	Agg_5 (2,4-D)	6.0	4.2
	Total	100%	26.1%			100%	70.8%

[a] Residue contained 3.1% of the total radioactivity.

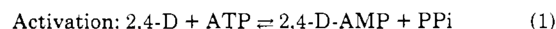
incubation and 2,4-D-Asp appears in significant concentrations in longer incubations (Feung *et al.*, 1972). Two other minor ether-soluble metabolites remain unidentified and may be conjugated amino acids, although not with any of the 20 major amino acids. All seven amino acid conjugates are physiologically active and stimulate growth (Feung *et al.*, 1973b). These conjugates generally appear in the callus tissue prior to the formation of large amounts of the water-soluble metabolites (Feung *et al.*, 1972).

Several water-soluble metabolites were formed; however, no effort has been made to isolate and identify the glycones. The entire water-soluble extract was subjected to enzymatic hydrolysis with Emulsin. Although this enzyme is generally used to cleave β-glucosidic linkages, this enzyme preparation contains other hydrolytic enzymes. For example, 2,4-D-Glu is hydrolyzed to 2,4-D and glutamic acid with Emulsin. Thus, it cannot be assumed that the unhydrolyzed metabolites are simply glycones, although for convenience Emulsin-treated metabolites are referred to as aglycones.

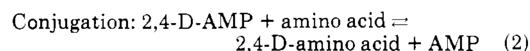
Three aglycone metabolites of 2,4-D have been identified, these are 4-OH-2,5-D (major), 4-OH-2,3-D (minor), and 2,4-D itself. The 2,4-D recovery in the aglycone fraction may indicate the presence of some glucose or sugar ester (Klämbt, 1961).

2,4-D-Glu is metabolized in 12 days by soybean callus tissue to 2,4-D, 2,4-D-Asp, 4-OH-2,5-D, and 4-OH-2,3-D. Thus, 2,4-D-Glu is converted into other amino acid conjugates such as 2,4-D-Asp, which is found in almost double the concentration of 2,4-D-Glu. Hydrolysis of 2,4-D-Glu does occur, since 2,4-D was present in almost equal proportion to 2,4-D-Glu. The water-soluble fraction consisted of 70.8% of the total radioactivity in the tissue when incubated for 12 days with 2,4-D-*1*-^{14}C-Glu. In contrast, this fraction was found in only about one-half this amount in the 2,4-D incubations: 33.8 and *ca.* 36% (Feung *et al.*, 1972) of the total radioactivity in the tissue in 8- and 12-day incubations, respectively. Since the water-soluble fraction consisted of *ca.* 75–80% 4-OH-2,5-D and 4-OH-2,3-D in all cases, the 2,4-D-Glu incubations resulted in approximately double the amount of hydroxylated metabolites found with 2,4-D incubations.

These results suggest that either several specific enzymes exist, each forming 2,4-D conjugates with a single amino acid, or a more general reaction of lower specificity exists. The latter reaction might be similar to amino acid activation with subsequent conjugation of the 2,4-D with an amino acid. The conjugation step would also allow interconversion of various amino acid conjugates. Thus, the steps would be



$$\text{Activation: 2,4-D + ATP} \rightleftharpoons \text{2,4-D-AMP + PPi} \quad (1)$$



$$\text{Conjugation: 2,4-D-AMP + amino acid} \rightleftharpoons \text{2,4-D-amino acid + AMP} \quad (2)$$

Both reactions could be catalyzed by the same enzyme. Although a conjugation enzyme has not yet been demonstrated, it is known that this type of reaction in other tissues requires induction (Venis, 1972). This would seem to rule out any of the normal activating enzymes involved in protein synthesis.

The hydroxylation reaction seems to prefer 2,4-D-Glu as a substrate rather than 2,4-D or 2,4-D-Asp. Since 2,4-D is converted rapidly into 2,4-D-Glu, it is conceivable that the hydroxylation reaction may even require 2,4-D-Glu as a substrate. Thus, glycosides of hydroxylated 2,4-D-Glu have so far not been ruled out as precursors of 4-OH-2,5-D or 4-OH-2,3-D obtained by Emulsin treatment of the water-soluble fraction. If the hydroxylation enzyme is somewhat substrate specific, it could selectively remove one of the conjugates and an accumulation of the other conjugates would

occur, as appears to be the case. It suggests that perhaps 2,4-D-Glu might be a good substrate for this hydroxylation reaction, resulting eventually in a lowering of its concentration due to this reaction as well as accumulation of the other conjugates such as 2,4-D-Asp.

Additional experiments are being performed to test this proposed mechanism of metabolism of 2,4-D. It is hoped that information gained from metabolism in callus tissue will be similar to the metabolism in the whole plant.

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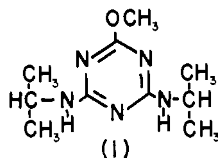
Rat Urinary Metabolites from 2-Methoxy-4,6-bis(isopropylamino)-*s*-triazine (Prometone)

Jerome E. Bakke* and Connie E. Price

Nine urinary metabolites from ^{14}C-ring-labeled prometone (I) were either identified or characterized by mass spectrometry of their trimethylsilyl derivatives. The major urinary metabolites were ammeline (31.5%) and 2-methoxy-4,6-diamino-*s*-triazine (10–14%). The other metabolites were dealkylation and oxidation products of prometone.

Bohme and Barr (1967) identified three rat urinary metabolites from prometone [2,4-bis(isopropylamino)-6-methoxy-*s*-triazine, I]. These metabolites were the mono- and di-*N*-dealkylated analogs of I and *N*-[2-methoxy-4-amino-*s*-triazinyl-(6)]alanine. Bakke *et al.* (1967) reported the excretion pattern and tissue residues resulting from a single oral dose of ^{14}C-ring-labeled prometone given to rats, and demonstrated the presence of at least 11 urinary metabolites from I by ion-exchange chromatography.



The present study reports the identification or mass spectral characterization of these rat urinary metabolites.

EXPERIMENTAL SECTION

The preparation of ^{14}C-ring-labeled prometone, animal treatment, radioanalysis, sample preparation, and ion-exchange chromatography of the urinary metabolites have been previously reported (Bakke *et al.*, 1967). The methods used to isolate and characterize the metabolites were identical with those reported for atrazine and 2-hydroxyatrazine urinary metabolites (Bakke *et al.*, 1972).

Rat urine (0–48 hr collections) was freeze dried and the radioactivity was extracted from the dry solids with methanol. Aliquots of this methanol extract were dried, taken up in 3 ml of water, adjusted to pH 3 with 6 *N* HCl, and applied to a 0.6 × 100 cm column of beadform, strong cation exchange resin (Chromabeads, Type A, Technicon, Inc., Column A). The column was jacketed with water circulating at 65°. The column preparation, equilibration, and regeneration, as well as buffer preparation (pH 2.875, 3.80, and 5.00 sodium citrate buffers) and buffer sequence in the variable-gradient device, were those given in the instruction manual for the automatic amino acid analyzer (Technicon Chromatography Corporation, 1962) and were similar to the system reported by Piez and Morris (1960), with the following exceptions. The column was monitored by a continuous-flow liquid scintillation detector; the detergent and bacteriostat were not included in the buffers; and after the end of the normal buffer cycle (675 ml), the elution was continued with a two-chamber gradient consisting of 75 ml of the pH 5 buffer in the first chamber and 75 ml of 0.2 *N* NaOH containing 0.6 mol/liter of NaCl in the second chamber.

The column flow rate was 0.5 ml/min, and 10-ml fractions were collected. The fractions containing activity were quantitated by liquid scintillation in counting solution A (Bakke *et al.*, 1967).

The radioactive fractions collected from column A were separately freeze dried and the residues were taken up in 5 ml of water, acidified to pH 3 with 6 *N* HCl, and applied to a 1- × 20-cm column of AG-50X8 cation-exchange resin in the ammonium form (column B). The citrate present from the column A buffer was eluted with water and the radioactivity was then eluted with 1 *N* NH_4OH. The recoveries from column B ranged from 71 to 95%. The citrate-free fractions were freeze-dried, and the residue from each fraction was dissolved in methanol and chromatographed on Whatman No. 1 paper. The chromatograms were developed in isoamyl alcohol-acetic acid-water (40:10:50, v/v/v). The radioactive fractions were extracted from the paper with methanol, the extracts were concentrated, and each was separately applied to a 0.9- × 100-cm column of methanol-equilibrated Sephadex LH-

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