Metabolism of 2,4-Dichlorophenoxyacetic Acid. IV. Mass Spectra and Chromatographic Properties of Amino Acid Conjugates

Chao-shieung Feung, Robert H. Hamilton, and Ralph O. Mumma*

Twenty L-form amino acid conjugates of 2,4-dichlorophenoxyacetic acid (2,4-D) were prepared and characterized by mass spectrometry, paper and thin-layer chromatography. Nineteen of the conjugates produced molecular ions and all gave characteristic spectra. The fragmentations of high m/e are few in number, usually intense, predictable, and characterized by the chlorine isotope peaks. Collectively these data have aided in the identification of several metabolites of 2,4-D and will be useful in identifying other similar metabolites of 2,4-D, as well as other acidic plant growth regulators. These studies suggest that 2,4-dichlorophenoxyacetyl derivatives of peptides may be desirable for mass spectral investigations similar to the commonly used long-chain fatty acyl derivatives.

Recently, Feung *et al.* (1971) demonstrated that soybean cotyledon callus tissue converts 2,4-dichlorophenoxyacetic acid (2,4-D) to numerous ether-soluble metabolites (pH 3) prior to the appearance of the characteristic ring-hydroxylated metabolites. Two of these eight ether-soluble metabolites have already been identified as the amino acid conjugates, 2,4-dichlorophenoxyacetylglutamic acid and 2,4-dichlorophenoxyacetylaspartic acid, by chemical degradative techniques and mass spectral examination of the methyl esters (Feung *et al.*, 1971, 1972). The conjugation of 2,4-D with amino acids has been shown to be a general reaction of other callus tissues, such as corn, tobacco, jackbeans, carrots, and sunflowers (Feung *et al.*, 1973b). Conjugation of externally applied acids to aspartic acid in plants has also been commonly observed (Andreae and Good, 1955; Sudi, 1964; Zenk, 1962); however, the significance and involvement of this conjugation to the metabolism of the phenoxy herbicides is generally unrecognized.

Biemann *et al.* (1961) have reported the mass spectra of the ethyl esters of amino acids. Several investigators have also studied the mass spectra of various peptides (Barber *et al.*, 1965a; Shemyakin, 1968b). Since the amino acid conjugates of 2,4-D play an important role in plant tissues, a known and predictable mass spectral fragmentation pattern of the amino acid conjugates would aid future studies in further characterizing the metabolites of 2,4-D as well as other herbicides. Therefore, we report the mass spectral fragmentation patterns as well as the thin-layer and paper chromatographic properties of 20 L-amino acid conjugates of 2,4-D. These collective data have already been used for the identification of seven amino acid conjugates of 2,4-D isolated from soybean callus tissue (Feung *et al.*, 1973a).

EXPERIMENTAL SECTION

Nineteen amino acid conjugates of 2,4-D were prepared by the reaction of 2,4-dichlorophenoxyacetylchloride (2,4-D-Cl) with the corresponding L-amino acid in aqueous sodium hydroxide (Wood and Fontaine, 1952). 2,4-D-Cl was synthesized in the same manner as reported by Freed (1946). The conjugates prepared in this manner were: N^{α}-(2,4-D)-L-glycine, N^{α}-(2,4-D)-L-alanine, N^{α}-(2,4-D)-L-serine, N^{α}-(2,4-D)-L-proline, N^{α}-(2,4-D)-L-valine, N^{α}-(2,4-D)-L-threonine, N^{α}-(2,4-D)-L-cysteine, N^{α}-(2,4-D)-L-leucine, N^{α}-(2,4-D)-L-isoleucine, N^{α}-(2,4-D)-L-hydroxyproline, N^{α}-(2,4-D)-L-aspartic acid, N^{α}-(2,4-D)-L-glutamic acid, N^{α}-(2,4-D)-L-methionine, N^{α}-(2,4-D)-L-histidine, N^{α}-(2,4-D)-L-phenylalanine, N^{α}-(2,4-D)-L-arginine, N^{α}-(2,4-D)-L-tyrosine, N^{α}-(2,4-D)-L-tryptophan, and N^{α}-(2,4-D)-L-cystine.

The N^{α}-lysine conjugate of 2,4-D was prepared in a modified procedure and is the first reported synthesis of this compound. N^{ϵ}-Carbobenzoxy-L-lysine (0.023 mol) was dissolved in 43 ml of 1 *N* NaOH to which was added 75 ml of H_2O. To this ice-cooled solution was added dropwise with rapid stirring 15 ml of benzene containing 2,4-D-Cl (0.046 mol). The reaction mixture was stirred for 30 min at room temperature and then transferred to a separatory funnel and extracted with ethyl ether. The aqueous layer was separated and acidified with 6 *N* HCl, which resulted in the desired product being precipitated. The aqueous suspension was filtered, and the precipitate was washed several times with distilled water and dried in a vacuum at 50°, giving N^{α}-(2,4-D)-N^{ϵ}-carbobenzoxy-L-lysine in *ca.* 75% yield. This white solid was dissolved in acetone–95% ethanol (2:1; v/v) and purified by thin-layer chromatography (tlc) employing solvent system II (Table III). The desired product (R_f 0.36–0.44, mp 128–129°) was eluted from the adsorbent with 95% ethanol.

The carbobenzoxy group was removed by hydrogenation. To the above ethanol solution containing N^{α}-(2,4-D)-N^{ϵ}-carbobenzoxy-L-lysine was added a few drops of acetic acid, 50 mg of platinum oxide, and a stream of hydrogen for 30–40 min with stirring. N^{α}-(2,4-D)-L-lysine was further purified with thin-layer chromatography employing solvent system II. The N^{α}-(2,4-D)-L-lysine, which was eluted from the tlc adsorbent, was recrystallized from ethanol, giving 0.013 mol (56% overall yield, mp 90–91°).

All the conjugates were purified by recrystallization or by tlc. The mass spectra were obtained employing an AEI Model MS902 mass spectrometer using a direct sample inlet system. Tlc was employed using Supelcosil 12A (Supelco, Inc.) as the adsorbent and a zinc phosphor for detection. Six thin-layer and one paper chromatographic solvent system was used (Table III). Descending paper chromatography on Whatman No. 1 was used.

RESULTS AND DISCUSSION

The mass spectra of the 20 amino acid conjugates of 2,4-D are given in Tables I and II. All the conjugates, except for histidine, exhibited molecular ions (P) and characteristic fragmentation patterns, including the corresponding chlorine isotopic ions. Prominent ions arising from only the 2,4-D portion of the molecule are the following: m/e 220, 219, 184, 175, 162, 161, 145, 133, 109, 98, 74, and 63 and their corresponding intense isotopic ions. An explanation of the origin of these common ions is shown in Figure 1.

The upper region of the spectra ($>m/e$ 223, Table II) is characteristic of the specific conjugate and particularly useful for identification. The main fragments, $>m/e$ 223, can be grouped into four types as follows: (a) parent-

Departments of Entomology and Biology, Pesticide Research Laboratory and Graduate Study Center, The Pennsylvania State University, University Park, Pennsylvania 16802.

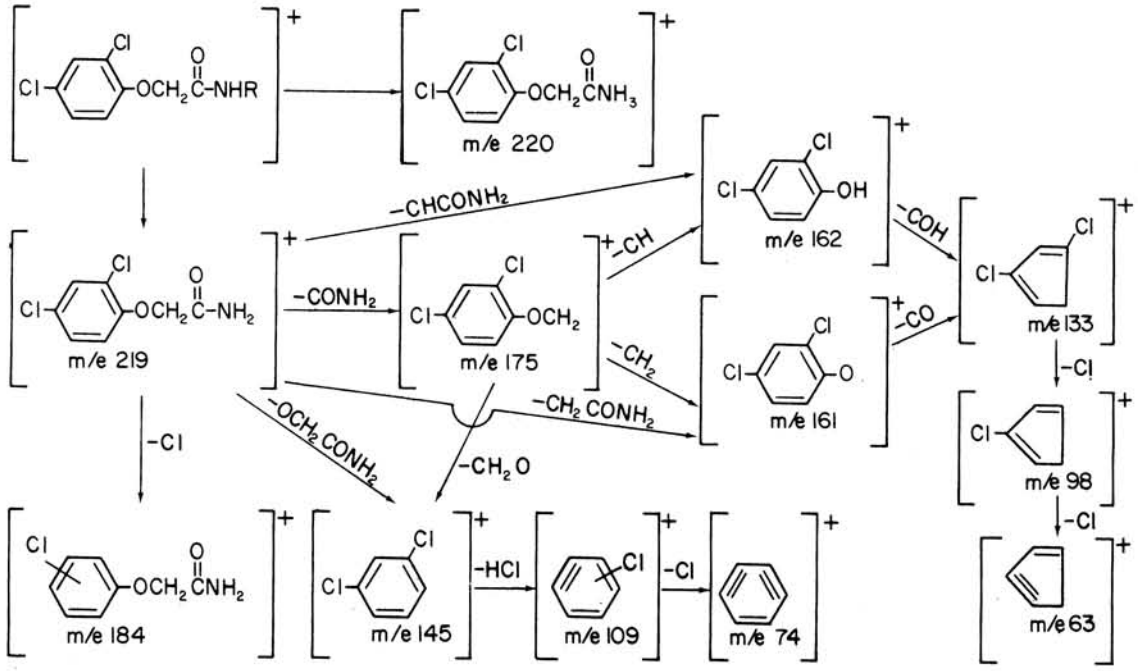

Figure 1. Prominent mass spectral ions arising from the fragmentation of the 2,4-D portion of the molecule of amino acid conjugates of 2,4-D.

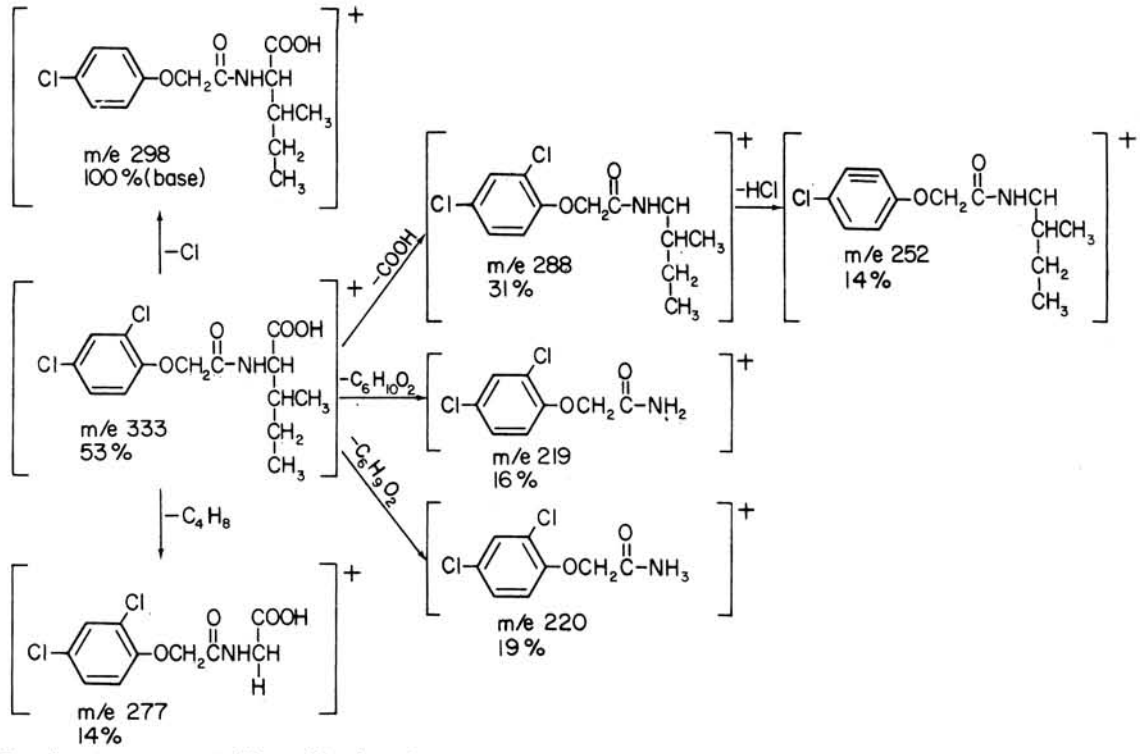

Figure 2. Prominent mass spectral ion arising from fragmentation of 2,4-D-Ile ($m/e > 219$).

Cl (P-35); (b) P-COOH (P-45); (c) P-H_2O (P-18); and (d) P-side chain fragmentation. The side chain fragmentation is similar to the side chain fragmentation previously reported for peptides and derivatives (Biemann *et al.*, 1966).

Figure 2 presents an interpretation of the mass spectral fragmentation pattern of 2,4-D-Ile and is typical of most of the conjugates. A prominent molecular ion is present (m/e 333, P, 53%) which readily looses chlorine (m/e 298, PCl, 100%), or a carboxyl group (m/e 288, PCOOH, 31%), or a carboxyl group and HCl (m/e 252, PHClCOOH, 14%), or chain fragmentation (m/e 277, PC_4H_8, 14%). An explanation of the fragmentation pattern of 2,4-D-Glu and 2,4-D-Asp has been given previously (Feung *et al.*, 1971, 1972). Similar characteristic and recognizable spectra would also be expected for the amino acid conjugates of indole-

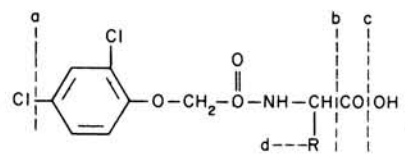

Table I. The Mass Spectra of Amino Acid Conjugates of 2,4-D <*m/e* 223

	Relative intensity of ions																			
m/e	Gly	Ala	Ser	Pro	Val	Thr	Cys	Leu	Ile	Hyp	Asp	Lys	Glu	Met	His	Phe	Arg	Tyr	Trp	Cys'
41			18	27	39	22		4	57	4	9	47	92	28	6	8	14			89
42	30	35	42	15	61	44	22	3	20	12	12	40	99	19	7	14	11	17	21	98
43	14	37	18	39	100	100	20	4	31	100	49	100	74	25	100	9	29	26	20	75
44	8	100	20	9		57	18		17	7		39	44	22	6		24		35	71
45					46		20		18			77	29	19	14					59
46												18								
47							20							19						
50																		7		
51												17				11		11	20	
52																			6	
53																			7	
54			27																	96
55				18	100				29			47					15	5		
56	21	59		16	54		28		17		9	41		37				7		57
57		25			44				47	3		21								
58	20					71	31			76	10	26			40	9	9			73
59					50		64			3		30			17					75
60	16											47								
61														100						71
62											7						8			73
63	20	21	26	29	61	26	51	3	20	2	29	15	83	28	11	18	38	25	35	98
64															7					
65												23				28		9		
66												23								
67																				
68				16									68				9			
69					33	19			49			21						5		
70		36		74	85	24					8	19	23				40			
72	9	48	15		98							16								
73	9	17	13	16	33	10	22	4			22		30		6		9			84
74	38	13	17	16	74	16	36	13	33		7		26	32	8	12			25	93
75	15	19	19	21	61	11	28				9		33	46		11			29	86
76							18													
77												55				20			100	
79												17				10				
80																				
82			19									24	47							68
83				38								55								
84					83			7			12	63	100				15			89
85													72							
86		23						13	22		7	30								
87							24				6							7		
88	33	37				6	45	8												
89																		5		
90																		6		
91																100				
92																20				
94																				
97						10						67								52
98	10	6	16	18	100	26	37	5	29	2	27	29	51	37	7	3	31	7	15	96
99	6	3	7	9	78	7	24	2	10	1	11	15	22	5	3	2	14	5	7	96
100	4	3	31	7	78	7	19	1	8	1	10	20	22	25	3	1	11	3	5	91
101																			16	
102		12											24						40	
103																14			81	
105																12				
107												27						100		
108												16						35		
109	17	16	22	24	65	10	21	6	14	1	12		32	15	5	13	5	18	21	91
110													26					8		43
111	20	19	29	27	78	11	25	9	22	2	17	20	42	21	6	19	9	26	28	96
112				32				4		2								9		
113					39							16						9		
114				24	65			6			11									
115					100														29	
116	23					9	21	10	29					46						80
117																			38	

Table I *(Continued)*

	Relative intensity of ions																			
m/e	Gly	Ala	Ser	Pro	Val	Thr	Cys	Leu	Ile	Hyp	Asp	Lys	Glu	Met	His	Phe	Arg	Tyr	Trp	Cys′
120							16									16				
123																		6		
126								4			21		21				12			50
127								6			6	19	7				4			
128			20					61		2	19	29	16				4		30	61
129								5											58	
130		23						33											100	
131																			100	
132																16				
133	12	10	19	27	59	7	16	17	94	2	12		24	4	7	9	5	15	16	82
134	2	1	2	2	6	1	5	2	16		2		5	1	1	1	1	7	3	9
135	8	7	12	19	41	5	11	11	65	1	8		15	2	5	6	5	10	12	52
136																		6		
139								6												
140								4												
141	14							6									12			
143																			34	
144								6				43							25	
145	21	21	31	29	85	12	33	64	22	2	17	9	35	23	7	21	5	33	36	96
146	4	4	6	5	13	2	7	10	4	1	2	3	9	6	1	7	1	6	10	21
147	18	16	26	24	70	10	26	49	20	1	14	5	29	20	5	28	4	29	27	93
148							28									28		10		41
149																8		8		
153																	12			
154								4												
155				25													28			
156											10								24	
158					24			9											19	
161	8	11	18	34	30	7	25	18	10	2	15		19		10	11		8	21	77
162	52	27	100	100	80	42	100	100	88	9	100	26	100	100	36	15	100	23	40	100
163	9	10	18	29	17	7	21	17	12	2	20	4	23	12	9	7	9	12	7	82
164	33	18	72	74	52	29	71	67	47	6	95	17	94	66	25	9	71	100	27	98
165				15				6										24		
166								11									10			41
170												29	100							
172								30												
173								4												
175	21	27	39	43	65	14	72	67	43	2	25	6	40	30	10	31	4	36	26	98
176	9	10	7	7	13	3	13	23	8	1	4	3	9	10	1	4	1	4	5	36
177	13	18	25	29	46	9	47	61	29	2	17	4	26	19	7	21	3	24	17	89
183	4																			
184	1		10	5	48		50	41	57	2	5				17	59	4	10		64
185	2		3	1	7		10	6	10	1	2				4	9	1	2		9
186			4	2	17		19	13	20	1	2				6	25	1	4		18
187																			100	
188																			46	
196	12																			
197	2																			
198	6																			
203																		13		
204																		5		
205																		4		
208																				18
210		6	10				6	7			22		12							27
211		1	1				1	1			3		2							5
212		2	3				2	2			7		4							9
216																		3		
219	1			59	7				16							31		6		
220		2	18	9	6	4	47	9	19	4	25	3	12	5	17	5	3	9		64
221			4	39	5	1	12	3	13	1	3	2	2	1	2	20	1	5		14
222		1	12			4	33	6	12	2	17	2	10	4	11		2	6		41

acetic acid, naphthylacetic acid, and 2,4,5-trichlorophenoxyacetic acid. The aspartic conjugates of indoleacetic acid and naphthylacetic acid have already been reported (Andreae and Good, 1955; Zenk, 1962).

Many times metabolic studies do not yield a sufficient quantity (<0.1 μg) or purity of a metabolite for mass spectral studies. Thus, other techniques must be utilized to characterize and identify unknown structures. Using radiochemical techniques, chromatographic properties can easily be obtained for a submicrogram quantity of metabolite.

Table II. The Mass Spectra of Amino Acid Conjugates of 2,4-D >m/e 222

Gly		Ala		Ser		Pro		Val		Asp		Lys		Glu		Met		His	
m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
224	25	256	59	254	60	243	6	238	13	254	4	262	19	258	8	277	68	282	2
225	6	257	9	255	8			239	2	256	2	263	5	259	2	278	9	284	1
226	10	258	20	256	19	245	4	240	4	282	24	280	22	260	6	279	46	357(P)	1
232	22	291(P)	9	272	32	271	7	274	28	283	4	281	5	268	6	290	22		
233	6			273	6			275	6	284	8	295	17	270	4	291	6		
234	15	293	4	274	12	273	4	276	19	300	37	296	5	297	30	292	15		
242	100			289	13	281	89	284	70	301	6	297	6	298	4	304	32		
243	14			290	3	282	16	286	13	302	13	314	2	299	9	305	12		
244	36			291	9	283	40	287	25	318	16	330	9	304	4	306	26		
277(P)	9			307(P)	3	317(P)	8	319(P)	6	319	2	331	5	305	1	351(P)	5		
278	2					318	5	320	2	320	12	332	7	306	2	352	2		
279	7			309	2	319	7	321	5	335(P)	1	348(P)	3	314	100	353	4		
										337	1	350	2	315	22				
														316	44				
														331	7				
														332	2				
														333	5				
														349(P)	7				
														350	2				
														359	4				

Thr		Cys		Leu		Ile		Hyp		Phe		Arg		Tyr		Trp		Cys	
m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%	m/e	%
224	7	270	49	232	11	252	14	298	3	286	1	281	9	281	2	360	21	242	71
226	2	271	8	234	7	253	3	300	1	322	2	282	2	283	1	361	22	243	11
242	15	272	18	246	5	254	6	333(P)	1	323	1	283	3	337	6	362	21	244	27
243	3	288	29	248	3	277	14	335	1	324	2	316	9	338	2	363	14	254	86
244	5	289	8	252	7	278	2			332	6	317	3	339	4	406(P)	2	255	16
259	4	290	10	254	3	279	9			333	2	318	7	365	1			256	30
261	3	323(P)	18	259	4	288	31			334	2	376(P)	3	367	1			270	46
268	16	324	7	260	5	290	19			367(P)	2	377	2	383(P)	5			271	11
269	3	325	15	272	6	298	100			368	1	378	2	384	2			272	25
270	6			274	4	299	18			369	1			385	3			277	7
277	4			277	32	300	33											278	4
279	3			278	4	333(P)	53											279	5
286	2			279	22	334	18											289	32
321(P)	1			288	36	335	35											290	14
				289	5													291	18
				290	25													323	6
				298	100													324	1
				299	23													325	2
				300	43													442(P)	1
				333(P)	5													444	1
				334	3														
				335	4														

Table III. R_f Values of Amino Acid Conjugates of 2,4-D

Compound	Solvent system[a] tlc I	II	III	IV	V	VI	pc VII	Compound	Solvent system[a] tlc I	II	III	IV	V	VI	pc VII
Gly	0.00	0.27	0.00	0.17	0.00	0.80	0.68	Asp	0.17	0.01	0.26	0.03	0.31	0.71	0.36
Ala	0.35	0.33	0.48	0.20	0.54	0.77	0.74	Lys	0.00	0.04	0.00	0.01	0.05	0.20	0.65
Ser	0.14	0.17	0.12	0.11	0.35	0.73	0.65	Glu	0.13	0.02	0.21	0.03	0.30	0.71	0.43
Pro	0.29	0.33	0.20	0.22	0.45	0.68	0.73	Met	0.37	0.43	0.47	0.28	0.51	0.74	0.74
Val	0.36	0.39	0.52	0.27	0.50	0.74	0.79	His	0.00	0.15	0.00	0.08	0.03	0.20	0.61
Thr	0.19	0.25	0.17	0.13	0.35	0.69	0.69	Phe	0.33	0.37	0.49	0.25	0.49	0.74	0.80
Cys	0.34	0.08	0.42	0.07	0.36	0.76	0.59	Arg	0.00	0.07	0.00	0.03	0.01	0.23	0.76
Leu	0.40	0.42	0.56	0.29	0.58	0.75	0.82	Tyr	0.20	0.24	0.27	0.15	0.38	0.74	0.75
Ile	0.42	0.43	0.56	0.27	0.58	0.74	0.82	Trp	0.27	0.28	0.32	0.18	0.42	0.80	0.80
Hyp	0.06	0.16	0.05	0.10	0.33	0.65	0.64	Cys'	0.17	0.10	0.45	0.07	0.37	0.82	0.60
								2,4-D	0.45	0.28	0.70	0.22	0.56	0.80	0.75

[a] 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); V, benzene–methanol–cyclohexane–formic acid (80:10:20:2, by vol); VI, 1-butanol–acetic acid–water (90:20:10, v/v/v); and VII, 1-butanol–95% ethanol–3 N ammonium hydroxide (4:1:5, v/v/v).

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

Chao-shieung Feung, Robert H. Hamilton, and Ralph O. Mumma*

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.

Departments of Biology and Entomology, Pesticide Research Laboratory and Graduate Study Center, The Pennsylvania State University, University Park, Pennsylvania 16802.

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)