Absorption, Translocation, and Metabolism of *p*-Nitrophenyl-α,α,α-trifluoro-2-nitro-*p*-tolyl Ether by Soybeans

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Soybeans growing in nutrient solution containing ^{14}C-C-6989 (*p*-nitrophenyl-α,α,α-trifluoro-2-nitro-*p*-tolyl ether) progressively absorbed radioactivity with time. There was limited translocation of the absorbed radioactivity out of the roots. If the site of action of this herbicide is in the shoots of plants, this limited mobility could be a major factor in the relative resistance of soybean to C-6989. When ^{14}C-C-6989 was applied to soybean leaves, translocation was limited to an acropetal direction. Metabolic studies revealed soybean rapidly degraded ^{14}C-C-6989. Metabolism was indicated to involve limited reduction of the nitro substituents and rapid cleavage of the ether linkage to form the corresponding phenolic derivatives. The rate of degradation was sufficient to serve as a protective mechanism to the soybean plant if the degradation products are relatively nonphytotoxic.

C-6989 (*p*-nitrophenyl-α,α,α-trifluoro-2-nitro-*p*-tolyl ether) is a relatively new herbicide which was introduced by CIBA Corporation. This compound shows considerable potential as a preemergence herbicide in a number of crops, including soybean at rates to 2 to 4 lb of active ingredient per acre. It is formulated as a 50% wettable powder, 15% granules, and as a 3 lb per gal emulsifiable concentrate. Its solubility in water is less than 2.0 ppm (CIBA, 1967).

Presently, extensive research is being conducted by several companies to determine the phytotoxicities of various analogues of the diphenyl ether moiety (Toyama, 1969). Thus, it is probable that additional herbicides of this type will soon be introduced. Matsunaka (1969) reported that the diphenyl ether type herbicides can be divided into two large groups. One of these groups requires light for activation, whereas the other exhibits herbicidal activity in the dark. The xanthophylls appear to be the pigments involved in this photoactivation. The former group is characterized by having at least one substituent in one of the ortho positions of one of the benzene rings. Those compounds which exhibit herbicidal activity in the dark strongly inhibit root emergence. Apparently no more specific information has been published concerning the basic mode of action of the diphenyl ether type herbicides.

Nitrofen (2,4-dichloro-4′-nitrodiphenyl ether) was the first compound of this type to be developed as a herbicide. Gutenmann and Lisk (1967) reported that nitrofen was rapidly reduced to 2,4-dichloro-4′-aminodiphenyl ether in the rumen of dairy cows. Walters *et al.* (1968) reported that no C-6989 was present in the seed of soybeans treated preemergence with 2 to 6 lb active ingredient per acre. The basic diphenyl ether moiety is thought to be quite stable in biological systems. The principal metabolite of diphenyl ether fed to rabbits was 4-hydroxydiphenyl ether which was excreted mainly as a glucuronide. The derivatives 4-methoxy- and 4,4′-dimethoxydiphenyl ether were converted to the corresponding hydroxy derivatives by the rabbit (Williams, 1959).

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METHODS AND MATERIALS

Experiments were conducted with ring labeled ^{14}C-C-6989 (1′ position, ring with *p*-nitro substituent) with a specific activity of 8.3 μc per mg. All radioassays were conducted with a Beckman Model LS 250 liquid scintillation system. Soybeans (*Glycine max* L. variety Lee) were used in all experiments. Experiments were conducted in a growth chamber programmed for a 16 hr day and 8 hr night. Temperatures were 32° and 24° C, respectively. Light intensity was about 1500 ft candles.

Absorption and Translocation. Initially ^{14}C-C-6989 root absorption and translocation were followed by growing 7-day old soybean plants in 300 ml of nutrient solution containing 1 ppm of ^{14}C-C-6989 for 0, 12, 24, 48 and 96 hr. Treatment solutions were contained in 500 ml glass bottles which were attached to a standard aeration system. There were four plants per replication and two replications per treatment. Foliar absorption and translocation were followed by spotting 2.5 μc of ^{14}C-C-6989 contained in 25 λ of acetone inside lanolin rings on the upper surface of the leaves. Treatment time was 48 hr. Upon termination of a treatment, the plants were positioned between two pieces of screen wire, frozen, freeze-dried in a Virtis Model 10-100 freeze drier, and exposed to Kodak No-Screen X-ray film for 2 weeks.

A second C-6989 absorption and translocation experiment was conducted by growing 7-day old soybean plants in 1 ppm ^{14}C-C-6989 solutions for 0, 1, 2, 4, 8, and 16 days. Treatments were applied as indicated in the initial study and were changed at 4-day intervals. The volume and radioactivity of the treatment solutions was determined at the initiation and termination of a treatment as well as when treatment solutions were changed. The fresh weights of the plants were also measured at the initiation and termination of treat-

ments. There were three plants per replication and two replications per treatment. The plants were sectioned into roots and shoots, the segments were weighed, and the quantity of radioactivity in the segments was determined by combusting 100 mg samples.

Metabolic Study. The metabolism of ^{14}C-C-6989 by soybeans was studied in conjunction with the latter absorption and translocation experiment. Thus, treatment rates, times, etc., are as indicated above. The roots and shoots of the treated plants were processed as indicated below. The extraction procedure is a modification of a procedure developed by Geissbuhler (1970).

The weighed plant segments were blended in 100 ml of acetonitrile-water (7:1 v/v) for 5 min. The homogenate was suction filtered and the process was repeated with the fibrous residue. The extracts were combined and radioassayed. The acetonitrile phase of the extract was evaporated, and the aqueous residue was standardized to a volume of 50 ml. The aqueous residue was then extracted twice with 100 ml of hexane and the hexane extracts were combined, radioassayed, concentrated, and chromatographed.

The pH of the aqueous residue was adjusted to 5.0, extracted twice with 50 ml of ethyl ether, and the ethyl ether extracts were combined, radioassayed, concentrated, and chromatographed.

The aqueous residue was then used to prepare 4*N* HCl solutions for hydrolysis of the corresponding fibrous residues. The hydrolysis was conducted for 4 hr at a slow boil in flasks attached to condensers. The slurry was suction filtered and the aqueous residue was extracted twice with 50 ml of ethyl ether. The ethyl ether extracts were combined, radioassayed, concentrated, and chromatographed.

The final aqueous residue was lyophilized; the residue was suspended in methanol, radioassayed, and chromatographed. The radioactivity remaining in the dried fibrous residue was determined by combustion.

Metabolite Identification. Solvent systems that would give adequate resolution of C-6989 and a number of synthesized probable metabolites were sought by spotting 10–100 μg samples of these compounds contained in acetone on 20 x 20 cm 200 μ Silica Gel G thin-layer plates. The compounds were spotted singly and as a group, and the plates were developed over a 15 cm area. The chromatograms were viewed in a chromatocab equipped with long wave and short wave uv lamps to determine the position of the compounds. Tentative identification of radioactive compounds in the plant extracts was attained by comparing their R_f values with R_f values of the reference compounds spotted on the same thin-layer plates. The compounds indicated to be present in the extracts by this comparison of R_f values were spotted with the extracts to determine if the areas of fluorescence from the reference compounds cochromatographed with the areas of radioactivity from the ^{14}C-compounds in the extracts.

RESULTS AND DISCUSSION

Absorption and Translocation. Autoradiographs of the ^{14}C-C-6989 treated soybeans indicated that after 12 hr considerable root uptake had occurred, but that there had been little or no translocation into the stems and leaves (Figure 1). After 24 hr some radioactivity was apparent along the veins of the leaves. With time there was a progressive increase in the quantity of radioactivity appearing in all plant segments, but the bulk of the radioactivity persisted in the roots at all times. Observation of the autoradiographs of leaf-treated plants indicated penetration of ^{14}C-C-6989 had occurred, but that translocation was restricted to an acropetal direction.

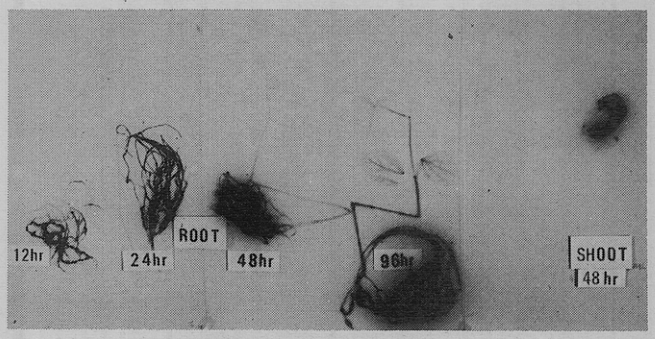

Figure 1. Autoradiographs of ^{14}C-C-6989 treated soybean plants

Table I. Absorption and Localization of Radioactivity from ^{14}C-C-6989 Solutions by Soybeans

Time (days)	Absorption (μg)	Localization	
		% Roots	% Shoots
1	74.6	98.3	1.7
2	136.4	94.7	5.3
4	222.0	89.6	10.4
8	500.1	82.6	17.4
16	874.6	79.0	21.0

Table II. Fractionation of Extracted Radioactivity During Processing of the Extracts

Time (days)		Fractions			
		Hexane (%)	1st Ether (%)	2nd Ether (%)	Aqueous Residue (%)
Roots	1	77.1	9.8	10.9	2.3
	2	73.2	4.0	11.0	11.8
	4	52.3	3.7	17.2	27.4
	8	18.6	6.9	25.7	48.9
	16	16.4	5.7	25.2	52.8
Shoot	1	45.3	22.3	34.9	0.0
	2	27.4	26.9	34.1	11.6
	4	24.5	13.6	35.0	27.1
	8	17.2	16.2	35.9	30.8
	16	8.7	9.6	52.1	28.6

Results of the second absorption and translocation experiment are shown in Table I. These data confirm that there was a significant increase in ^{14}C-C-6989 uptake with time, and that the bulk of the radioactivity was retained in the roots of root-treated plants at all treatment times, *i.e.*, after 16 days 75% of the absorbed radioactivity still persisted in the roots which accounted for only 30% of the plant fresh weight. Thus, it would appear that C-6989 quite readily moves into plant roots and shoots, but that its mobility within the plant is very limited. The practical importance of this depends upon the basic mode of action of C-6989. Observations by the author indicate that there is a rather striking difference in the relative susceptibility of soybean to preemergence applications of C-6989, as contrasted to postemergence applications. Thus, the limited translocation of C-6989 into soybean shoots may be a major factor in the relative resistance of this species to preemergence applications of C-6989.

Metabolic Study. The percentages of the total radioactivity extracted from the treated plants, as indicated by radioassays on the initial acetonitrile-water extracts, which appeared in the various fractions of the processed extracts are shown in Table II. In this processing procedure, the intact herbicide and pos

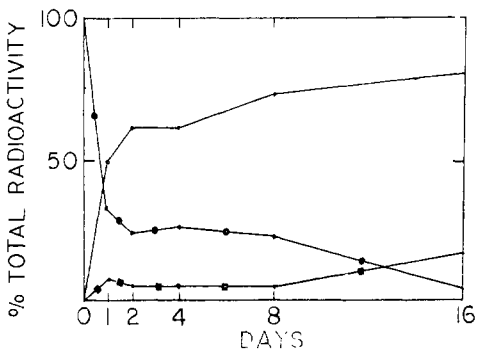

Figure 2. Distribution of radioactivity in extracts of soybean shoots from plants growing in a 1 ppm ^{14}C-C-6989 solution

Legend: ——— *p*-nitrophenol
—●— C-6989
—■— unidentified

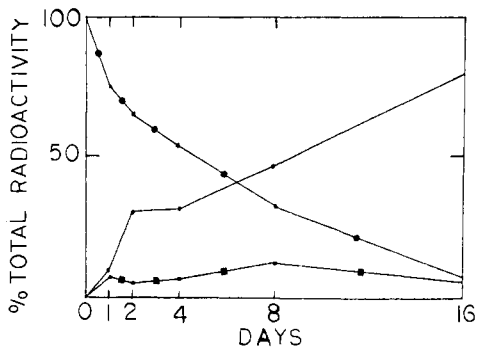

Figure 3. Distribution of radioactivity in extracts of soybean roots from plants growing in a 1 ppm ^{14}C-C-6989 solution

Legend: ——— *p*-nitrophenol
—●— C-6989
—■— unidentified

sible amino derivatives appear primarily in the hexane fraction, whereas phenolic derivatives formed by cleavage of the ether linkage appear primarily in the ethyl ether fractions, and more polar materials are retained in the aqueous residue (Geissbuhler, 1970). It is apparent that there was a general decrease in the quantity of radioactivity appearing in the hexane and first ethyl ether fractions with time, while the converse was true of the second ethyl ether and aqueous residue fractions. Thus, the extraction data suggested cleavage of the ether linkage was involved in C-6989 metabolism in soybeans. Progressive increases in the quantity of radioactivity appearing in the second ethyl ether fraction indicated the degradation products were bound in the plant in some form whereby they could be partially extracted as a phenolic derivative upon acid hydrolysis. A balance sheet on the extraction of absorbed radioactivity from the treated plants is shown in Table III.

The R_f values of the radioactive components of the extracts of ^{14}C-C-6989 treated soybeans and of several synthesized probable metabolites are shown in Table IV. A comparison of the R_f values of the ^{14}C-compounds in the extracts with those of the synthesized probable metabolites indicated the radioactive components of the roots and shoots were C-6989, 4-trifluoromethyl-2-amino-4′-nitrodiphenyl ether, 4′,2-diamino-4-trifluoromethyldiphenyl ether, and *p*-nitrophenol. When the indicated reference compound(s) were spotted with the extracts, the reference compounds cochromatographed with the respective components of the extracts in both

Table III. Balance Sheet of Radioactivity from ^{14}C-C-6989 Treated Soybeans

Time (days)	Absorbed (μg)	Acetonitrile Extract (μg)	Fibrous Residue (μg)	Recovery (%)
1	74.6	49.6	38.6	118.5
2	136.4	66.2	70.9	100.6
4	222.0	118.8	125.9	108.5
8	500.1	126.5	221.4	78.2
16	874.6	236.8	702.6	107.4

Table IV. R_f Values of Several Synthesized Probable Metabolites and of Radioactive Constituents of Extracts of ^{14}C-C-6989 Treated Soybean Plants

Compounds	R_f Values: Benzene:1,2-dichloroethane (1:1 v/v)	R_f Values: Chloroform:ethanol:acetic acid (90:5:5 v/v)
Extract Components		
1	0.69	0.91
2	0.35	0.82
3	0.28	0.60
4	0.11	0.75
5	0	0
Reference Compounds		
C-6989	0.65	0.93
CFA-170	0.35	0.85
CFA-171	0.28	0.61
E-192	0.15	0.75

C-6989—*p*-nitrophenyl-α,α,α-trifluoro-2-nitro-*p*-tolyl ether
CFA-170—4-trifluoromethyl-2-amino-4′-nitrodiphenyl ether
CFA-171—4′-2-diamino-4-trifluoromethyldiphenyl ether
E-192—*p*-nitrophenol

solvent systems. However, identification *via* thin-layer chromatography should be regarded as tentative. A small amount of radioactivity was retained at the origin and was not identified. The first three ^{14}C-compounds appeared primarily in the hexane extracts, while *p*-nitrophenol and the unidentified fraction appeared in the ether and aqueous residue extract fractions. The most abundant ^{14}C-compound in the shoots was *p*-nitrophenol at all treatment times, while C-6989 was the most abundant constituent of root extracts until the eighth day. The relative quantities of radioactivity which appeared in these component fractions of the root and shoot extracts are shown in Figures 2 and 3. The mono- and diamino derivatives were present in only trace amounts, and are not represented in these figures. The former metabolite was observed more consistently and in larger quantities than the latter. Thus, these data indicate that metabolism of C-6989 in the soybean plant primarily involves a cleavage of the diphenyl ether linkage. Reduction of the nitro substituents was of minor importance. Reduction of nitro substituents has been reported to be involved in the degradation of other herbicides (Gutenmann and Lisk, 1967; Probst *et al.*, 1967). Cleavage of a diphenyl ether type moiety has not been reported to occur with other herbicides of this type. A similar scheme of degradation of C-6989 has been observed by Geissbuhler (1970) and by Boyd (1969).

CONCLUSIONS

Soybeans growing in nutrient solution containing ^{14}C-C-6989 will progressively absorb the herbicide with time. The absorbed radioactivity exhibits little mobility within the plant, with limited quantities of radioactivity being present in shoots of soybean plants grown in nutrient solution containing

^{14}C-C-6989 for 16 days. Metabolic studies revealed most of this radioactivity to be a ^{14}C-C-6989 metabolite. ^{14}C-C-6989 applied to soybean leaves was translocated to a limited extent in an acropetal direction.

Metabolic studies revealed soybeans readily degrade ^{14}C-C-6989. C-6989 metabolism was observed to primarily involve a cleavage of the ether linkage. Reduction of the nitro substituents of the rings was of minor importance. The rate of metabolism of C-6989 was sufficient to serve as a protective mechanism to the soybean plant if the degradation products are relatively nonphytotoxic.

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