Metabolism of 4-(2,4-Dichlorophenoxy)butyric Acid in Soybean and Cocklebur

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The metabolism of ^{14}C-ring-labeled 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) at 1 and 7 days after treatment of one mature leaf of soybean [*Glycine max* (L.) Merr. var. Lee] and cocklebur (*Xanthium* sp.) was investigated at 3, 5, 7, and 11 weeks after planting. Thin-layer chromatographic, autoradiographic, and liquid scintillation analyses of plant extracts indicated that the predominant compound isolated from both species was 2,4-DB. Two pathways for transformation of the parent molecule were detected. Both species β-oxidized the 2,4-DB to (2,4-dichlorophenoxy)acetic acid (2,4-D) at every stage of growth, but degradation was less rapid in soybean. At equivalent stages of growth, cocklebur contained higher concentrations of 2,4-D than did the soybean. The 2,4-D was readily translocated in cocklebur, whereas only trace quantities were observed in the leaves, stems, and roots of soybean. The 4-(2,4-dichlorophenoxy)crotonic acid was identified as an intermediate in the β-oxidation pathway and was found only in the treated leaves. A second transformation pathway involved elongation of the fatty acid side chain and formation of 10-(2,4-dichlorophenoxy)decanoic acid which was found in the treated leaf cuticle.

Cocklebur (*Xanthium* sp.) is a major weed problem in soybean (*Glycine max* L. Merr.). This weed reduces yields, lowers quality, and increases harvesting costs of soybean. Cocklebur is relatively resistant to preemergence herbicides but can be controlled by application of 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) at 7 to 10 days prior to soybean bloom up to the midbloom stage. However, soybeans are occasionally injured by this treatment and injury is especially likely if 2,4-DB is applied at an earlier stage of growth.

The 2,4-DB was first used to control weeds in legumes and it was found to be a nonphytotoxic chemical (Wain, 1955). The herbicidal property was derived by degradation of 2,4-DB to 2,4-D. Investigations by Wain (1955, 1964), Gutenmann and Lisk (1963, 1964), Linscott (1964), Linscott *et al.* (1968), and Robertson and Kirkwood (1970) revealed that β-oxidation was one mechanism of 2,4-DB degradation and that the primary metabolic product was (2,4-dichlorophenoxy)acetic acid (2,4-D). Wain and Wightman (1954) suggested that selectivity of this herbicide was associated with differential β-oxidation capabilities between crops and weeds. The 2,4-DB would be an effective herbicide for the weed species if the chemical could be degraded to form the toxic 2,4-D in the weed but not in the crop. However, recent investigations cast some doubt on previous descriptions of 2,4-DB metabolism in both crops and weeds. The 2,4-D has been detected in limited amounts in both crops and weeds (Linscott *et al.*, 1964, 1968; Robertson and Kirkwood, 1966, 1970).

Newcomb and Stumpf (1953) established that β-oxidation of fatty acids could take place in the tissues of plants. Subsequently, information concerning the aliphatic side chain of fatty acid herbicides such as ω-phenoxyalkanoic acids was obtained to confirm the β-oxidation of herbicides in plants. Experiments by Wain and Wightman (1954) and Fawcett *et al.* (1954, 1958a,b, 1960, 1961) indicated that all members of the ω-phenoxyalkanoic acid series with an even number of side-chain methylene groups were partially converted to phenol within the plant, whereas members with an odd number of methylene groups, except 9-phenoxynonane-1 carboxylic acid, yielded negligible amounts of phenol. Furthermore, studies by Fertig *et al.* (1964) and Webley *et al.* (1955, 1957, 1958) confirmed the presence of β-oxidation in microorganisms as well as in plant tissues. The results were consistent with the idea that this mechanism occurred in plants and in microorganisms.

At present there is no clear evidence of the site of β-oxidation in leaf tissue and the location of β-oxidation enzymes is a controversial issue (Robertson and Kirkwood, 1970). The enzymes which β-oxidize ω-phenoxyalkanoic acids have not been isolated or characterized. However, it is assumed that the aliphatic side chain of phenoxy compounds should respond to a similar enzyme system as fatty acids. In addition, the inherent differences in the rate of metabolism of fatty acids may vary among plant species. This experiment was conducted to study the effect of plant age and treatment time on the metabolism of 2,4-DB in soybean and cocklebur, and to correlate metabolic potential with species selectivity.

MATERIALS AND METHODS

Seeds of soybean [*Glycine max* (L.) Merr. var. Lee] and cocklebur (*Xanthium* sp.) were planted in pots of Goldsboro sandy loam soil and were grown in the greenhouse essentially as described previously (Wathana, 1970). The seedlings were thinned to two plants per pot after 1 week. Supplementary light (15-hr photoperiod) was used to sustain vegetative growth during the first 7 weeks. A 12-hr photoperiod was used at 7 weeks to initiate flowering and was maintained during the last 4 weeks of the experiment. Plants were transferred from the greenhouse to the growth chamber 1 day before treatment with 2,4-DB at 3, 5, 7, and 11 weeks after planting, respectively. The growth chamber photoperiods were identical to those used in the greenhouse

The 2,4-DB (114 μg) was applied to a single mature leaf near the shoot apex. Only the terminal leaflet of a soybean

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trifoliate was treated at 5, 7, and 11 weeks. A unifoliate soybean leaf was treated at 3 weeks. Plants were harvested at 1 and 7 days after treatment for evaluation of metabolites.

The 2,4-DB used was either the unlabeled high-purity chemical or the uniformly ring-labeled analytical grade ^{14}C-2,4-DB (sp. act. 9.98 mCi/m*M*). The stock solution contained 6 mg of ^{14}C-2,4-DB, 140 mg of unlabeled 2,4-DB, and 1 g of dimethylamine in 100 ml of water. Carboxyl-labeled ^{14}C-2,4-D was used as a reference standard for identification of metabolites on thin-layer chromatograms. Other analytical standards used were 4-(2,4-dichlorophenoxy)crotonic acid (2,4-DC), 10-(2,4-dichlorophenoxy)decanoic acid (2,4-DD), 8-(2,4-dichlorophenoxy)octanoic acid, and 6-(2,4-dichlorophenoxy)caproic acid.

Immediately after harvesting, the treated leaves were washed for 3 min in water and 3 min in chloroform. Then each plant was sectioned into component parts (upper leaves, lower leaves, upper stem, lower stem, and roots) and frozen in dry ice. All samples were stored in a freezer prior to extraction and analysis. Samples were removed from the freezer, sectioned into 0.5 cm lengths, and immersed into 30 ml of boiling water. After cooling, the sample was homogenized in a stainless steel blending cup in a mixture of 2-propanol and acetone (2:1, v/v) followed by additional grinding in a ground glass homogenizer. The homogenate was filtered under vacuum and the plant residue was rinsed several times with 2-propanol and acetone. The solvents were evaporated almost completely under vacuum at 40° C and the extract was transferred to a separatory funnel. The metabolites were partitioned into 0.4 *M* K_2HPO_4 (pH >9.0). The K_2HPO_4 was extracted several times with $CHCl_3$ to remove soluble pigments and other impurities and a radioactive count was made to assure partitioning of metabolites into the K_2HPO_4. The aqueous K_2HPO_4 solution was acidified with 4 *N* H_2SO_4 (pH <2.0). The metabolites which were now in the free acidic form were partitioned into $CHCl_3$. The $CHCl_3$ fraction was filtered through anhydrous Na_2SO_4 and excess solvent was evaporated under vacuum at 40° C.

Samples were assayed by liquid scintillation counting in a solution which contained 667 ml of toluene, 333 ml of Trition X-100 (scintillation grade), 5.0 g of 2,5-diphenyloxazole (PPO), and 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl POPOP) per liter of solution. Vials were counted on two consecutive days for 10 min each time. Counting efficiencies were determined by the channels ratio technique. The data from three replications were analyzed by computer to detect differences in metabolism, plant species, plant age, and treatment time.

Identification of radioactive metabolites was by thin-layer chromatography on 0.025 cm silica gel G glass plates (Merck). Unknown samples were spotted at the origin (1.5 cm from the plate edge) and cochromatographed with authentic standards. Chromatographic systems employed were chloroform/acetic acid (19/1) and hexane/chloroform/acetic acid (13/6/1). Plates were positioned in the developing tank at an angle of 130° between the front of the plate and the developing solvent, according to the technique of Abbott and Wagstaffe (1969). The plates were developed to a 15.0 cm solvent front, allowed to dry, and exposed to Kodak no-screen X-ray film for a minimum of 1 month. The metabolites were located on the plates, and the silica gel spots were transferred to 20-ml vials containing the scintillation liquid described above. Nonradioactive analytical standards were applied to thin-layer plates and developed in a similar fashion to the labeled compounds. The plates were developed, dried, and sprayed with 2′,7′-dichlorofluorescein. Acidic metabolites appeared as bright yellow spots under ultraviolet light.

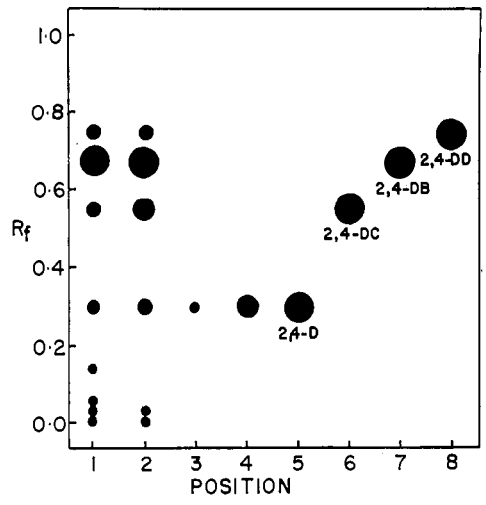

Figure 1. Drawing of a summary of the thin-layer chromatographic analyses of 2,4-DB, metabolites of 2,4-DB, and related standards. Positions 1 to 4 are extracts from: the soybean treated leaf; cocklebur treated leaf; soybean root, stem, and upper leaves; and cocklebur root, stem, and upper leaves, respectively; positions 5 to 8 are authentic standards

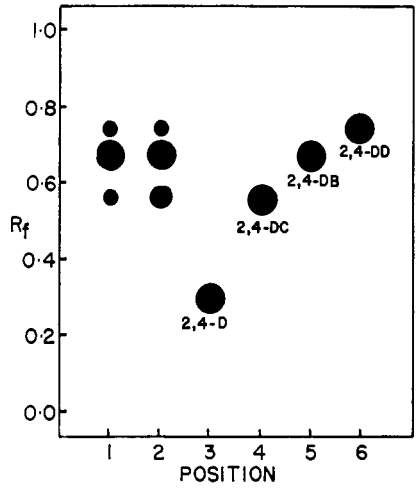

Figure 2. Drawing of a summary of the thin-layer chromatographic analyses of 2,4-DB, metabolites of 2,4-DB, and related standards. Positions 1 and 2 are extracts from the cuticular fraction of soybean and cocklebur treated leaves, respectively; positions 3 to 6 are authentic standards

The concentrations of all standard stock solutions were verified by analysis on a recording spectrophotometer at 282 and 228 nm.

RESULTS AND DISCUSSION

A summary of the thin-layer chromatographic analysis is presented in Figures 1 and 2. The treated leaf extracts of both cocklebur and soybean contained various radioactive products but more metabolites were found in soybean than in cocklebur (Figure 1). The 2,4-DB was the predominant radioactive compound in both species at all growth times and treatment periods, and the major metabolic product was 2,4-D. An intermediate with an R_f similar to (2,4-dichlorophenoxy)crotonic acid (2,4-DC) was observed in both species at various stages, which indicated that 2,4-DB was metabolized

Table I. Recovery of 2,4-DB and its Metabolites Following Application of 2,4-DB to a Single Leaf of Cocklebur and Soybean Plants

Plant age, weeks	Treatment time, days	2,4-DB TLE, μg	2,4-DB TLI, μg	2,4-D TLI, μg	2,4-D Translocated, μg	2,4-DC TLE, μg	2,4-DC TLI, μg	2,4-DD TLE, μg	2,4-DD TLI, μg
				Cocklebur					
3	1	154.3	9.7	0.9	3.1	3.4	0	1.8	0
5	7	139.9	9.2	1.6	1.8	2.9	0.9	1.6	0
5	1	153.9	22.6	0.6	2.2	1.4	1.1	0.8	0
	7	142.1	16.2	1.0	2.0	1.2	0.7	0.7	0
7	1	115.8	18.5	2.0	3.4	2.3	0	3.2	0
	7	112.4	23.0	0.2	6.1	6.4	1.0	1.3	0
11	1	131.9	20.5	0.7	3.5	3.8	2.0	3.2	0.3
	7	159.3	24.6	0.3	2.2	4.5	0.5	2.5	0
				Soybean					
3	1	175.3	10.7	0.6	0.6	0	0.7	0	0
	7	150.4	21.6	0.5	0.3	1.5	0.7	0.8	0
5	1	174.3	20.2	0.4	0.4	0.5	1.8	1.3	0.3
	7	163.4	17.7	0.5	0.4	1.2	0.5	0.7	0
7	1	173.7	18.1	0.4	0.2	1.0	0.5	0	0
	7	135.6	27.3	1.0	T	1.5	2.6	1.6	0.2
11	1	153.9	13.9	0.4	T	1.4	1.2	0.3	0
	7	153.6	33.9	0.3	T	3.9	1.2	2.5	0
LSD 0.05		1.5	1.5	0.5	0.2	0.5	0.5	0.5	0.2

114 μg was applied to each plant. Two plants were combined for each extraction for a total of 228 μg. TLE = those components removed by brief immersions (3 min) of treated leaves in chloroform. TLI = those components determined after extraction of homogenized plant parts in 2-propanol/acetone (2/1).

by β-oxidation (Gutenmann and Lisk, 1964; Linscott *et al.*, 1968). In addition, a product was identified with an R_f similar to that of the long chain 2,4-DD which Linscott *et al.* (1968) proposed to result from fatty acid synthesis in *Medicago sativa* (alfalfa) (Figures 1 and 2).

Both cocklebur and soybean contained enzymes capable of degrading 2,4-DB, and the metabolite 2,4-D was obtained at all stages of growth, but the degree of this capability was higher in cocklebur (Table I). Furthermore, the intermediate 2,4-DC was detected at higher concentrations in cocklebur than in soybean. In addition, both plants contained other unknown products, but there was a distinguishable difference between these two plant species in the total number of metabolic products. Additional metabolism of 2,4-D in soybean apparently altered the chemical before it reached the meristematic tissues. Only sharp spots at R_f 0.0 and 0.05 were observed in cocklebur-treated leaf extracts at all growth times and treatment periods, whereas there were spots overlapping from R_f 0.0 to R_f 0.1 at 7 days of all growth times in soybean (Figure 1). Moreover, a product was observed at R_f 0.15 at 7 days of 3, 5, 7, and 11 weeks soybean, as shown in Figure 1. This unknown intermediate was not observed at any time in cocklebur, which indicates that in soybean further reduction in length of the side chain of 2,4-D may take place after β-oxidation including ring hydroxylation (Webley *et al.*, 1955; Wilcox *et al.*, 1963). One can reasonably presume that the herbicide was adsorbed to or conjugated with other cellular components and was converted into a bound form which would neither diffuse rapidly nor exchange from the tissues (Sargent and Blackman, 1962). Although adsorption to nonactive sites is not likely to be the sole source of the difference in species response (Brian, 1958; Fang, 1958), the degree of this occurrence was less in cocklebur than in soybean. This partially explains the higher concentration of 2,4-D in cocklebur than in soybean. Moreover, the detection of radioactive components in the root and lower stem of cocklebur and soybean revealed that more 2,4-D was translocated in cocklebur than in soybean (Table I). In both species, 2,4-D was the only metabolic product found to be translocated out of the treated leaf.

To evaluate further differences in metabolism of 2,4-DB between soybean and cocklebur, the chloroform extracts of treated leaf cuticles were subjected to chromatographic analysis in a similar manner to the internal extracts of treated leaves. Results from cuticular fraction chromatographs are presented in Figure 2 and Table I. The 2,4-DB was the predominant radioactive substance, but 2,4-DC and 2,4-DD were also identified. An unknown product at R_f 0.92 was detected at various stages in soybean, whereas it was observed in cocklebur only at 7 weeks.

An interesting aspect of this experiment was the synthesis of the long chain metabolite 2,4-DD. This reaction occurred in both soybean and cocklebur and the highest concentrations were found in the older plants. Linscott *et al.* (1968) provided convincing evidence that this synthesis process represented one mechanism of resistance of legumes to 2,4-DB. Our experiments add support to Linscott's hypothesis, but some doubt exists concerning the relative importance of this mechanism in the selectivity of soybean and cocklebur to the herbicide. However, our experiments were limited to 7 days after treatment and we treated only one leaf. Availability of the substrate should not be a limiting factor because high concentrations of 2,4-DB were always present within the treated leaves.

We can only speculate at this time about the relative importance of each of the metabolic products identified in this experiment. The total concentration of 2,4-DC exceeded that of 2,4-D at several stages of growth and this was especially true in the older soybeam plants. In addition, the relatively high concentrations of 2,4-DC in the cuticular fraction of the treated leaf were unexpected. Several possibilities exist: the 2,4-DB was oxidized to 2,4-DC by microorganisms on the leaf surface; the 2,4-DC was formed on the outer surface of cell membranes and was thus readily accessible for extraction in the cuticular fraction; the 2,4-DB was absorbed into the leaf cells and was oxidized to 2,4-DC; then the 2,4-DC was

excreted to the cuticle with other fatty acids. The first alternative seems the least likely, since the concentrations of 2,4-DC were consistently higher in cocklebur than in soybean. The third possibility cannot be ruled out until sufficient control experiments are performed. The second alternative certainly seems possible and is in agreement with previous suggestions that extracellular enzymes may be responsible for β-oxidation of phenoxy–butyric compounds (Garraway and Wain, 1962).

We did not find 2,4-D in the cuticular fraction of the leaves at any growth stage of either species. This indicates that the 2,4-DC was absorbed into the leaf cells prior to conversion to 2,4-D. Linscott *et al.* (1968) suggested that plants may have a tolerance potential to 2,4-DB if they lack the capacity for β-oxidation, if the reaction proceeds too slowly, or if the resultant 2,4-D is subsequently inactivated. A comparison between soybean and cocklebur at 7 weeks and 7 days gives one an excellent example of the difference in the rate of the β-oxidation reaction. At this stage, we extracted 13.7 μg of total β-oxidized product (2,4-DC + 2,4-D) from cocklebur, but only 5.1 μg from soybean. In addition, the high 2,4-DC to 2,4-D ratio in the older soybean plants indicates that the enzymatic transformation of 2,4-DC to 2,4-D was reduced.

In addition to the above mechanisms, other possibilities undoubtedly will be proposed. Additional experiments at the molecular and at the cellular level will be required to determine the relative contributions of each of the metabolic mechanisms described above in plant selectivity to 2,4-DB. However, one may conclude from this experiment that in cocklebur the 2,4-DB was absorbed into the cuticle more rapidly, was metabolized to 2,4-DC and 2,4-D in higher concentrations, and the 2,4-D was distributed to meristematic tissues throughout the plant in higher concentrations than in soybean.

ACKNOWLEDGMENT

Appreciation is extended to Rhodia Inc., Chipman Division, New Brunswick, N. J., for providing the ^{14}C-labeled 2,4-DB and the analytical ω-phenoxyalkanoic acids referred to in the text. We thank D. L. Linscott, Cornell University, Ithaca, N. Y., for providing some analytical standards. The graduate study program of the senior author was sponsored by the Agency For International Development. Statistical analysis was under the supervision of L. A. Nelson, Institute of Statistics, North Carolina State University, authorized under N.I.H. Grant No. FR-00011.

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Received for review May 21, 1971. Accepted July 23, 1971. This study was supported in part by a grant from Rhodia, Inc., Chipman Division, New Brunswick, N.J. Paper number 3475 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N.C.