Metabolism of 2-Methoxy-3,6-dichlorobenzoic Acid (Dicamba) by Wheat and Bluegrass Plants

NORMAN A. BROADHURST, MARVIN L. MONTGOMERY, and VIRGIL H. FREED

Department of Agricultural Chemistry, Oregon State University, Corvallis, Ore.

The metabolism of dicamba by wheat and bluegrass plants yielded a major metabolite, which was 90% of the total metabolic products, and two minor metabolites, each constituting 5% of the total metabolic products. The major metabolite after hydrolysis has been identified as 5-hydroxy-2-methoxy-3,6-dichlorobenzoic acid. One of the minor metabolites after hydrolysis was the parent compound; the other being 3,6-dichlorosalicylic acid. Wheat and bluegrass plants give the same metabolites in the same proportions.

THE CHEMICAL, 2-methoxy-3,6-dichlorobenzoic acid (dicamba), has been shown to be an effective selective herbicide for use on wheat and forage plants such as bluegrass (*1*, *3*). This selectivity is largely lost when the methoxy group is replaced by a chlorine atom to give the herbicide 2,3,6-trichlorobenzoic acid. These observations suggested that the presence of the methoxy group in dicamba makes the molecule subject to attack by the resistant plants. Selective metabolism has been shown to be responsible for the selective action of a number of herbicides (*4*, *6*, *7*). The studies in this report were carried out to determine if the metabolism of dicamba by wheat and bluegrass plants is a dominant factor in their resistance to the chemical.

Materials and Methods

Wheat (var. Gaines) and bluegrass (var. Newport) plants were germinated and grown in sand. After the plants attained a height of 4 to 6 inches, they were transferred to jars containing nutrient solution. A week was allowed for adjustment to the new growing conditions, and the plants were then exposed to nutrient solution containing 1 p.p.m. ^{14}C-carboxyl-labeled dicamba (1.89 mc. per mmole). After a 6-day exposure period, the plants were returned to nutrient solution. Plants were harvested at time intervals varying from 2 to 29 days following removal of the ^{14}C-dicamba. Plants harvested up to 10 days following exposure to dicamba were separated into tops and roots for separate extraction. Subsequent extractions were performed on whole plants since it was found that 90% of the radioactivity had been translocated to the tops of the plants, and the metabolites produced by both portions of the plants were the same.

The extraction was performed by first macerating the plants with 95% ethanol in a blender. The macerated tissue was then extracted for 6 hours in a Soxhlet extractor with 95% ethanol. This crude extract was chromatographed on Whatman No. 1 paper in four developing systems. Since organic acid herbicides are known to form conjugates with plant constituents (*5*), the alcohol was evaporated and the residue hydrolyzed for 4 hours on a steam bath with 4*M* hydrochloric acid. In a preliminary experiment, 4*M* sodium hydroxide was also used for hydrolysis. Following the acid digestion, the extracts were extracted with chloroform until there was no detectable radioactivity in the aqueous phase. The chloroform extract then was evaporated to a convenient volume and spotted on Whatman No. 1 paper for development in the four solvent systems. In addition to the alkaline and acidic digestions, an enzymatic hydrolysis with β-glucosidase (Sigma Chemical Co.), in a pH 4.5 citric acid–phosphate buffer, was carried out to ascertain whether it would liberate the metabolites from the plant constituents.

Wheat plants which were growing in soil were sprayed with ^{14}C-dicamba at a level equivalent to 2 pounds per acre when the plants were about 6 inches tall, after which the plants were allowed to grow to maturity. When the heads were ripe, the plants were cut into heads, stalks, and roots and extracted with 95% ethanol. The extracts were analyzed for dicamba and metabolites.

Experiments were carried out to determine if the plants were able to metabolize the dicamba to carbon dioxide. Plants were placed in a closed system in which a stream of carbon dioxide–free air was passed. The carbon dioxide given off by the plants was trapped in carbonate-free 1*M* sodium hydroxide. The carbonate collected was precipitated as barium carbonate and checked for radioactivity. The experiment was performed on plants which had been exposed to dicamba for 3 and 8 days.

The authors suspected that the first step in the detoxification process was a demethylation reaction, hence, 3,6-dichlorosalicylic acid could be a metabolic product of dicamba. To check this theory, the plants were treated with ^{14}C-methoxy-labeled dicamba to see if the same metabolites would be produced. This compound was prepared by the reaction of ^{14}C-diazomethane with nonlabeled 3,6-dichlorosalicylic acid. The methylated product was saponified to remove the ^{14}C in the ester portion of the molecule.

The dicamba and hydrolyzed metabolites were purified by column chromatography and by paper chromatography using Whatman No. 1 paper in the solvent system, benzene–acetic acid–water (2:2:1). Essentially, all of the radioactivity in the chloroform extract could be extracted with two portions of 0.5*M* sodium hydroxide. Most of the neutral and colored material remained in the chloroform. After acidification of the alkaline extract, the ^{14}C compounds were extracted with chloroform. This extract was concentrated to 50 ml., put on a basic alumina column (1.5 × 12 cm.), washed with chloroform (200 ml.), ether (200 ml.), and dried by drawing air through the column. The radioactivity then was eluted from the column with 1% sodium bicarbonate solution (200 ml.). Most of the colored material remained at the top of the alumina column. The bicarbonate eluate was acidified and again extracted with chloroform. Solutions of the individual metabolites were obtained from this purified extract by development on Whatman No. 1 paper with benzene–acetic acid–water, and subsequent extraction of the radioactive bands with methanol in a Soxhlet extractor.

The first test with the purified metabolites was an attempt to deter-

mine whether the aromatic ring was still intact. This was done by using different esterifying reagents. Benzoic acids substituted in the 2,6-position apparently are hindered sterically to esterification by BF_3 methanol reagent, but are esterified readily by diazomethane, which is planar. Aliphatic acids, which would result if the ring was opened, would be esterified with either reagent. The metabolites were treated with both reagents and chromatographed on paper to see if the esters were formed.

To establish whether one or two chlorine atoms were still on the molecule, a standard amount of radioactive metabolite was methylated with diazomethane, and the amount of chlorine present in the molecule was determined on a microcoulometric gas chromatograph. A standard amount of nonlabeled dicamba was used to determine the percentage recovery of chlorine obtainable with the instrument. The peak attributed to the major metabolite was trapped as $^{14}CO_2$ following combustion in this instrument. This was done to make certain the peak was due to the metabolite rather than a naturally occurring chlorine-containing compound.

The substituent groups present on the aromatic ring indicate an activated 5 position and a deactivated 4 position. If the plant was to hydroxylate the dicamba in the detoxification process, the 5 position would be the favored position for substitution.

The 5-hydroxy compound was prepared after the method of Faulkner and Woodcock (*2*). This method consists of esterification and nitration of dicamba. The nitro group then is reduced using platinum oxide, diazotized, and the diazonium substituent replaced with a hydroxyl group. A small amount of ^{14}C 5-hydroxy dicamba also was prepared in a similar manner.

The major metabolite and synthetic products were not sufficiently pure to permit application of physical methods of identification. The purification was accomplished by the use of a preparatory gas chromatograph using a 2-foot column containing 5% SE 30 on Gas Chrom Z at 130° C. A thermal detector was employed, and the fraction between 5.5 and 8.0 minutes was collected.

After purification of the major metabolite and synthetic products, the retention times of the compounds on a number of gas chromatographic columns were determined. Both microcoulometric and electron-capture methods of analyses were used.

KBr pellets were made of the purified methylated metabolite and 5-hydroxy dicamba, and infrared spectra were obtained from these pellets. Fragment spectra of these two compounds were obtained from a single focusing mass spectrometer.

Results and Discussion

The hydrolysis of the crude ethanolic extract from wheat plants, by acid and alkali, yielded the same metabolites in similar proportions. Four paper chromatography systems were used in separating and identifying the metabolites, whose R_f values are shown in Table I.

The enzymatic hydrolysis by β-glucosidase also yielded the metabolites, with nearly quantitative hydrolysis being achieved. This indicated that the acid did not alter the metabolites in any way, and suggested that the metabolites are conjugated as glucosides also.

The time study on the extent to which the dicamba was metabolized by wheat plants revealed a reasonably rapid change from the parent compound. After 18 days, there was no detectable free dicamba present in the plants; however, a small amount persisted as a conjugate after 29 days. Table II indicates the conversion of the dicamba to a major metabolite, which reaches a high percentage of total metabolites, after which it remains constant. Likewise, the concentration of the minor metabolite reaches a certain level after which it also remains fairly constant. The rate of metabolism of dicamba by bluegrass was quite similar.

The barium carbonate, containing the CO_2 which was trapped from the wheat plants, had no appreciable amount of radioactivity. This meant that the dicamba was not decarboxylated to any measurable extent. Thus, no aromatic metabolite was being neglected because of a loss of the ^{14}C label through decarboxylation.

Esterification of the metabolites was obtained upon treatment with diazomethane but not with BF_3 methanol. This indicated that the aromatic ring still was intact. After saponification,

Table I. R_f Values of Dicamba and Metabolites

	Dicamba	Conjugated Products	Hydrolyzed Metabolites: Major	Hydrolyzed Metabolites: Minor
Butanol–1.5*M* ammonium hydroxide	0.65	0.07	0.07	0.49
Benzene–acetic acid–water, 2:2:1	0.87	0.04	0.42	0.76
Isopropanol–ammonium hydroxide–water, 4:1:1	0.91	0.66	0.52	0.65
2,6-Lutidine–water 65:35	0.72	0.56	0.76	0.84

Table II. Rate of Dicamba Metabolism by Wheat Plants

Days Following Exposure		Percentage of Radioactivity Present as: Dicamba	Conjugated products	Major metabolite	Minor metabolite
2	Crude extract	16	84		
	hydrolyzed	30		58	11
7	Crude extract	14	86		
	hydrolyzed	20		71	9
10	Crude extract	13	87		
	hydrolyzed	14		76	7
18	Crude extract	0	100		
	hydrolyzed	5		90	5
29	Crude extract	0	100		
	hydrolyzed	5		90	5

Table III. GLC Retention Times of Dicamba, Metabolite, and 5-Hydroxy Dicamba

Method of Detection[a]	Type of Column	Temperature, ° C.	Compound	Retention Time, Min.
MC	5% Carbowax 20M on Chrom Q, 4 ft.	155	Dicamba	4.0
			Metabolite	17.5
			5-Hydroxy dicamba	17.4
MC	5% QF1, 5.5 ft. 5% Dow 11, 1.2 on Chromosorb W	139	Dicamba	4.8
			Metabolite	16.6
			5-Hydroxy dicamba	16.6
MC	5% Dow 11 on Chromosorb W, 4 ft.	139	Dicamba	3.2
			Metabolite	9.4
			5-Hydroxy dicamba	9.4
EC	5% SE 30 on Chrom Z, 4 ft.	130	Dicamba	1.9
			Metabolite	6.0
			5-Hydroxy dicamba	6.0

[a] MC = Microcoulometric. EC = Electron-capture.

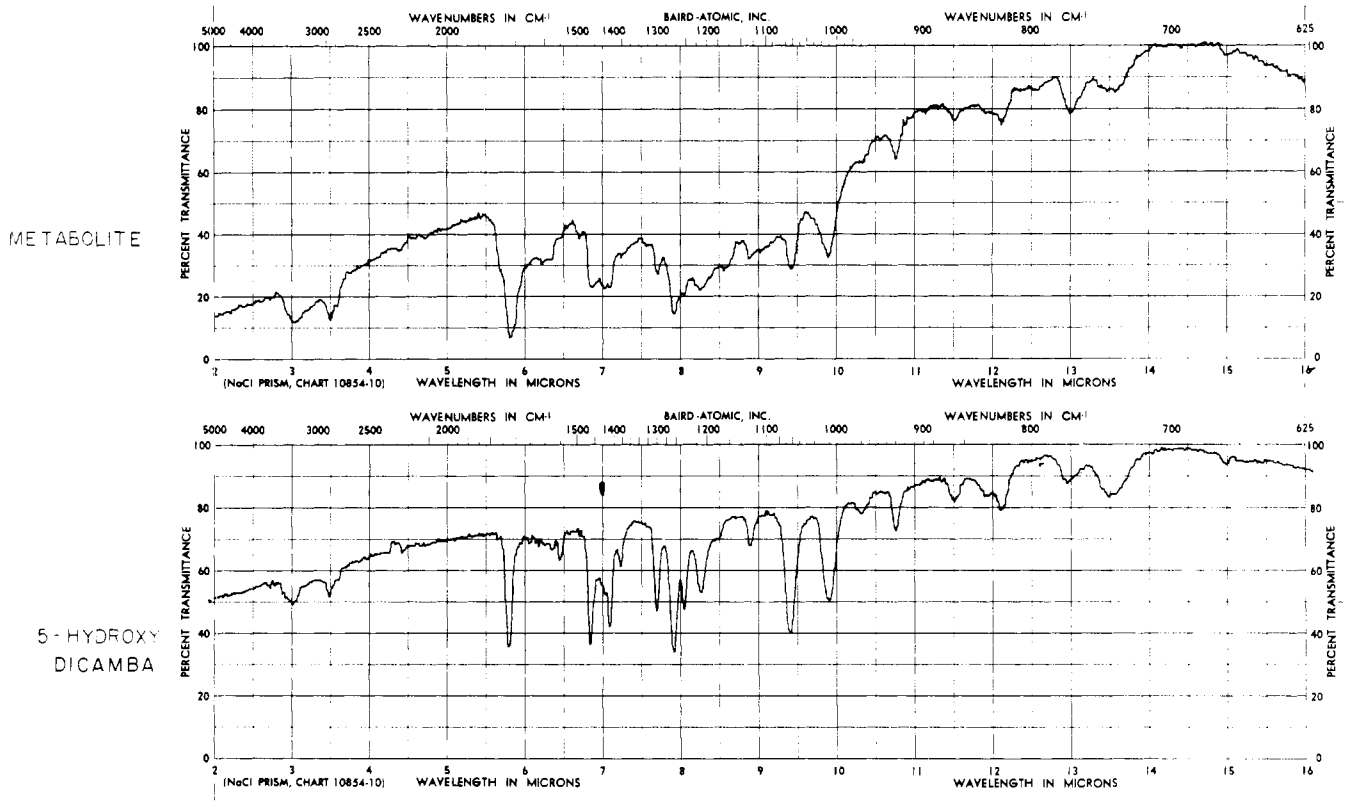

Figure 1. Infrared spectra of the methylated major metabolite and methylated 5-hydroxy dicamba in KBr pellets

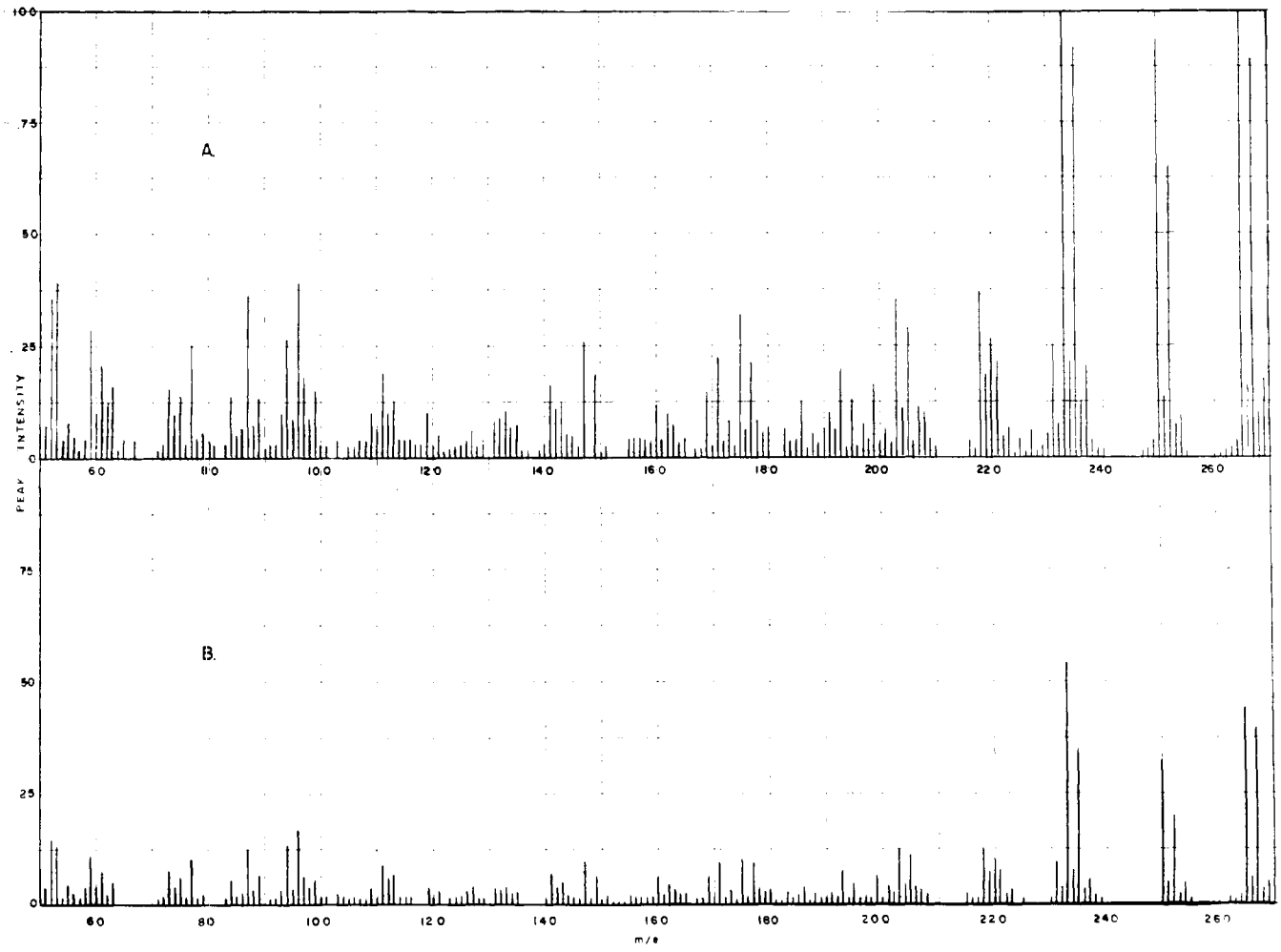

Figure 2. Mass spectra of (A) methylated 5-hydroxy dicamba and (B) methylated major metabolite

the R_f values of the metabolites in butanol–ammonia–water indicated that the minor metabolite had been converted to dicamba. In the case of the major metabolite, the saponification yielded a compound which differed from the original. This suggested that the aromatic ring had been hydroxylated.

By the microcoulemetric method for chlorine determination, the ratio of ^{14}C to chlorine for the major metabolite was approximately 80% of dicamba. This indicated that if the benzoic acid structure still was intact, the major metabolite, contained two chlorine atoms per molecule. By trapping the $^{14}CO_2$ from the combustion of the major metabolite, the chlorine, measured by the microcoulometric gas chromatography, was found to come from this metabolite.

The identification of the minor metabolite was afforded further by comparing its R_f values, as detected on a strip scanner, with those of nonlabeled 3,6-dichlorosalicylic acid, which was detected via a phenolic spray reagent. This reagent consisted of mixing equal volumes of 1% ferric chloride solution with a 1% potassium ferricyanide solution. The ^{14}C-methoxy-labeled dicamba gave no labeled minor metabolite at the R_f's expected for dichlorosalicylic acid on the four chromatography systems, which indicated that the methoxy group was present in the major metabolite.

The intermediate product in the synthesis of 5-hydroxy dicamba from dicamba was isolated and elemental analysis obtained. Methyl-5-amino-2-methoxy - 3 - 6 - dichlorobenzoate gave pale yellow plates, m.p 108° C., from ethanol. Found 43.2;3.7. $C_9H_9O_3NCl_2$ requires C = 43.2 and H = 3.6.

Gas liquid chromatography, infrared spectrophotometry, and mass spectrometry were used to identify the major metabolite as being 5-hydroxy-2-methoxy-3, 6-dichlorobenzoic acid. The retention times of the methylated metabolite and methylated 5-hydroxy dicamba are found in Table III. To obtain an IR spectrum, a sufficient amount of the methylated metabolite was purified further by means of a GLC fraction collector. This spectrum was compared with the IR spectrum of the methylated 5-hydroxy dicamba (Figure 1).

Figure 2 gives the mass spectra for the methylated metabolite and methylated 5-hydroxy dicamba.

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INSECTICIDE ABSORPTION

Parathion Absorption, Translocation, and Conversion to Paraoxon in Bean Plants

ALI EL-REFAI[1] AND T. L. HOPKINS

Department of Entomology, Kansas State University, Manhattan, Kan.

Parathion deposits on bean plant leaves, *Phaseolus vulgaris,* had half lives of 1 day on leaf surfaces and 2.4 days for the total plant under controlled environmental conditions. Disappearance was initially more rapid from leaves than from glass surfaces owing to combined evaporation and absorption, but later the cuticular residues became more persistent. Leaf absorption of parathion occurred rapidly with about 30% of the original deposit within the plant by 2 days. Parathion degradation products accumulating on both glass and leaf surfaces were similar and included paraoxon, *p*-nitrophenol, and possibly *S*-ethyl parathion. Total paraoxon residues remained small at approximately 1% of the original application. Parathion was readily absorbed from nutrient solution by the roots, but less than 2% was translocated to the foliage. Small paraoxon residues also accumulated in roots and foliage.

THE BEHAVIOR OF PARATHION deposits on roots and foliage and the mechanisms of loss from plants have not been elucidated clearly, although many data exist on parathion residues in various crops. Parathion apparently penetrates into living plants to a limited extent, and volatilization from foliage surfaces has been suggested as a major loss factor, as well as hydrolysis in or on the plant (*6, 17*). Translocation of parathion in plant tissue has been indicated by several studies showing mortality of insects feeding on plants grown in parathion-treated soil. However, volatilization of parathion from the soil to the foliage was not ruled out as a possible cause of toxicity (*5, 17*). David and Aldridge (*7*) demonstrated that the guttation fluid from wheat plants treated with parathion by root application was toxic to mosquito larvae and contained an anticholinesterase with properties similar to paraoxon. They concluded that parathion was oxidized in the roots close to the site of absorption as no parathion could be detected in the foliage by bioassay techniques. Zeid and Cutkomp (*18*) also showed by bioassay that paraoxon was translocated in plants, but evidence for parathion movement in plant tissue was inconclusive.

[1] Present address, Insect Control Section, Ministry of Public Health, Cairo, U. A. R.