Metabolism of Hydroxysimazine by Corn Plants

M. L. Montgomery, D. L. Botsford, and V. H. Freed

The metabolic products of hydroxysimazine in corn plants were studied. The primary metabolite was a result of dealkylation, yielding 2-hydroxy-4-amino-6-ethylamino-*s*-triazine. This nonvolatile product was determined by gas chromatography as its trimethylsilyl derivative. Paper chromatography of the other metabolites indicated that a second dealkylation occurs, giving rise to ammeline. In addition, there is also a product which corresponds to ammelide. Cotton, which is able to dealkylate diuron, is also capable of dealkylating hydroxysimazine. The dealkylation of herbicides appears to be an important pathway of detoxification.

Resistance of corn to the triazine simazine [2-chloro-4,6-bis (ethylamino)-*s*-triazine] appears to be a result of this plant's ability to degrade the herbicide. Simazine is rapidly metabolized by the corn plant with the production of a number of metabolites and eventual oxidation of the ring and chain carbons to carbon dioxide (Davis *et al.*, 1959; Montgomery and Freed, 1961; Funderburk and Davis, 1963). The first degradation product of simazine to be identified was hydroxysimazine (Castelfranco *et al.*, 1961). Since hydroxysimazine has no appreciable biological activity, the replacement of the chlorine by a hydroxyl group inactivates the herbicide. This conversion can be effected by simply incubating simazine with expressed corn sap. In corn, the reaction is primarily nonenzymatic, being mediated by the naturally occurring 2,4-dihydroxy-3-keto-7-methoxy-1,4-benzoxazine or its glucoside (Hamilton and Moreland, 1962). Subsequently, other metabolites are formed through metabolism of hydroxysimazine (Plaisted and Thornton, 1964). The first product of hydroxysimazine metabolism accumulates to the greatest extent, so we have concentrated our efforts on its identification.

EXPERIMENTAL

Corn plants (*Zea mays*, var. Tendermost) were germinated in sand and transferred to nutrient media after they reached a height of 6 inches. Upon reaching a height of about 10 inches, they were exposed to a 1-p.p.m. solution of ring labeled simazine, specific activity 5.5 μc. per mg., for three days. The plants were then transferred to untreated nutrient solution and harvested after 3, 14, and 28 days.

Upon harvesting, the plants were macerated and extracted with methanol overnight in a Soxhlet extractor. The extent of metabolism was determined by paper chromatography in either isoamyl alcohol saturated with 3*M* hydrochloric acid or butanol saturated with 1.5*M* ammonium hydroxide.

As indicated earlier, the first product of hydroxysimazine metabolism accumulated to the greatest extent. This metabolite will be referred to as Metabolite II. This metabolite was purified by paper chromatography and subjected to hydrolysis, since many herbicides have been shown to undergo conjugation with plant constituents. The metabolite was heated at 60° C. for four hours with either 3*M* hydrochloric acid or 3*M* ammonium hydroxide. The digestion mixture was evaporated to dryness under vacuum, redissolved in methanol, and paper chromatographed to determine whether any alteration had occurred.

The chlorotriazine herbicides are known to be metabolized through dealkylation both in fungi and plants (Kearney *et al.*, 1965; Shimabukuro, 1968). The possibility that Metabolite II was a dealkylated product of hydroxysimazine was evaluated by comparison of the metabolite to different hydroxytriazines. Ammeline (2-hydroxy-4,6-diamino-*s*-triazine) and ammelide (2,4-dihydroxy-6-amino-*s*-triazine) were purchased from commercial sources. Geigy Chemical Company supplied 2,4-dihydroxy-6-ethylamino-*s*-triazine. 2-Hydroxy-4-amino-6-ethylamino-*s*-triazine was prepared by acid hydrolysis of 2-chloro-4-amino-6-ethylamino-*s*-triazine, which was synthesized by the method of Pearlman and Banks (1948).

The only reagent which was found to be sensitive to the aminotriazines was that described by Whitenberg (1967). The dried chromatograms were sprayed with 5% butyl hypochlorite in cyclohexane and the excess reagent was evaporated. The strips were then sprayed with 1% starch, followed by 1% potassium iodide solution. The R_f values of Metabolite II and reference compounds were determined in three developing solvents.

To conduct confirmatory tests, it became necessary to collect more metabolite than was obtained from ^{14}C-treated plants. Therefore, large amounts of corn were treated with nonlabeled simazine through nutrient media. Three weeks following exposure, the plants were extracted with chloroform and then 80% ethanol, which extracts the desired metabolite. This extract was purified by passage through a column of Dowex 50 resin, H^+ form, and the metabolites were eluted with 4*M* ammonium hydroxide in 50% ethanol (Plaisted and Thornton, 1964). After evaporation of the eluate to dryness, the residue was dissolved in dilute acid solution, pH 4, and again passed through the Dowex 50 resin. The metabolites were eluted from the resin with 1*M* hydrochloric acid. Some ^{14}C metabolites from previous extracts were added to the initial extract to serve as markers.

At this point the extract was still colored and contained metabolites other than the major one of interest. Therefore, Metabolite II was separated from the others by paper chromatography using the butanol:ammonia solvent. The metabolite was extracted from the papers with methanol in a Soxhlet extractor and finally purified by passage through a 2 × 10-cm. column of Woelm basic alumina, activity grade V. The metabolite was eluted from the column with 95% ethanol, and was sufficiently pure for further tests.

Although the hydroxytriazines are nonvolatile, they can be gas chromatographed as their trimethylsilyl derivatives. These were prepared by evaporating the solvent from solutions of metabolite or reference standards in 1-ml. volumetric flasks. After the addition of 0.6 ml. of pyridine, 0.2 ml. of hexamethyldisilazane, and 0.1 ml. of trimethylchlorosilane, the flasks were held at 40° C. for 1 hour, and centri-

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

Table I. Compounds Containing ^{14}C in Corn Following Exposure to ^{14}C Simazine

Days following exposure	Percentage of ^{14}C in: Simazine	Hydroxysimazine	Metabolite II	Low R_f band
3	6	94	0	0
14	0	50	50	0
28	0	20	60	20

Table II. Paper Chromatography R_f Values and Gas Chromatography Retention Times of Synthetic Triazines and Metabolite II in Corn Plants

Compounds	R_f Values PNW[a]	BNW[b]	BAW[c]	Retention time,[d] minutes
Ammeline (2-hydroxy-4,6-diamino-triazine)	0.07	0.05	—	7.6
Ammelide (2,4-dihydroxy-6-amino-triazine)	0.08	0.05	—	4.3
2,4-Dihydroxy-6-ethylaminotriazine	0.36	0.12	0.40	4.6
2-Hydroxy-4-amino-6-aminotriazine	0.40	0.32	0.42	6.6
Metabolite II	0.38	0.33	0.42	6.6

[a] *n*-Propanol–ammonium hydroxide–water, 73:20:7. [b] *n*-Butanol saturated with 1.5*M* ammonium hydroxide. [c] *n*-Butanol–acetic acid–water, 90:29:10. [d] Compounds were chromatographed on a 5-foot, 5% SE-30 on Gas Chrom Q column at 167° C.

fuged. An aliquot of the supernatant was analyzed using a hydrogen flame detector on a 5-foot, 1/8-inch o.d., 5% SE-30 on Gas Chrom Q, column at 167° C.

Infrared spectra were obtained using potassium bromide pellets. The metabolite extract was evaporated to dryness and taken up in a small amount of methanol. This solution was evaporated on potassium bromide, from which a pellet was made. The spectrum of the metabolite was compared to those of the reference standards.

RESULTS AND DISCUSSION

The extracts of the tops of plants harvested 3 days following exposure contained radioactivity equivalent to approximately 1 p.p.m. of simazine. Since there was a limited exposure to herbicide, the concentration at subsequent harvests declined because of growth dilution. Approximately 70% of the radioactivity absorbed was translocated to the tops of the plants.

Paper chromatography of the extracts in the isoamyl alcohol developer showed that the metabolism of simazine was quite rapid. In this developer simazine has an R_f of 0.9, while that of hydroxysimazine is 0.55. Three days following exposure, less than 10% of the absorbed simazine was still parent herbicide. The remainder of the absorbed radioactivity was present as hydroxysimazine.

In later harvest, metabolite II and a band containing more than one metabolite were detected. In the isoamyl solvent, the metabolite II had an R_f value of 0.2, while there was a broad band from the origin to 0.12. In the butanol-ammonia solvent, both simazine and hydroxysimazine have R_f values of about 0.9. Metabolite II has an R_f of 0.33, while the low-R_f metabolites run from near the origin to 0.15. Table I shows the percentages of metabolites found at the different harvest times.

It is apparent from Table I that the degradation of hydroxysimazine is not very rapid. However, the rate will be dependent on the vigor of the plants. In this particular time study, the plants did not grow as rapidly as expected. In a preliminary study in which a single harvest at 15 days was made, the plants were growing more rapidly and there was an appreciable amount of the low-R_f component.

The rapid appearance of hydroxysimazine and its decline with the appearance of other metabolites strongly indicated that subsequent metabolites resulted form the metabolism of hydroxysimazine. To make certain that hydroxysimazine was an intermediate, corn plants were exposed to this compound. The absorption and translocation of the compound was much less than with simazine, but chromatography indicated that the same metabolites were formed.

Attempts to hydrolyze Metabolite II were unsuccessful. The R_f values of the metabolite in the acid and the alkaline solvent did not change following hydrolysis. In addition to showing that the metabolite was not a conjugate, the attempted hydrolysis indicated that the triazine nucleus was still intact. It was anticipated that if the ring had been opened, giving rise to a substituted biguanide or biuret, some alteration of R_f or loss of radioactivity would have been found.

Metabolite II was suspected to be a dealkylated product, since it has been shown that plants are capable of carrying out *N*-demethylation of herbicides (Lemin, 1966; Shimabukuro *et al.*, 1966; Smith and Sheets, 1967). The initial attempts to detect the aminotriazines were not always reproducible. Frequently, the compounds would not give the characteristic blue color. This problem was overcome by thoroughly drying the strips in an oven at 50° C. prior to spraying. After spraying, the excess hypochlorite was evaporated with the aid of a fan. The paper chromatography R_f values of the authentic compounds and major metabolite are given in Table II.

The R_f values in Table II indicate that Metabolite II is the dealkylated hydroxysimazine, 2-hydroxy-4-amino-6-ethylamino-*s*-triazine. The movement of reference standards and metabolite agree in all three developers. The compound which would result from replacement of the ethylamino substituent by a hydroxyl group agrees quite well in two solvents. However, it is quite different in the butanol-ammonia solvent.

As noted earlier, it was necessary to obtain more metabolite in order to determine its infrared spectra and retention time in gas chromatography. This was obtained from about 3 kg. of corn, 21 days following exposure to 2 p.p.m. of nonlabeled simazine. The extract following column and paper chromatography was nearly colorless.

The first analysis was by gas chromatography. Since methoxy triazines are volatile, the metabolite was treated with diazomethane in an attempt to prepare the methoxy derivative. However, using a preparatory gas chromatograph, no detectable ^{14}C was found in the effluent following injection of diazomethane-treated ^{14}C metabolite. Subsequently, the metabolite was treated with hexamethyl disiliazane and chlorotrimethylsilane. Radioactivity was found in the effluent. Therefore, the trimethyl silyl derivatives of the metabolite and synthetic products were prepared and their retention times determined (Table II). Again the data show the metabolite to be the dealkylated product of hydroxysimazine.

The infrared spectra of all the compounds in Table II were determined following their incorporation into potassium bromide pellets. The infrared spectra in Figure 1 confirm the observation that the first metabolite of hydroxysimazine in corn is 2-hydroxy-4-amino-6-ethylaminotriazine.

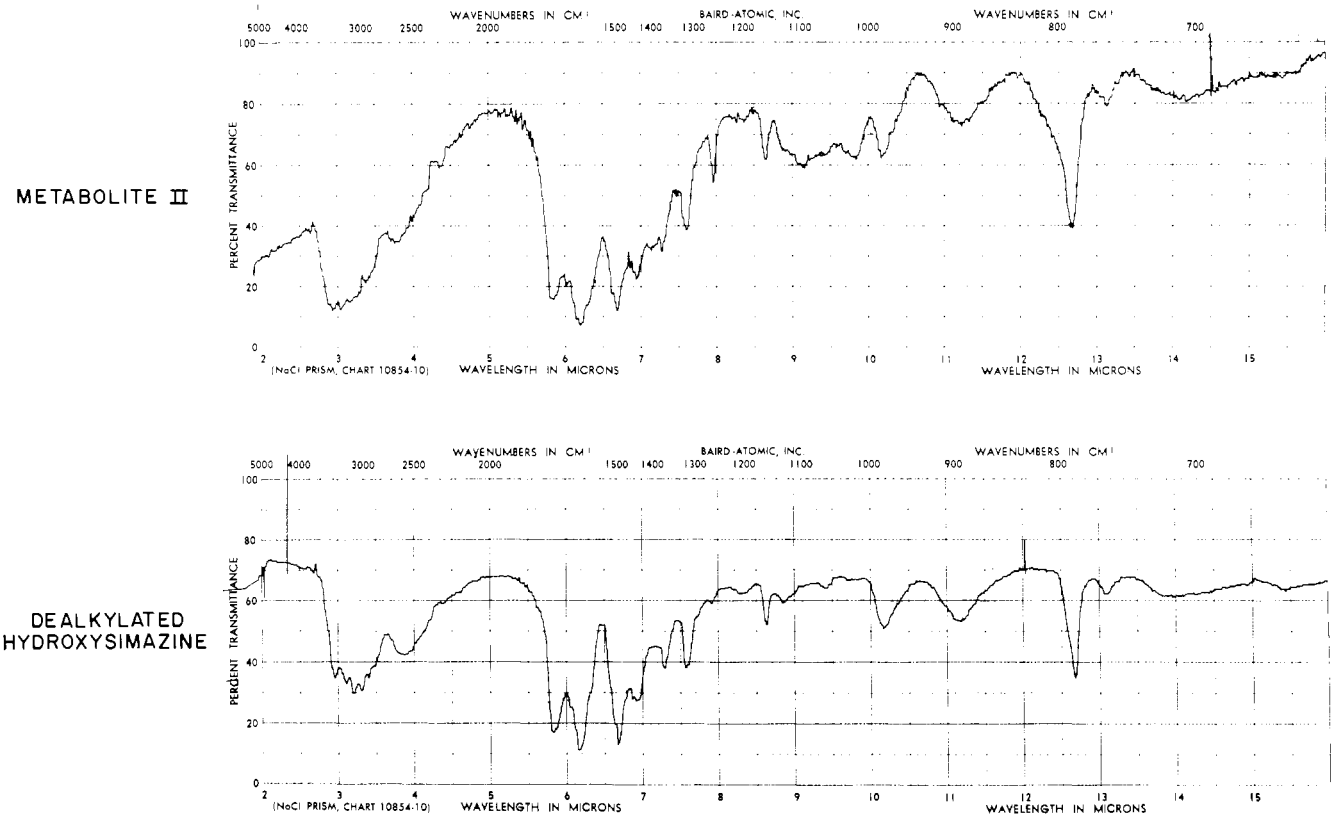

Figure 1. Infrared spectra of Metabolite II and dealkylated hydroxysimazine (2-hydroxy-4-amino-6-ethylamino-*s*-triazine)

Metabolites of low R_f were not sharply separated. However, one of the components was sufficiently well resolved to obtain R_f values which were found to agree with ammeline (Table II), the product which would be formed by removal of both the alkyl groups of hydroxysimazine. In addition, there was a component in the low R_f band which corresponded to ammelide in the developers listed in Table II and in the solvent lutidine-water, 65 to 35.

The results of this study show that in the corn plant, hydroxysimazine is metabolized by removal of one of the alkyl groups. Chromatographic behavior of the metabolites also suggests that the second alkyl group is removed and the resulting amino group is replaced by a hydroxyl substituent. Since $^{14}CO_2$ is evolved from corn treated with ^{14}C ring labeled simazine, the ring must ultimately be opened. Whether this occurs following di-dealkylation or following conversion to ammelide or cyanuric acid has not been established.

The above data show that metabolism of hydroxysimazine involves a dealkylation step. Since it has been shown that cotton dealkylates diuron (Smith and Sheets, 1967), it was of interest to determine if this species would also dealkylate hydroxysimazine. Fourteen days following exposure of cotton plants to ^{14}C hydroxysimazine, the extracts of the plants contained unchanged hydroxysimazine, a compound which corresponded to Metabolite II in corn and a low R_f band. The relative percentages of ^{14}C in the different components were 40, 40, and 20, respectively. Thus, it would appear that *N*-dealkylation is a common metabolic process in plants.

ACKNOWLEDGMENT

The authors thank Geigy Chemical for gifts of chemicals and support of this research.

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Received for review June 20, 1968. Accepted July 16, 1969. Published with the approval of the Director of the Oregon Agricultural Experiment Station. Work was supported by U. S. Public Health Service Grant ES 00040.