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FUNGAL METABOLISM OF HERBICIDES

Metabolites of Simazine by *Aspergillus fumigatus*

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At least two Cl^{36}-labeled simazine metabolites were detected in culture solutions of the soil fungus *Aspergillus fumigatus* Fres. One of these was identified as 2-chloro-4-amino-6-ethylamino-*s*-triazine by spectral methods of analysis. The second Cl^{36}-labeled metabolite possessed an intact *s*-triazine ring but no alkyl substituents associated with the ring. A new pathway of metabolism, which does not involve the hydroxy analog reported to occur in higher plants, is proposed for degradation of the herbicide, simazine.

THE isolation and identification of a soil fungus, *Aspergillus fumigatus* Fres., which degrades the herbicide 2 - chloro - 4,6 - bis(ethylamino) - *s*-triazine (simazine) have been reported (*9*). The organism reduced the amount of radioactivity in culture solutions containing C^{14}-chain-labeled simazine more rapidly than in culture solutions containing C^{14}-ring-labeled simazine. Subsequent investigations (*8*) revealed that $C^{14}O_2$ was evolved only from the chain-labeled simazine. This result suggested that the ring portion of the molecule was still intact.

Several new metabolites, unreported from simazine metabolism by higher plants (*5, 10, 15*), were detected in culture solutions of *A. fumigatus*. The purpose of the research reported in the present paper was to examine some of these metabolties and establish a metabolic pathway in *A. fumigatus*.

Materials and Methods

Source of Materials. The relative mobility of several authentic substituted *s*-triazines was determined on paper and thin-layer chromatograms in order to identify simazine metabolites from *A. fumigatus* solution by cochromatography. The substituted *s*-triazines examined were

A. 2 - Hydroxy - 4,6 - bis(ethylamino)-*s*-triazine (hydroxysimazine)
B. 2 - Chloro - 4 - amino - 6 - ethylamino-*s*-triazine
C. 2 - Chloro - 4 - hydroxy - 6 - ethylamino-*s*-triazine
D. 2,4 - Dihydroxy - 6 - ethylamino-*s*-triazine
E. 2,4 - Dihydroxy - 6 - amino - *s*-triazine (ammelide)
F. 2,4,6 - Trihydroxy - *s* - triazine (cyanuric acid)

To facilitate discussion, these compounds are alphabetically designated. Compounds E and F were labeled with C^{14} in the ring. Compound A was synthesized from simazine C^{14} by a method similar to that of Castelfranco, Foy, and Deutsch (*2*). These suspected metabolites and Cl^{36}-labeled simazine (specific activity 0.324 μc. per mg.), C^{14}-ring-labeled simazine (specific activity 5.06 μc. per mg.), and C^{14}-chain-labeled simazine (methylene-labeled, specific activity 4.97 μc. per mg.) were generously supplied by the Geigy Chemical Corp., Ardsley, N. Y.

Silica gel G for thin-layer chromatography was purchased from the Research Specialties Co., Richmond, Calif. Florisil previously activated at 1200° F. was purchased from Floridan Co., Hancock, W. Va. Nuclear magnetic resonance and mass spectra and some of the infrared spectra were determined by the Stanford Research Institute, Menlo Park, Calif. Gas-liquid chromatography was performed with a flame ionization detector on a RSCo 600 series instrument.

C^{14}-Labeled Simazine Experiments. Culture conditions for *A. fumigatus* with respect to time, temperature, media composition, and inoculation have been described (*8, 9*). All solutions containing labeled simazine were prepared by first dissolving the compound in 5 ml. of chloroform and then adding the chloroform solution to the nutrient solution. Chloroform was removed by bubbling sterile air through the nutrient solution. The final concentration of simazine in each solution was 5 p.p.m. and contained the following amounts of radioactivity in 100 ml. of solution: Cl^{36} simazine (0.162 μc.), C^{14}-ring-labeled simazine (2.53 μc.), and C^{14}-chain-labeled simazine (2.48 μc.). Aliquots of the culture solution were removed periodically after inoculation and extracted three times with chloroform, and the chloroform and aqueous phases were assayed for radioactivity. Chloroform extracts were reduced in volume and chromatographed on Whatman No. 1 paper and thin-layer plates (250 microns thick). Metabolites that appeared in the chloroform phase of Cl^{36} simazine solutions were compared to metabolites recovered from ring- and chain-labeled simazine C^{14} solutions.

Large-Scale Incubation Experiments. Mass culture experiments were conducted to obtain sufficient quantities of a

chloroform-soluble metabolite for identification by spectral methods. Fifty milligrams of simazine was dissolved in 10 liters of culture solution (5 p.p.m.). After an 8-day incubation, the fungal mycelia were harvested on cheesecloth and washed with distilled water; then the wash was combined with the original supernatant nutrient solution. The fungal mycelia were stored in the freezer for studies directed at isolating the catalyst responsible for altering simazine. The nutrient solution was concentrated in vacuo to a 500-ml. volume in a rotary flash evaporator at 10° C.

Extraction and Purification of Chloroform-Soluble Metabolites. The concentrated solution containing the simazine metabolites was extracted four times with 500 ml. of chloroform and the aqueous phase discarded. The chloroform extract was reduced in vacuo, transferred to 12-ml. pointed test tubes, and again carefully reduced; then the chloroform extract (ca. 4 ml.) was spotted on silica gel G–coated plates. Thin-layer chromatograms were developed with benzene–acetic acid (1 to 1). After development, the silica gel between R_f 0.70 and 0.85 was carefully removed, placed on a sintered glass filter, and eluted with pure methanol. Previous studies with the labeled intermediates indicated that a major simazine metabolite appeared in this region of the chromatograph.

The methanol extract containing the metabolite was evaporated to dryness and redissolved in chloroform. A Florisil column (10 × 2 cm.) was prepared, and the chloroform extract (5 ml.) was added to the column. A stepwise elution was carried out with 100-ml. volumes of chloroform-ether solutions (0, 5, 10, and 50% ether, respectively). The metabolite in the eluates was determined by chromatographing a small aliquot (10 μl.) on silica gel. The benzene–acetic acid solvent system (*17*) was used, and the developed chromatograms were sprayed with 0.1*M* $AgNO_3$.

Results

The R_f values (averages from three determinations) of several *s*-triazine derivatives on paper chromatograms are shown for five solvent systems (Table I). Some of the compounds were detected by C^{14} and others by appropriate spray methods. Study of several detection methods revealed that compound D (2,4 - dihydroxy - 6 - ethylamino - *s*-triazine) reacts with bromophenol blue if the spot is first sprayed with dilute NH_4OH and allowed to dry before being sprayed with the indicator dye. The dihydroxy-*s*-triazine acts as an anion and gives a yellow spot on a blue background in solvent systems I and IV (Table I).

Compound B at fairly high concentration (50 μg. or above) can be seen on chromatograms with ultraviolet light, but the spot is too faint for the method to be of value for detection purposes. Solvent system I generally gave the best resolution of the metabolites from *A. fumigatus* culture solutions. However, paper chromatography for identification of *s*-triazine derivatives is generally limited because of the difficulty of detecting nonlabeled compounds.

Chromatography of the chloroform and aqueous phases in solvent system I showed the appearance of at least two metabolites containing Cl^{36}. Chloroform extracts from day-6 samples contained a major radioactive spot in the region R_f 0.66 to 0.74; activity in samples taken on day-8 and subsequent days was located in the region R_f 0.84 to 0.85 with an occasional shoulder at R_f 0.75 to 0.77. The aqueous phase after chloroform extraction contained a Cl^{36} metabolite (R_f 0.22) that appeared 10 days after inoculation. As previously reported (*8*, *9*), hydroxysimazine was not detected on any chromatograms. Cochromatography of the Cl^{36} metabolites with metabolites from C^{14}-ring- and chain-labeled simazine culture solutions revealed that the Cl^{36}-metabolite in the chloroform phase (R_f 0.84, unknown I) contained an intact ring and at least one side chain intact and that the Cl^{36} metabolite in the aqueous phase contained an intact ring and no alkyl side chains. Thin-layer chromatography of unknown I with simazine and suspected metabolites B and C on silica gel was carried out with a benzene–acetic acid solvent (1 to 1) (*17*). Unknown I migrated to the same area as B (R_f 0.73) and beyond C (R_f 0.33), but the presence of several contaminants from the culture solution plus small impurities in the authentic samples necessitated further investigation into the identity of the unknown. A mass culture experiment was initiated to isolate enough of the chloroform-soluble metabolite (unknown I) for identification purposes.

Simazine-C^{14} and unknown I-C^{14} were separated on a Florisil column by means of chloroform and chloroform plus increasing concentrations of ether (Figure 1). Unknown I, eluted from silica gel G and passed through Florisil columns, was detected in tubes 36, 37, and 38. A yellow contaminant from the nutrient media was detected in tubes 1 through 10. Small amounts of the impurity also occurred in later tubes after concentration of the chloroform-ether solutions.

A comparison of unknown I with simazine and suspected metabolite B by mass spectral analysis revealed that unknown I was 2-chloro-4-amino-6-ethylamino-*s*-triazine (B). Our sample of unknown I, after purification, also contained two small impurities. A prominent parent peak occurred at m/e = 173, with a parent +2 peak (33% of the parent peak at 173) due to the isotopic contribution of Cl^{37}. The odd number molecular weight would indicate the presence of an odd number of nitrogens, and would be consistent with the molecular weight of 2-chloro-4-amino-6-ethylamino-*s*-triazine. A large peak was also observed at m/e = 158 (173−15) due to the loss of a methyl group from B during fragmentation. The appearance of this compound supports the thesis that unknown I is compound B.

One impurity in the material recovered from the Florisil column had a mass of 280 or higher and probably represents some of the material excreted by the microorganism into the nutrient solution. A small peak at m/e = 201 probably represents some residual simazine.

Nuclear magnetic resonance spectra of 2 - chloro - 4 - amino - 6 - ethylamino-*s*-triazine and unknown I were in good agreement. Protons associated with CH_3, CH_2, NH, and NH_2 are discernible; but an undesirable signal-to-noise ratio, due to the low solubility of the compound in deuterated chloroform, made quantitative interpretation difficult. The NH_2 peak appears as a broad band in the region 4.4 tau, which would be in agreement with the published value for the structurally related amino group in 2-aminopyridine at 4.28 tau (*1*).

The infrared spectrum of unknown I, isolated from the mass culture experiment and purified on silica gel and Florisil, is shown in Figure 2. The failure to detect a strong band in the 8.5- to 10.0-micron region probably rules out the possibility of a hydroxy- or alkoxy-substituted *s*-triazine as a metabolite. The bands appearing in the

Table I. R_f Values of Substituted *s*-Triazines in Five Solvents

Substituents at 2, 4, and 6 Positions			Solvent[a] Systems				
R_1	R_2	R_3	I	II	III	IV	V
Cl	$C_2H_5NH_2$	$C_2H_5NH_2$	0.95	0.93	0.55	0.99	0.90
OH	$C_2H_5NH_2$	$C_2H_5NH_2$	0.62	0.78	0.85	0.95	0.79
OH	OH	$C_2H_5NH_2$	0.24	...	...	0.87	...
OH	OH	NH_2	0.06	0.29	0.76	0.00	0.46
OH	OH	OH	0.20	0.36	0.73	0.93	0.59

[a] I. Isoamyl alcohol saturated with 0.1*N* HCl.
II. 1-Butanol–acetic acid–water (4:1:5).
III. 30% Acetic acid on kerosine-soaked paper.
IV. Dioxane-water (1:1).
V. 2,6-Lutidene–water (6.5:3.5).

region 2.8 to 3.0 microns probably represent the NH and —NH_2 groups attached to the *s*-triazine ring. The weak carbonyl band at 5.75 microns is probably due to the high molecular weight impurity noted in the mass spectral studies (*16*).

Gas-liquid chromatography was employed to determine whether 2-chloro-4-amino - 6 - ethylamino - *s* - triazine could have been an impurity in the original starting material. The liquid phase Ucon L B 550-X on 60- to 80-mesh, HMDS-treated Chromosorb W effectively separated the authentic compounds (relative retentions at 172° C., simazine = 1.00 and 2 - chloro - 4-amino - 6 - ethylamino - *s* - triazine = 1.45; resolution, 2.2; carrier gas, nitrogen; injector temperature, 215° C.). A sample of the original simazine, sufficient to produce full scale deflection of the recorder at the retention time for simazine with the attenuator set at 30, produced no deflection at the retention time for compound B with the attenuator set at 10. This analysis should have readily detected less than 1% of an impurity in the original simazine. No 2 - chloro - 4 - amino - 6 - ethylamino-*s*-triazine was detected in the original simazine by this method.

Discussion

At least two major Cl^{36}-labeled metabolites from simazine-Cl^{36} were detected in culture solutions of *A. fumigatus*. One of these metabolites (unknown I) appeared in the chloroform extracts of nutrient solutions; and by cochromatography with ring- and chain-labeled-C^{14} simazine, unknown I was shown to have an intact ring; one intact alkylamino moiety; and a chlorine atom, bonded to the ring. Mass, infrared, and nuclear magnetic resonance spectra indicated that the compound has a molecular weight of 173 and an NH_2 group. Based on these observations unknown I was identified as the 2-chloro-4-amino-6-ethylamino-*s*-triazine.

A second Cl^{36}-labeled metabolite appeared in the aqueous phase after the appearance of 2-chloro-4-amino-6-ethylamino-*s*-triazine; it is known to have an intact ring; a chlorine bonded to the ring; and no alkyl moiety in the side chains. Isolation and purification of this second metabolite are now under way.

Other metabolites from C^{14}-labeled simazine were separated by paper chromatography. *A. fumigatus* cells, concentrated by centrifugation and incubated with C^{14}-ring-labeled simazine for 3 hours, produced a metabolite that cochromatographed with ammelide-C^{14} in three solvent systems (I, II, and III). Ammelide probably is produced by a nonenzymatic reaction involving a cellular constituent; the halide is probably removed from one of the simazine metabolites.

The unique capacity of *A. fumigatus* to remove the alkyl group from simazine provides evidence for a route of metabolism different from that reported in higher plants (*6*). Certain higher plants possess a cyclic hydroxyamate (2,4-dihydroxy - 7 - methoxy - 1,4 - benzoxazine-3-one) which catalyzes the nonenzymatic conversion of simazine to hydroxysimazine. The nonphytotoxic hydroxysimazine is further decomposed in plants with the evolution of $C^{14}O_2$ from ring-labeled simazine (*10*). Hydroxysimazine has also been detected in soils. Harris (*7*) found it in four soils incubated for 32 weeks at 30° C. Since it was also found in simazine-treated soils incubated for 6 days at 60° C., the conversion in soils may also be catalyzed by nonbiological systems.

Demethylation was recently reported (*4*) as a detoxification mechanism for other alkylated amine herbicides. The *N'* - (4 - chlorophenoxy)phenyl - *N*,*N*-dimethylurea (chloroxuron) was converted in plants and soils to the corresponding *N'*-(4-chlorophenoxy)phenyl-*N*-methylurea.

Activation of the phosphoramidate insecticide, octamethylpyrophosphoramide (schradan), to a potent anticholinesterase metabolite apparently proceeds by oxidation of one of the methylamine groups to the corresponding

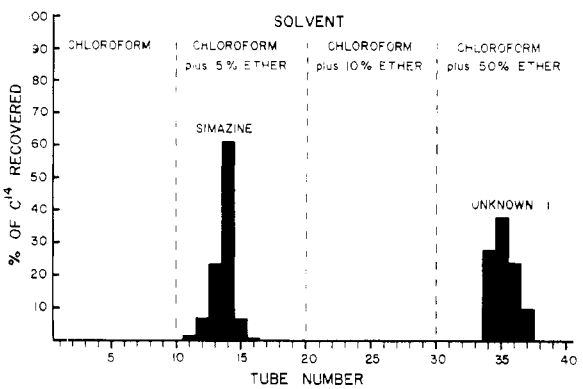

Figure 1. Elution pattern of simazine-C^{14} and unknown 1-C^{14} on Florisil using chloroform and various concentrations of chloroform plus ether

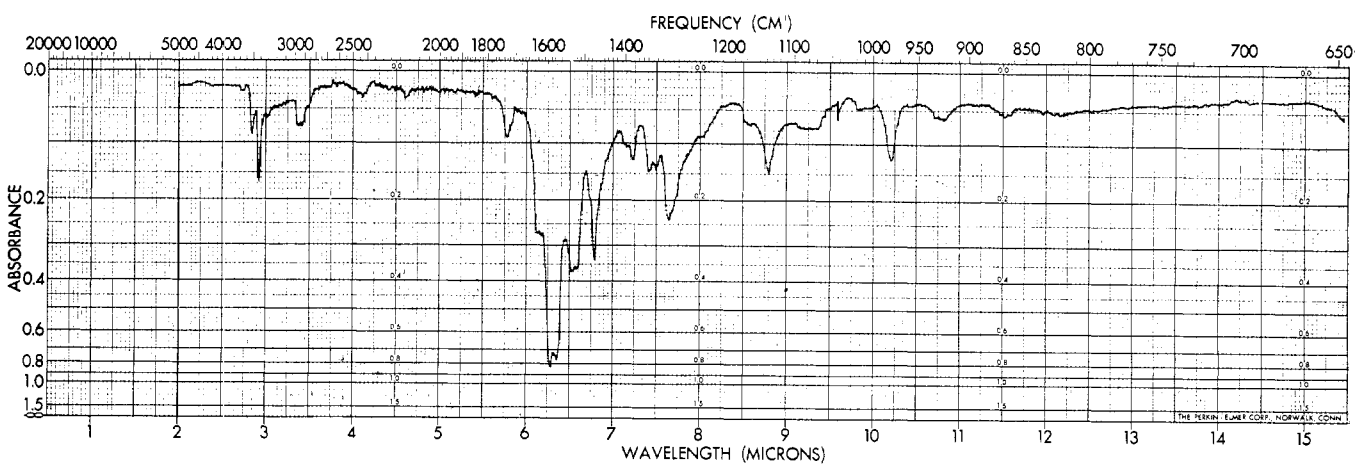

Figure 2. Infrared spectrum of 2-chloro-4-amino-6-ethylamino-*s*-triazine isolated from *A. fumigatus* culture solutions

3.5 mg. dissolved in 0.5 ml. of chloroform; resolution 928, scale 1 ×, thickness 0.4 mm.

hydroxymethyl derivative (*13*). Oxidation of schradan to its hydroxy alkyl derivative has been demonstrated in rat liver homogenates fortified with reduced pyridine nucleotides (DPNH or TPNH), magnesium, and nicotinamide (*3, 11, 12*). Further chemical decomposition of hydroxymethyl schradan yields heptamethylpyrophosphoramide and formaldehyde. However, the ethyl analog of schradan is not activated by the same process, nor is *O,O*-diethyl *S*-2-(diethylamino)ethylphosphorothioate (tetram), which contains a diethylamino group. The system described above appears to be specific for dimethyl amides, but a similar type of mechanism could be operative at the ethylamino group of simazine.

The mechanism by which simazine is converted to 2-chloro-4-amino-6-ethylamino-*s*-triazine has not been determined. Fractionation of the fungal mycelia by several methods has failed to yield an active preparation for dealkylating simazine. Concentration of the cellular components in the external media by reverse dialysis at reduced temperature has also failed to produce a catalyst. Since the majority of the metabolites were found in the external solution, several possibilities exist to explain the mode of breakdown. Extracellular enzymes might catalyze the degradation of simazine. Another possible method involves the metabolism of simazine on the surface of the cells, with subsequent release of the dealkylated *s*-triazine to the external media.

The following pathways, based on our own observations as well as those reported by others (*5, 7, 10, 14*), are proposed for the alterations of simazine in soils (Figure 3). The identity of the second Cl^{36} metabolite has not been established, but obvious possibilities include the diamino or hydroxyamino derivatives of simazine. The low frequency with which ammelide was detected in these experiments suggests that it occurs only after extensive metabolism of other simazine metabolites.

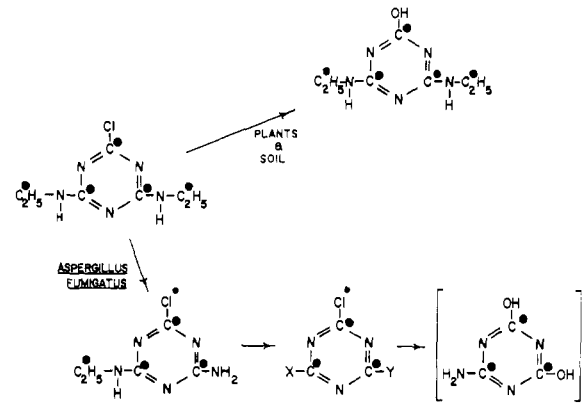

Figure 3. Proposed pathway of simazine degradation in soils

Dots indicate positrons of various radioactive atoms in molecule

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