Table II. Residual Radioactivity (ppm) in Sugarcane Plants 8 Weeks after Foliar Treatment with Mixture of ^{14}C-Labeled and Nonlabeled Chlorocholine Chloride in October 1972[a]

Plant part		Radio-anal.	GLC anal.
Leaves	Methanol extract	18.8	20.4
	Marc	2.0	–[b]
	Total	20.8	–
Stalks	Methanol extract	0.75	0.59
	Marc	0.11	–
	Total	0.86	–

[a] Treated at rate of 4 lb of active ingredient per acre. [b] Not determined.

0.013 ppm. The limited number of chemical assays with the GLC method was in agreement with the radioassays as is evident from the data in Table I. Because this experiment used such a low treatment rate compared to actual field conditions, another experiment was conducted in October using a dosage rate of diluted chlorocholine-^{14}C chloride equivalent to 4 lb of active ingredient per acre, which corresponds to the rate for commercial usage. Table II shows the distribution of radioactivity found in leaves and stalks 8 weeks after application. The residues of extracted radioactivity found were 18.8 ppm in leaves and 0.75 ppm in stalks. When these same samples were analyzed by the chemical procedure, the values of 20.4 and 0.59 ppm were found. Only negligible amounts of radioactivity were found in the marc.

Removal of plant pigment from sugarcane leaves before thin-layer chromatography was achieved by treatment of the methanol–acetonitrile solution with animal charcoal. Only 3% of the radioactivity was adsorbed by the charcoal and alumina, while the bulk of the radioactivity was recovered in the filtrate. The nature of the adsorbed radioactivity is not known but presumed to be parent Cycocel.

Investigations with various organic solvents using silica gel plates revealed that acetonitrile–water–acetic acid (60:40:2) gave the best separation of chlorocholine chloride from its reported metabolites (choline chloride and betaine) using one-dimensional chromatography.

An example of the autoradiograms obtained from thin-layer analysis of leaf extracts and mixtures of control extracts with authentic chlorocholine-^{14}C chloride is shown in Figure 1. Only one spot was observed demonstrating that only the labeled chlorocholine chloride was present.

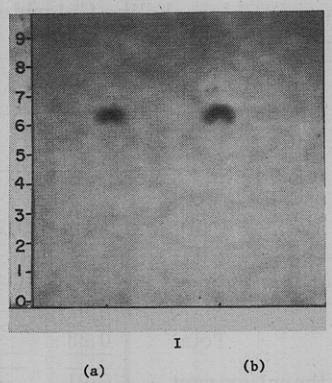

Figure 1. Thin-layer chromatography of the methanol-extractable radioactivity found in sugarcane leaves 8 weeks after treatment (second experiment, Oct 1972): (a) sample; (b) fortified control.

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Indole-3-acetic Acid. Mass Spectra and Chromatographic Properties of Amino Acid Conjugates

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Twenty amino acid conjugates of indole-3-acetic acid were synthesized and characterized by mass spectrometry and paper and thin-layer chromatography. Molecular ions and a base peak of *m/e* 130 were observed for most conjugates. The mass spectral fragments of high *m/e* (>175) are few in number, predictable, and correlated with specific amino acid derivatives.

A number of plant species convert indole-3-acetic acid (IAA) into several ether-insoluble (at neutral pH) metabolites. One of these metabolites has been identified as the amino acid conjugate, indole-3-acetylaspartic acid (IAA-Asp). Indole-3-acetylysine (IAA-Lys) has also been isolated from certain strains of *Pseudomonas savastanoi* (Hutzinger and Kosuge, 1968). IAA-Asp has been identified in most cases through its chromatographic properties, color tests, and, in a few instances, biological activity (Andreae and

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Table I. Mass Spectra of Amino Acid Conjugates of IAA <*m/e* 176

m/e	IAA	Gly	Ala	Ser	Pro	Val	Thr	Cys	Leu	Ile	Hyp	Asp	Lys	Glu	Met	His	φAla	Arg	Tyr	Trp	Cys′
77	26	18	30	24	33	18	26	24	47	30	91	36	41	13	24	15	53	61	28	21	77
91				4						30			34			9	74		11		
92				5			20				41										
93								15													
94																					100
102	13	9	11	11	21	8	22	13	13	15	38	17	8	16	15	16	35	20	13	15	17
103	19	13	19	15	21	13	21	14		12	49	23	19	11	16	12	45	16	17	15	21
104		8	6		19	8	18		35	15	23		56		11	8	27				24
105									29				37								
107																			100		
108																			100		
109								23													
115																		52			
116							16														
117	4	4	6	5	21	11	17	15	39	21	22	4	100	15	14	64	45	28	11	17	24
118																					
119		8	10		20		21				80		25					74			
128	10	7	8			7	16	9			27	9	14	17	12		27	20	10	12	21
129	12	11	18	17	35	13	24	17	18	18	73	16	14	19	25	33	50	46	19	19	24
130	100	100	100	100	100	100	100	100	100	100	100	100	72	100	100	100	100	100	100	100	100
131	25	19	43	27	23	27	25	23	24	24	62	33	37	21	24	51	53	33	17	19	25
132	7		6			9	13				21			17			12	15	8		9
133							23		41				69	15			14	24	9	15	26
134																					
137																		36			
139								43													
142							17														
143							17														
144			5	5									17			14					24
145			6								16		16	8		15					22
146							11							7							
147			6				22				45		19								
149							14	12							17			54			
150																				10	
155																	16				
156									14							5	16			6	
157	1	5	5	16	21	8	20	10	15	10	44	4	14	17	29	5	32	59	17	13	24
158														15						15	
160													20								
162																					16
164								13										83			11
165															13						
167																	7				
168																	8			7	
169					12						7						8				
170					9					5											13
172		3	5			3	7				6										
173			3			2	5				5										
174				10		3	5	10											62	14	15
175	63			22			15	11			6	33	9	17	11				14		

Good, 1955; Good et al., 1956; Andreae and van Ysselstein, 1956, 1960; Fang et al., 1959; Bennet-Clark and Wheeler, 1959; Klämbt, 1961; Thurman and Street, 1962; Wightman, 1962; Zenk, 1963; Sudi, 1964, 1966; Winter and Thimann, 1966; Olney, 1968; Robinson et al., 1968; Morris et al., 1969; Beyer and Morgan, 1970; Lau et al., 1974).

Conjugation of externally applied plant growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid with aspartic acid has also been commonly observed (Beyer and Morgan, 1970; Veen, 1972; Venis, 1972; Goren and Bukovac, 1973). Feung et al. (1971, 1972, 1973b) have detected 2,4-D conjugates with aspartic acid, glutamic acid, alanine, valine, leucine, phenylalanine, and tryptophan in soybean callus. These 2,4-D conjugates have also been shown to possess significant biological activity (Feung et al., 1974). Recently in a preliminary report Hamilton et al. (1974) detected the presence of some unidentified amino acid conjugates of IAA in *Parthencissus tricuspidata* crown gall tissue. Other investigators have reported a number of unidentified conjugated metabolites of IAA (Thurman and Street, 1962; Wightman, 1962). Therefore, 20 amino acid conjugates of IAA were synthesized and their mass spectral fragmentation patterns and chromatographic properties were determined in order to further our knowledge concerning amino acid conjugates and to aid in metabolism studies with IAA.

Table II. Mass Spectra of Amino Acid Conjugates of IAA >*m/e* 176

Gly		Ala		Ser		Pro		Val	
m/e	%	*m/e*	%	*m/e*	%	*m/e*	%	*m/e*	%
186	4	200	3	185	11	185	3	185	2
214	11	228	11	198	8	198	13	195	2
232 (P)	26	229	2	200	12	225	21	228	4
		246 (P)	33	214	3	227	18	244	1
				226	5	254	3	256	14
				233	2	272 (P)	4	274 (P)	12
				244	17				
				262 (P)	5				

Thr		Cys		Leu		Ile		Hyp	
m/e	%	*m/e*	%	*m/e*	%	*m/e*	%	*m/e*	%
185	4	185	3	182	22	242	5	198	29
212	6	191	6	184	7	270	13	217	6
214	16	192	11	185	5	288 (P)	3	224	2
217	6	200	3	197	14			244	3
232	23	226	4	198	8			270	10
233	5	244	2	240	7			288 (P)	21
258	10	260	4	258	7				
276 (P)	3	278 (P)	4	270	11				
				288 (P)	7				

Asp		Lys		Glu		Met		His	
m/e	%	*m/e*	%	*m/e*	%	*m/e*	%	*m/e*	%
185	1	181	5	185	1	185	5	188	4
200	2	240	3	202	4	191	9	217	5
227	3	257	3	214	1	192	11	233	2
245	1	285	3	245	2	214	6	245	29
272	17	303 (P)	0.2	258	3	245	8	256	11
				259	4	258	9	270	3
				260	3	259	7	312 (P)	0.2
				286	14	262	4		
				304 (P)	3	306 (P)	5		

ϕAla		Arg		Tyr		Trp		Cys′	
m/e	%	*m/e*	%	*m/e*	%	*m/e*	%	*m/e*	%
185	3	177	4	185	6	184	4	185	14
217	5	182	7	214	1	185	3	198	16
218	6	185	2	231	22	215	3	200	15
232	6	191	6	292	3	256	3	397 (P)	0.1
233	10	192	14	293	4	269	2		
257	10	220	5	320	9	271	3		
258	6	234	12	338 (P)	15	297	4		
276	7	271	25			315	6		
278	4	272	7			331	2		
304	15	285	5			343	1		
305	5	287	6			361 (P)	0.4		
322 (P)	5	313	2						
		331 (P)	0.6						

EXPERIMENTAL SECTION

The preparation of the amino acid conjugates of IAA is basically a modification of the method used by Mollan et al. (1972). The nitrophenol ester of IAA and the corresponding L-amino acid dissolved or suspended in 50% methanol containing 50 μM tetramethylguanidine were stirred for various times (2–28 hr) at room temperature. To this reaction mixture containing the crude amino acid conjugates was added 50 ml of water and the entire solution was extracted twice with 50 ml of diethyl ether. The aqueous fraction (Aq_1) was acidified to pH 5 with concentrated HCl and the resulting solution extracted three times with 50 ml of diethyl ether. The aqueous fraction (Aq_2) was further acidified to pH 1 with HCl and the resulting solution extracted twice with 1-butanol (40 ml and 10 ml). The 1-butanol fraction was washed four times with 10–15 ml of water and then the 1-butanol fraction was evaporated to dryness resulting in colored residues. N^{α}-(IAA)-L-aspartic acid and N^{α}-(IAA)-L-glutamic acid were directly recrystallized from ethanol. The valine, leucine, isoleucine, methionine, and tryptophan derivatives were purified by thin-layer chromatography (TLC) of the 1-butanol extract fraction (solvent system II, Table III). The desired product was eluted from the absorbent with 80% ethanol which was subsequently evaporated to dryness. The other amino acid conjugates (Gly, Ala, Ser, Pro, Thr, Cys, Hyp, ϕAla, Trp,

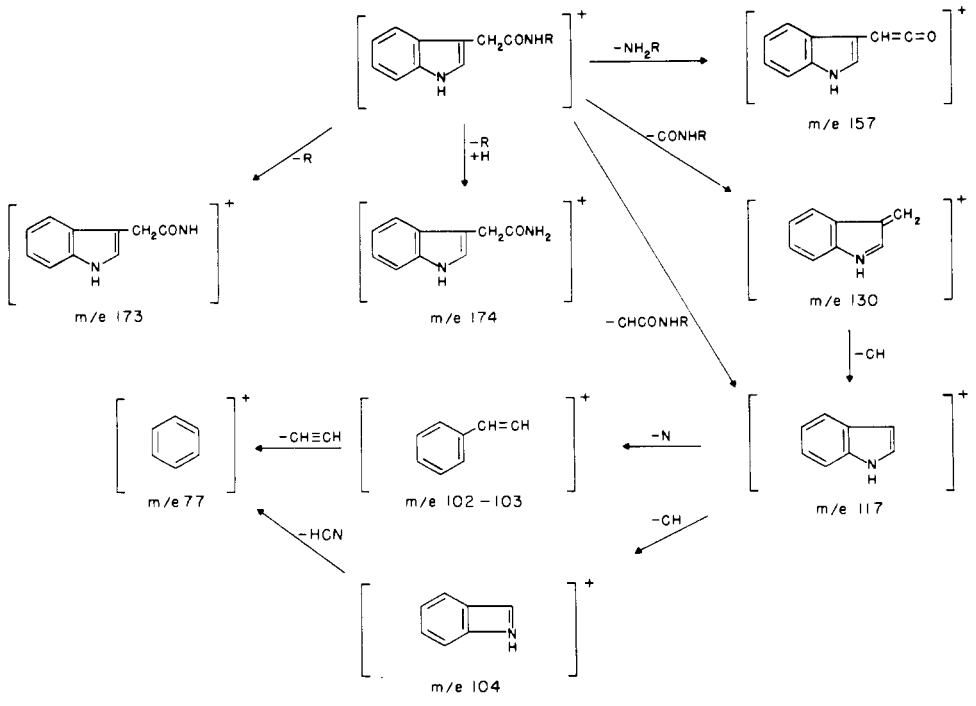

Figure 1. Prominent mass spectral ions arising from the fragmentation of the IAA portion of the molecule of amino acid conjugates of IAA.

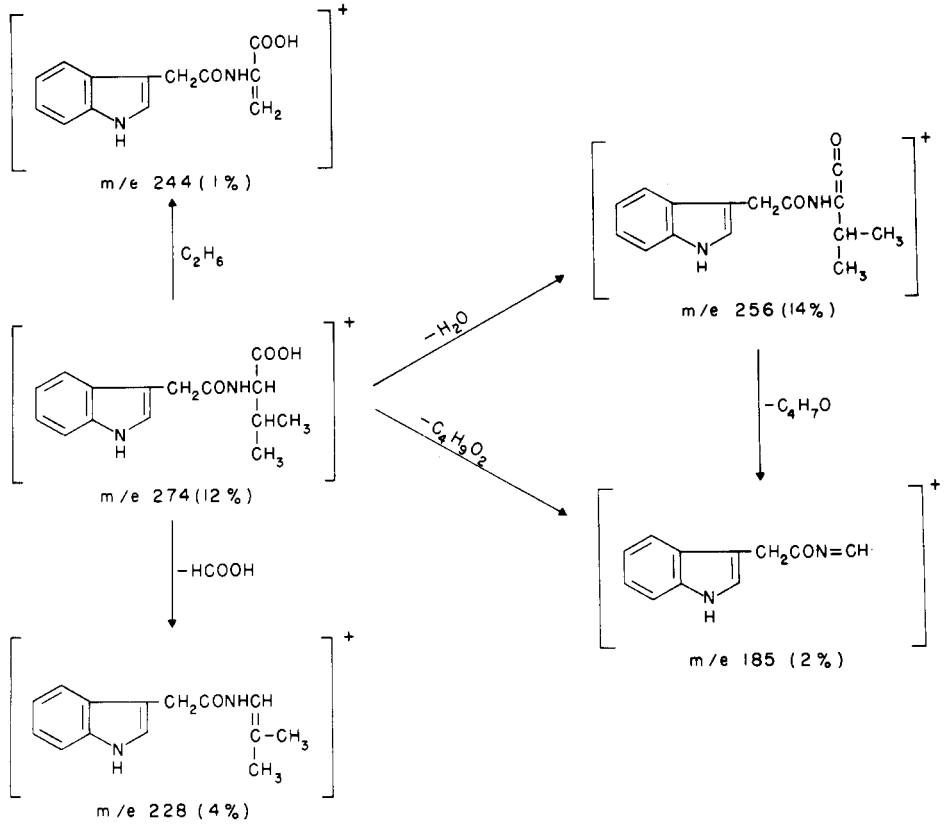

Figure 2. Prominent mass spectral ions arising from fragmentation of IAA-Val (*m/e* >175).

Cys′, Lys, His, and Arg) were isolated by TLC directly from the ether and 1-butanol phases.

Supelcosil 12A (Supelco, Inc.) was used as the absorbent for TLC and a zinc phosphor was used for detection. The mass spectra were obtained employing an AEI Model MS902 mass spectrometer using a direct inlet system. Three TLC and four paper chromatographic (PC) solvent systems were used (Table III) in characterizing the amino acid conjugates and to check their purity.

RESULTS AND DISCUSSION

The mass spectra of 20 amino acid conjugates of IAA are given in Tables I and II. All of the conjugates exhibited molecular ions and characteristic fragmentation patterns, and all conjugates except IAA-Lys have *m/e* 130 as their base peak, presumably the 3-methylene indole ion. Prominent ions arising from only the IAA portion of the molecule are *m/e* 174, 173, 130, 117, 104, 103, 102, and 77 and are inter-

Table III. R_f Values of Amino Acid Conjugates of IAA

		Solvent system[a]						
		TLC			PC			
Compd	Mp, °C	I	II	III	I	IV	V	VI
Gly	220–221	0.52	0.43	0.35	0.39	0.23	0.25	0.23
Ala	200–203	0.57	0.52	0.43	0.48	0.48	0.35	0.32
Ser	195–200	0.36	0.27	0.09	0.30	0.08	0.24	0.18
Pro	210–215	0.47	0.57	0.37	0.40	0.10	0.33	0.41
Val	168–170	0.53	0.63	0.47	0.56	0.77	0.45	0.65
Thr	215–220	0.36	0.30	0.10	0.41	0.10	0.27	0.20
Cys	100–102	0.30	0.30	0.27	0.18	0.07	0.14	0.10
Leu	178–181	0.57	0.67	0.48	0.60	0.85	0.49	0.65
Ile	168–170	0.57	0.67	0.48	0.60	0.85	0.49	0.65
Hyp	210–211	0.37	0.30	0.19	0.30	0.05	0.16	
Asp	165–166	0.15	0.30	0.27	0.18	0.06	0.03	0.12
Lys	124–127	0.27	0.02	0.00	0.31	0.00	0.16	0.40
Glu	182–184	0.21	0.37	0.28	0.07	0.05	0.04	0.10
Met	165–168	0.55	0.62	0.45	0.53	0.45	0.45	0.40
His	228–232	0.41	0.04	0.01	0.07	0.05	0.03	0.03
Phe	174–176	0.50	0.64	0.49	0.55	0.85	0.48	0.02
Arg	144–146	0.21	0.05	0.01	0.31	0.00	0.24	0.34
Tyr	222–223	0.51	0.47	0.41	0.48	0.23	0.28	0.28
Trp	262–264	0.59	0.61	0.45	0.42	0.58	0.45	0.46
Cys′	127–128	0.37	0.30	0.27				
IAA		0.43	0.65	0.56	0.30	0.78	0.24	0.75

[a] I, 2-propanol–concentrated ammonia hydroxide–water (8:1:1, v/v/v); II, chloroform–methanol–acetic acid (75:20:5, v/v/v); III, chloroform–ethyl acetate–formic acid (35:55:10, v/v/v); IV, benzene–1-butanol–acetic acid–water (95:5:5:1, v/v/v/v); V, ethyl acetate–2-propanol–acetic acid–water (90:50:1:1, v/v/v/v); VI, petroleum ether–diethyl ether–methanol–acetic acid–water (60:60:15:1:2, v/v/v/v/v).

preted in Figure 1. Their elemental composition has been confirmed by high-resolution mass spectrometry. The prominent ion *m/e* 175 common to nine of the conjugates is derived from free IAA, a minor contaminant of these conjugates.

The upper region of the mass spectra of all the conjugates (*m/e* >175) can be grouped into three types as follows: (a) molecular ions minus H_2O (M − 18); (b) M − HCOOH or M − COOH or M − CO_2; (c) M − side-chain fragmentation. The side-chain fragmentation is similar to that previously reported for peptides and 2,4-D amino acid derivatives (Biemann et al., 1961; Feung et al., 1973a).

An interpretation of the upper region (*m/e* >175) of the mass spectral fragmentation pattern of IAA-Val is shown in Figure 2 and the pattern is also typical of most of the conjugates. The mass spectrum of IAA-Asp has been reported by Mollan et al. (1972). Similar characteristic and recognizable spectra would also be expected for the amino acid conjugates of naphthaleneacetic acid.

Because metabolites are not always available in pure form or in sufficient amounts other techniques must sometimes be employed in characterization and identification of unknown metabolites. Using radiochemical techniques, chromatographic properties can easily be obtained for a submicrogram quantity of metabolites. Therefore, the chromatographic properties of the 20 amino acid conjugates of IAA were determined. The R_f values of these synthetic conjugates in three TLC solvent systems and in four paper chromatographic solvent systems are given in Table III along with their melting points. The Lys, His, and Arg conjugates were well separated by TLC in the solvent system of 2-butanol–formic acid–water (75:5:5). The Cys and Cys′ conjugates and the Val, Leu, Ile, and Phe conjugates have similar chromatographic properties and cannot be easily separated (Table III); however, they can be easily identified by mass spectrometry.

In the characterization and determination of the structure of amino acid conjugates of IAA, mass spectral and chromatographic data collectively provide additional information for the identification of structurally unrecognized amino acid conjugates of IAA. Furthermore, these data should be useful for the characterization of amino acid conjugates of other plant growth regulators. The mass fragmentation products of amino acid conjugates of IAA of high *m/e* (>175) are few in number, predictable, and correlated with the specific amino acid derivative.

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