Metabolism of 2,4,5-Trichlorophenoxyacetic Acid. Evidence for Amino Acid Conjugates in Soybean Callus Tissue

Masood Arjmand, Robert H. Hamilton, and Ralph O. Mumma*

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) is metabolized by soybean cotyledon callus tissue to ether- and water-soluble metabolites after 6 and 15 days incubation. The ether fraction consisted of ca. 93% of the extractable ^{14}C label and was separated into seven bands on thin-layer chromatography. Only one major band was present (84.8 to 89.2%) besides unmetabolized 2,4,5-T, which was identified as a mixture of the glutamic acid (major) and aspartic acid (minor) conjugates of 2,4,5-T. The high recovery of radioactivity (99.1 to 91.9%) precludes appreciable decarboxylation in this tissue.

Nearly 300 million lbs of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Byrnes and Holt, 1976) have been applied to control unwanted woody species on utility rights-of-way, along roadside ditch banks, on rangeland, industrial sites, water sheds, and forest lands. Despite the wide use of 2,4,5-T, little work has been done on its metabolism by plants. Primer (1967) reported a small rate of decarboxylation of 2,4,5-T by Winesap and Staymann Winesap cultivars. Beans, sunflowers, and barley converted 2,4,5-T to 2,4,5-trichlorophenol (Chkanikov et al., 1965). Fitzgerald (1966) also detected 2,4,5-trichlorophenol in sweetgum and southern red oak leaves 1 month after application with 2,4,5-T. Hamilton et al. (1971) reported ring hydroxylation in bean plants to form glycosides of 4OH-2,5-D.

Recent investigations on the fate of 2,4-D in plants and plant tissue cultures (Feung et al., 1971, 1972, 1973) revealed the presence of 2,4-D amino acid conjugates as major metabolites of this compound. Also plants form amino acid conjugates of indole-3-acetic acid (Feung et al., 1976). The purpose of this study was to investigate the possibility of the formation of amino acid conjugates as metabolic products of 2,4,5-T. Because of the advantages of using plant tissue cultures, this study was conducted on soybean cotyledon callus tissue rather than whole plants.

EXPERIMENTAL SECTION

Soybean (*Glycine max* L. Merrill var. Acme) cotyledon callus stock cultures were grown on an agar solidified medium of Miller (1963) with 3% sucrose, α-naphthalene acetic acid (2.0 mg/L), and kinetin (0.5 mg/L). The stock cultures were maintained under low intensity fluorescent light at 27 °C and were routinely subcultured asceptically on the same medium once a month.

Four-week-old callus clumps were transferred to a sterile petri dish and sliced with a sterile scalpel. Approximately 8–10 g of the callus pieces was asceptically placed into each 125-mL erlenmeyer flask containing 50 mL of sterile liquid medium with 7 μCi of 2,4,5-T-*1*-^{14}C (sp act. 4.30 mCi/mM) in place of NAA. The labeled 2,4,5-T was dissolved in 140 μL of ethanol and added to the autoclaved nutrient medium (15 min, 121 °C, 15 psi). The tissues were then incubated at room temperature with gentle shaking with 2,4,5-T-*1*-^{14}C for 6 to 15 days. Following incubation the tissue was surface rinsed with cold distilled water on filter paper in a Buchner funnel. The rinsed solution was removed by suction and the medium was saved and analyzed. The surface rinsed tissue was weighed and stored in plastic bags at −20 °C.

The frozen callus was thoroughly ground in 95% ethanol with a VirTis homogenizer. The homogenate was filtered in a Buchner funnel with suction, and the residue was rinsed repeatedly with 80% ethanol. The procedure of extraction and fractionation were similar to methods previously described (Hamilton et al., 1971). The ethanol fraction was concentrated and was extracted three times with diethyl ether (pH 3.0). The acidic 2,4,5-T metabolites were extracted out of the ether with 5% $NaHCO_3$. Following acidification (pH 3.0) of this bicarbonate solution, the metabolites were reextracted into diethyl ether. The aqueous fraction remaining after the initial ether extraction was washed three times with 1-butanol. The 1-butanol fraction was concentrated to dryness and incubated with Emulsin (β-glucosidase) in an aqueous buffer (pH 4.5). The aglycons were recovered by subsequent acidification (pH 3.0) and extraction into diethyl ether. The scheme of extraction is presented in Figure 1.

The radioactivity in each fraction was measured by liquid scintillation counting (Aquasol). The residue was combusted by oxygen flask method of Kalberer and Rutschman (1961) prior to liquid scintillation counting in Brays solution (Bray, 1960). All counts were corrected for quenching by the external standard channels ratio method. Initial separation of the ether-soluble metabolites were accomplished by thin-layer chromatography (TLC) using the solvent system, diethyl ether–petroleum ether–formic acid (70:30:3). The relative amount of each radioactive metabolite, as detected by autoradiography, was determined by eluting the appropriate region of chromatograms and counting the radioactivity of the eluent. Further separation of 2,4,5-T-Asp and 2,4,5-T-Glu was achieved by high-pressure liquid chromatography (LC). A μBondapak C_{18} column, 30 cm × 4 mm i.d. (Waters Associates), was used with methanol–water–PIC Reagent A as the solvent.

Partial characterization of the most abundant ether-soluble metabolites after 6 and 15 days of incubation was accomplished by TLC of the unknowns before and after acid hydrolysis (6 N HCl at 65 °C for 24 h). Chromatograms were examined by autoradiography, and released amino acids were detected by a ninhydrin spray. The synthetic 2,4,5-T-Asp and 2,4,5-Glu (Arjmand et al., 1978a) and the isolated ether-soluble unknowns (Et_3) were esterified with diazomethane and the dimethylated products were characterized by mass spectrometry (LKB Model 9000).

RESULTS AND DISCUSSION

After 6 days of incubation, ca. 89% of the applied 2,4,5-T was taken up by soybean tissue (8–10 g). The amount of radiolabel in the alcohol extract, the diethyl ether, and

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

0021-8561/78/1426-1126$01.00/0

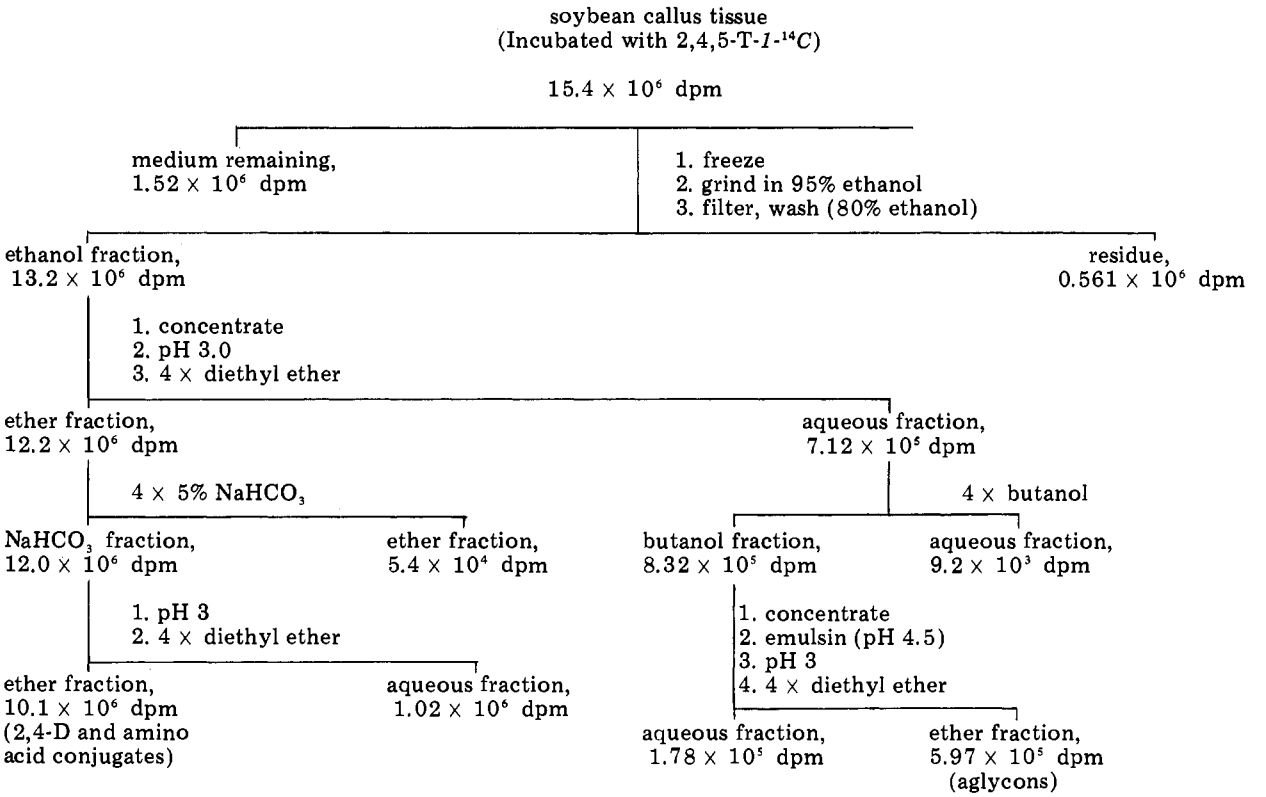

Figure 1. Extraction procedure and radioactivity in various fractions from soybean cotyledon callus tissue incubated with 2,4,5-T-*1*-^{14}C for 6 days.

Table I. Relative Composition of Various Fractions of Soybean Cotyledon Callus Tissue Incubated with 2,4,5-T-*1*-^{14}C

fraction	6-day incubation		15-day incubation	
	% in tissue	% of applied	% in tissue	% of applied
alcohol soluble	95.9	85.6	90.8	44.5
ether fraction	89.2	79.6	84.8	41.5
1-butanol fraction	6.0	5.4	8.8	4.3
residue	4.1	3.6	9.2	4.4
incubation medium		9.9		43.0

aqueous soluble fractions as well as the residue was determined, and the results of a typical experiment are presented in Table I. The ether-soluble fraction (which should contain all the amino acid conjugates) consisted of ca. 93% of the ^{14}C-label extractable from the tissue and thus attention was focused on the analysis of this ether extract. The ^{14}C-labeled metabolites of this fraction were separated into seven regions or bands on TLC which were

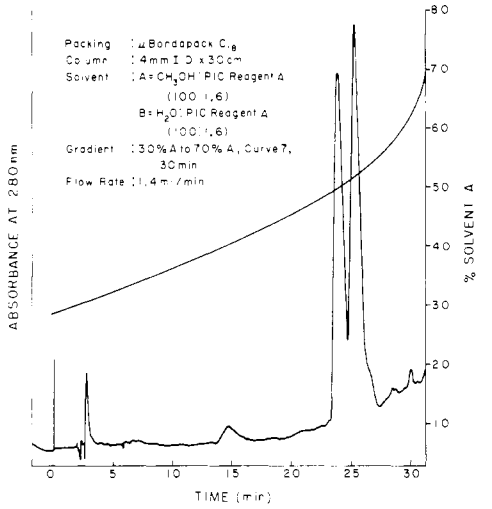

Figure 2. High-pressure liquid chromatography separation of 2,4,5-T-Glu and 2,4,5-T-Asp.

Table II. Relative Composition of Diethyl Ether Soluble Extract of Soybean Cotyledon Callus Tissue Incubated with 2,4,5-T-*1*-^{14}C

R_f region[c]	designation	6-day incubation[a]		15-day incubation[b]	
		% in fraction	% in tissue	% in fraction	% in tissue
0.03-0.08	Et_1	6.3	4.5	5.8	5.1
0.10-0.13	Et_2	3.9	2.9	3.9	3.4
0.22-0.24	Et_3 (2,4,5-T-Glu 2,4,5-D-Asp)	66.6	48.8	55.3	48.7
0.42-0.45	Et_4	2.0	1.6	0.8	0.7
0.50-0.53	Et_5	0.5	0.4	0.9	0.8
0.56-0.59	Et_6	1.3	0.9	1.1	1.0
0.64-0.73	Et_7 (2,4,5-T)	19.4	14.2	32.0	28.2
	Total	100%	73.3%	100%	87.9%

[a] Four-week-old soybean cotyledon callus tissue. [b] Five-week-old soybean cotyledon callus tissue. [c] TLC employing the solvent system diethyl ether-petroleum ether (38:46)-formic acid (70:30:3, v/v/v).

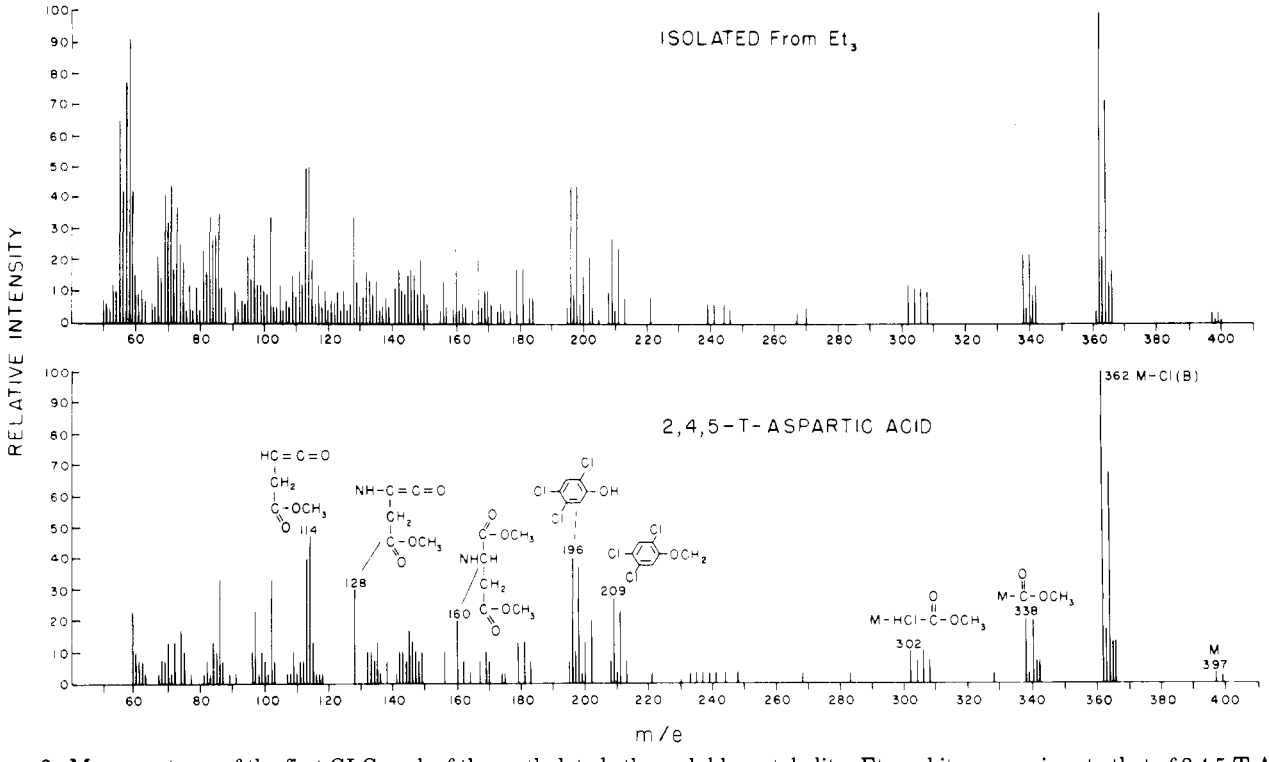

Figure 3. Mass spectrum of the first GLC peak of the methylated ether soluble metabolites Et_3 and its comparison to that of 2,4,5-T-Asp.

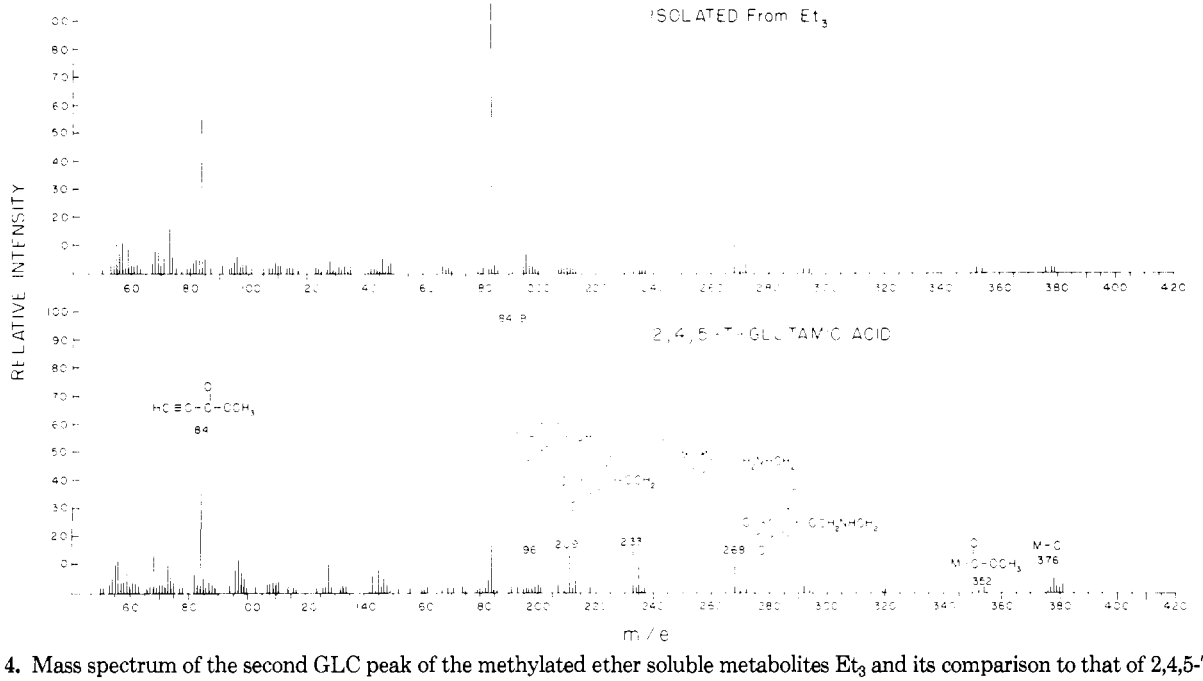

Figure 4. Mass spectrum of the second GLC peak of the methylated ether soluble metabolites Et_3 and its comparison to that of 2,4,5-T-Glu.

arbitrarily designated Et_1 through Et_7. The relative TLC mobility and abundance of these metabolites from tissue incubated with 2,4,5-T-*1*-^{14}C for 6 and 15 days are presented in Table II. Two major radiolabeled bands (Et_3 and Et_7) are present in both incubations and consist of 86.0–87.5% of the ether fraction. Et_7 (19.4–32.0%) cochromatographs on TLC, LC, and gas–liquid chromatography (GLC) with standard 2,4,5-T and therefore Et_7 represents unmetabolized 2,4,5-T in the plant tissue. This is consistant with previous studies on the metabolism of 2,4-D in which significant amounts of free 2,4-D were found in the plant tissue (Feung et al., 1973, 1975).

The TLC mobility of the major ether-soluble metabolite Et_3 (55.3–66.6%), corresponds with the general mobility of two amino acid conjugates, 2,4,5-T-Asp and 2,4,5-T-Glu (Arjmand et al., 1978a) and suggested that Et_3 might be a mixture of these two conjugates. Et_3 was subjected to acid hydrolysis (6 N HCl, 65 °C, 24 h), and the products were analyzed by TLC indicating the presence of at least 2,4,5-T and glutamic acid in the hydrolyzate. Et_3 from the 6-day incubation was added to 1 mg/mL of synthetic 2,4,5-T-Glu and 2,4,5-T-Asp and the sample subjected to LC (Figure 2). The 2,4,5-T-Glu, 2,4,5-T-Asp, and the void volume of solvent were collected, and each fraction was analyzed for radioactivity. The 2,4,5-T-Glu peak contained 89% of the ^{14}C label, while the 2,4,5-T-Asp peak contained 11% of the label. To further confirm the TLC and LC evidence for the presence of the aspartic and glutamic conjugates in Et_3, a sample of Et_3 was methylated and analyzed by GLC and mass spectrometry employing an

SP2100 column. The retention time of the two detectable components of Et_3 was identical with the retention time of the methyl esters of 2,4,5-T-Glu and 2,4,5-T-Asp. Figures 3 and 4 show the mass spectra of the methyl esters of the two components of Et_3 and compare these spectra with that of the synthetic compounds. As is evident, the major fragmentation ions of the isolated compounds are congruent with that of 2,4,5-T-Glu and 2,4,5-T-Asp. Collectively these data indicate that the major metabolites of 2,4,5-T in 4-week-old soybean callus tissue cultures incubated with 2,4,5-T for 6 days are 2,4,5-T-Glu (major) and 2,4,5-T-Asp (minor).

A major portion (ca. 73%) of water-soluble metabolites was recovered in the diethyl ether fraction upon Emulsin treatment (Figure 1). This indicates the presence of aglycons which were released by enzymatic hydrolysis (β-glucosidase) (Hamilton et al., 1971). Although the compounds found in the water fraction are generally referred to as glycosides, they might also contain conjugates of sugar esters since Emulsin is a crude preparation of different hydrolytic enzymes. For example, 2,4-D-Glu is hydrolyzed to 2,4-D and glutamic acid with Emulsin (Feung et al., 1973). The hydrolyzate contained only trace amounts of 2,4,5-T, so the carboxylic glycoside was not present in any significant extent as has been found for 2,4-D (Feung, et al., 1975).

Ring hydroxylation has been thought to be a means of detoxification of 2,4-D and a number of hydroxylated derivatives of 2,4-D have been identified. With 2,4,5-T only 4OH-2,5-D has been reported as a minor metabolite in bean (Hamilton et al., 1971). If hydroxylation is a method of reducing biological activity then the relatively small amounts of water-soluble metabolites in soybean callus tissue indicate that detoxification through hydroxylation takes place at a much slower rate for 2,4,5-T than for 2,4-D. On the other hand, the biological activity of 2,4,5-T and its amino acid conjugates, which are present in significant amounts, are considerably less biologically active than 2,4-D or its amino acid conjugates (Arjmand et al., 1978b).

These data indicate that 2,4,5-T is metabolized by soybean cotyledon callus tissue to ether- and water-soluble metabolites in a similar manner to that of 2,4-D. Only a small portion of the ^{14}C label is found in the callus tissue residue; therefore, little 2,4,5-T is associated with macromolecular fractions. Also, the high recovery of radioactivity precludes appreciable decarboxylation in the tissue. The main portion of the ^{14}C label was recovered in the ether fraction which contained at least eight different compounds of which 2,4,5-T-Glu and 2,4,5-T-Asp consisted of 81–82% of the metabolites of this fraction. No attempt was made to identify any of the other minor metabolites.

ACKNOWLEDGMENT

The authors wish to thank Jerry Porter for mass spectral analyses.

LITERATURE CITED

Arjmand, M., Hamilton, R. H., Mumma, R. O., *J. Agric. Food Chem.* **26**, 898 (1978a).
Arjmand, M., Hamilton, R. H., Davidonis, G. H., Mumma, R. O., *J. Agric. Food Chem.* **26**, in press (1978b).
Bray, G. A., *Anal. Biochem.* **1**, 279 (1960).
Byrnes, W. R., Holt, H. A., in "Herbicides in Forestry", Department of Forestry and Natural Resources, Purdue University, Lafayette, Ind., 1976, p 47.
Chkanikov, D. I., Pavlova, N. N., Gertsuskii, D. G., *Khim. Selsk. Khoz.* **4**, 56 (1965).
Feung, C. S., Hamilton, R. H., Witham, F. H., *J. Agric. Food Chem.* **19**, 475 (1971).
Feung, C. S., Hamilton, R. H., Witham, F. H., Mumma, R. O., *Plant. Physiol.* **50**, 80 (1972).
Feung, C. S., Hamilton, R. H., Mumma, R. O., *J. Agric. Food Chem.* **21**, 637 (1973).
Feung, C. S., Hamilton, R. H., Mumma, R. O., *J. Agric. Food Chem.* **23**, 373 (1975).
Feung, C. S., Hamilton, R. H., Mumma, R. O., *Plant Physiol.* **58**, 666 (1976).
Fitzgerald, C. H., *Diss. Abstr. B* **27**, 1772 (1966).
Hamilton, R. H., Hurter, J., Hall, J. K., Ercegovich, C. D., *J. Agric. Food Chem.* **19**, 480 (1971).
Kalberer, F., Rutschmann, J., *Helv. Chim. Acta* **44**, 1956 (1961).
Miller, C. O., in "Moderne Methoden der Pflanzenanalyze", Vol. 6, Linskens, H. F., Tracey, M. V., Ed., 1963, p 194.
Primer, P. E., *Diss. Abstr. B* **28**, 415 (1967).

Received for review February 27, 1978. Accepted May 8, 1978. Authorized for publication as Paper No. 5430 in the Journal Series of the Pennsylvania Agricultural Experiment Station. Supported in part by Northeastern Regional Project NE-53 and Regional Research Funds.

Metabolism of *cis*- and *trans*-[^{14}C]Permethrin by the Tobacco Budworm and the Bollworm

Walter S. Bigley and Frederick W. Plapp, Jr.*

The fate of ^{14}C-acid- and -alcohol-labeled cis and trans isomers of permethrin (3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) was studied after topical treatment of larvae of the tobacco budworm, *Heliothis virescens* (F.), and the bollworm, *Heliothis zea* (Boddie). All permethrin preparations were very stable in *Heliothis* larvae under in vivo conditions, but older larvae detoxified the insecticide more rapidly than younger larvae. Permethrin metabolism was both hydrolytic and oxidative and the trans isomer was metabolized more rapidly than the cis isomer. Metabolism was more rapid in the tobacco budworm than in the bollworm, a factor probably responsible for the twofold greater tolerance of the former to permethrin.

The tobacco budworm, *Heliothis virescens* (F.), and the bollworm, *Heliothis zea* (Boddie), are major pests of cotton in the United States, Mexico, and Central America. The tobacco budworm is resistant to essentially all insecticides registered for use on cotton (Adkisson, 1968; Nemec and Adkisson, 1969; Plapp, 1971, 1972). Studies with resistant tobacco budworms indicate resistance is due primarily to

Department of Entomology, Texas A&M University, College Station, Texas 77843.

0021-8561/78/1426-1129$01.00/0