Residues of Atrazine, Cyanazine, and Their Phytotoxic Metabolites in a Clay Loam Soil

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The degradation of 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine (atrazine) and 2-chloro-4-(1-cyano-1-methylethylamino)-6-ethylamino-*s*-triazine (cyanazine) was investigated in a Perth clay loam soil. Atrazine and cyanazine were applied at rates of 1, 2, and 3 lb/A of active ingredients as pre- and postemergence treatments. Soil was sampled from plots after 0, 2, 3.5, 5, and 12 months at 0–2.5, 2.5–5, and 5–10 in. depths. Atrazine, cyanazine, and their phytotoxic metabolites were extracted with a 65% acetonitrile–water mixture and quantitated on a Coulson conductivity detection system. Presented data indicate that atrazine is converted into deethylated atrazine (2-chloro-4-amino-6-isopropylamino-*s*-triazine) as a major and deisopropylated atrazine (2-chloro-4-amino-6-ethylamino-*s*-triazine) as a minor phytotoxic metabolite and that cyanazine is changed to deisopropylated atrazine as a major phytotoxic metabolite. Cyanazine amide [2-chloro-4-(1-carbamoyl-1-methylethylamino)-6-ethylamino-*s*-triazine] was found. It is proposed that the hydrolysis precedes the microbiological degradation to the deisopropylated atrazine.

Atrazine and cyanazine are selective herbicides used for the control of annual broadleaf and grassy weeds in corn and sorghum. Both herbicides may be applied at the rates of 1–4 lb/A active Cyanazine, developed at the Woodstock Agricultural Research Centre in England, is marketed by the Shell Chemical Co. under the trade name Bladex. It is claimed by the manufacturer to be less persistent in soil than atrazine and therefore less detrimental on the subsequent crops. The unstable nature of the cyanazine molecule also allows it to be used as a herbicide in potatoes, peas, barley, and wheat (Beynon *et al.*, 1970).

Atrazine and cyanazine are related structurally, the difference being that in cyanazine the isopropyl-hydrogen is replaced by a nitrile group. The addition of the nitrile group has changed the physical properties. The vapor pressure is reduced from 3.0×10^{-7} to 1.6×10^{-9} at 20°, and the solubility in organic solvents, including water, is increased fivefold. More important are the changes which occur in the hydrolysis pattern because they affect the herbicidal properties. The usual replacement of the chlorine atom in the 2 position with a hydroxyl group, found by Harris (1967), Roeth *et al.* (1969), and Skipper and Volk (1972) as common to all 2-chloro-4,6-alkylamino-*s*-triazines, is delayed. The initial attack is on the functional nitrile group within the cyanomethylethylamine. This reaction after the incubation period proceeds rapidly, producing 2-chloro-4-(1-carboxyl-1-methylethylamino)-6-ethylamino-*s*-triazine which loses chlorine and follows the regular *s*-triazine breakdown pattern (Figures 1 and 2) (Beynon, 1972; Beynon *et al.*, 1970, 1972a,b; Wright, 1970).

The sequential degradation of cyanazine to cyanazine amide and then cyanazine acid with subsequent decarboxylation has led to speculations that cyanazine, through the activity of microorganisms, may be converted to atrazine as one of the degradation products (Corke, 1972).

The phytotoxicity of cyanazine in the amide form is reduced and completely disappears as the hydrolysis continues. The ease of this conversion process separates cyanazine from many other *s*-triazines and is the basis for the manufacturer's claim as to why the herbicidal activity disappears rapidly (Beynon *et al.*, 1970).

The aim of our investigation was to determine the persistence of cyanazine and its phytotoxic metabolites, while at the same time comparing them with atrazine and its metabolites. For this purpose soils from an existing field experiment were sampled at varying time intervals following atrazine and cyanazine application.

MATERIALS AND METHODS

Field Experiment. Field experiments were conducted in 1970 and 1971 at Exeter, Huron County, Ontario. Plots were 12 ft × 50 ft and replicated four times. The soil was a Perth clay loam (32% clay) at a pH of 7.0, containing 4.8% organic matter and with a cation exchange capacity of 22.8 mequiv/100 g. The previous year's crop (1969) was seed corn treated with 1.5 lb/A ai atrazine. Plots were planted in corn on May 27. The weather conditions during the preemergence herbicide applications were cool and wet (May 27) but changed to fair and dry at the time of the postemergence applications (June 13). Treatments of 1, 2, and 3 lb/A ai were made with a 12-ft boom attached to a bicycle sprayer applying 21 gal/A of spray solution.

Soil samples were taken at the time of application and at 2, 3.5, 5, and 12 months after applications, using the standard 0.75–1.5-in. diameter corers. The cores were stored in plastic bags at −10° until delivered to the laboratory for analysis (Nov 7, 1972). Air-dried soil samples were crushed in a mortar, passed through a 20-mesh sieve, and kept in sealed glass jars at ambient temperatures. The soil was plowed and white beans were planted in June, 1971. The crop was harvested in the fall and the weight of grain was determined (Table I).

ANALYTICAL PROCEDURE

The analytical method was based on that of Purkayastha and Cochrane (1973), with some modifications. The solvents used were nanograde quality supplied by Caledon Laboratories, Georgetown, Ontario. Diazomethane for esterification was produced in our laboratory using a kit and reagents supplied by Aldrich Chemical Co.

The standards (i) 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine, (ii) 2-chloro-4-(1-cyano-1-methylethylamino)-6-(ethylamino)-*s*-triazine, (iii) 2-chloro-4-(1-carbamoyl-1-methylethylamino)-6-ethylamino-*s*-triazine, (iv) 2-chloro-4-(1-cyano-1-methylethylamino)-6-amino-*s*-triazine, (v) 2-chloro-4-(1-carbamoyl-1-methylethylamino)-6-amino-*s*-triazine, (vi) 2-chloro-4-amino-6-ethylamino-*s*-triazine, (vii) 2-chloro-4,6-diamino-*s*-triazine, (viii) 2-chloro-4-amino-6-isopropylamino-*s*-triazine, (ix) 2-chloro-4-(1-carboxy-1-methylethylamino)-6-ethylamino-*s*-triazine, (x) 2-hydroxy-4-(1-cyano-1-methylethylamino)-6-ethylamino-*s*-triazine, (xi) 2-hydroxy-4-(1-carboxy-1-methyleth-

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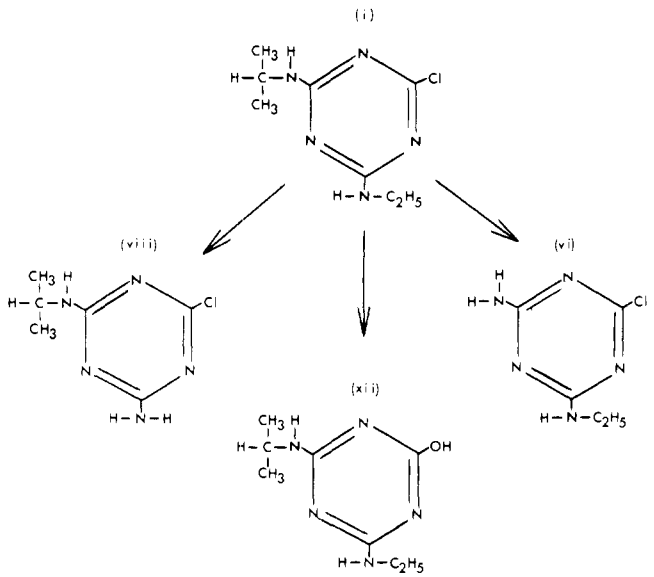

Figure 1. Atrazine phytotoxic (viii and vi) and nonphytotoxic (xii) metabolites in soil.

ylamino)-6-ethylamino-*s*-triazine and (xii) 2-hydroxy-4-(ethylamino)-6-(isopropylamino)-*s*-triazine were obtained from the Shell Chemical Co. through the courtesy of C. T. Corke, University of Guelph, and CIBA-Geigy Agricultural Chemicals.

For gas–liquid chromatography, a Tracor 550 apparatus with a Coulson conductivity detection system operative in nitrogen mode was used. Two columns, 6 ft × 0.25 in. o.d. glass packed with 6% Carbowax 20M on Aeropak 30 80–100 mesh and 2 ft × 0.25-in. o.d. glass packed with 1.5% cyclohexane dimethanol succinate (CHDMS) on Gas Chrom Q 80–100 mesh, were operated at 210°. Other temperatures were: injector, 245°; transfer line, 220°; and pyrolyser, 860°. Bridge potential was set at 30 V.

The quartz pyrolyser tube was 0.25-in. × 12-in. with a 0.5-in. long strontium hydroxide scrubber. The carrier gas was purified helium set at a flow rate of 70 ml/min for 6% Carbowax 20M column and 200 ml/min for 1.5% CHDMS column. Hydrogen was used in the pyrolyser at a flow rate of 50 ml/min.

The ion-exchange resin used was AG50W-X8 with a mesh size of 20–50 in the hydroxide and hydrogen form. The water flow rate through the cell was 3 ml/min.

For spectrophotometric analysis, a Unicam ultraviolet spectrophotometer was used.

LABORATORY PROCEDURE

One-hundred-gram samples of soil were extracted in a liter boiling flask with 600 ml of a 2:1 acetonitrile–water mixture by employing a mechanical shaker. The initial shake was 40 min, carried out in late afternoon. After standing overnight a second shaking for 40 min was given. Five-hundred milliliters of the extract was filtered off through a coarse porosity sintered glass funnel into a second 1-l. boiling flask. The acetonitrile was evaporated on a rotary evaporator and the water phase was transferred into a liter separatory funnel with 600 ml of water. The pH was adjusted to 9 by the addition of 0.4 ml of a 1:2 ammonium hydroxide–water mixture. The chlorine-containing herbicides and their metabolites were partitioned into two 100-ml portions of chloroform. The chloroform extracts were passed through a chloroform-washed and dried cotton filter into a 300-ml boiling flask and evaporated almost to dryness on a rotary evaporator at 50°. Ten milliliters of isooctane was added as retainer and the last remnants of chloroform were evaporated off. The residue was dissolved in 5 ml of methanol and 5–25 μl was injected into a glc for qualitative and quantitative analysis.

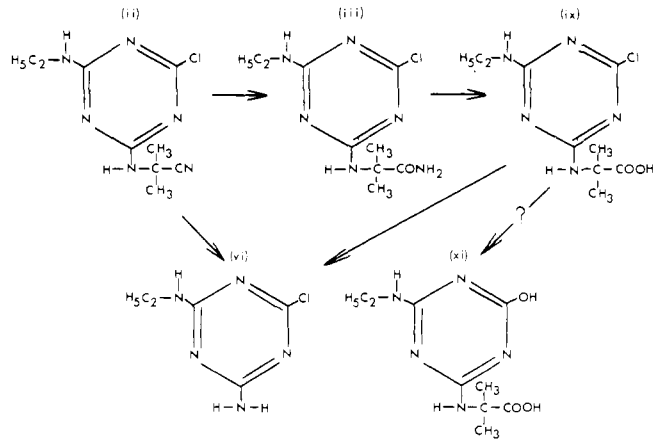

Figure 2. Cyanazine phytotoxic (vi and iii) and nonphytotoxic (ix, xi) metabolites in soil.

Table I. Yield of White Beans Harvested in 1971 from Plots Treated with Atrazine and Cyanazine in 1970

Herbicide, lb/A	Yield, bushels/A[a]	
	Preemergence	Postemergence
Atrazine 0 + cyanazine 0	23.4 bc[b]	23.0 b
Atrazine 1 + cyanazine 1	23.2 bc	23.0 b
Atrazine 2 + cyanazine 0	22.9 bc	16.5 ab
Atrazine 3 + cyanazine 0	8.4 a	9.3 a
Atrazine 0 + cyanazine 2	25.7 c	24.1 b
Atrazine 0 + cyanazine 3	22.0 bc	22.1 b

[a] Corrected for 3% moisture. [b] Means followed by the same letter do not differ significantly at the 1% level (Duncan's Multiple Range Test). Beans harvested Sept 3, 1971; threshed Sept 18, 1971.

The aqueous phases of selected samples were evaporated to dryness on a rotary evaporator using a water bath at 70° and the residues were dissolved in 5 ml of methanol. These fractions were first scanned on an ultraviolet spectrophotometer between 210 and 270 nm for hydroxy-*s*-triazines and then methylated with diazomethane. For the methylation step the samples were evaporated to dryness in a flow of nitrogen and were dissolved in 5 ml of ethyl ether containing 4–6% diazomethane. The reaction occurred in 20 min at room temperature with occasional shaking. Excess diazomethane and ethyl ether were removed under a flow of nitrogen. Samples were dissolved in 5 ml of methanol and analyzed for chlorinated carboxyl and diamino compounds. The glc results of both the chloroform and aqueous phases were confirmed using different glc columns.

Standard recovery curves were prepared from 100-g soil samples fortified to 0.10, 0.15-, and 0.20-ppm levels with methanolic solutions of standards i–xii 24 hr before extraction (Figures 3 and 4). Recoveries of deethylated atrazine were acceptable but those of deisopropylated atrazine were low due to its greater solubility in the aqueous phase (Table II).

RESULTS AND DISCUSSION

The degradation of atrazine and cyanazine produces three phytotoxic metabolites: 2-chloro-4-amino-6-isopropylamino-*s*-triazine (deethylated atrazine); 2-chloro-4-amino-6-ethylamino-*s*-triazine (deisopropylated atrazine); and 2-chloro-4-(1-carbamoyl-1-methylethylamino)-6-ethylamino-*s*-triazine (cyanazine amide). Of these three compounds the deethylated atrazine is a potential herbicide, which might explain the phytotoxicity of soils where analytical data indicate low levels of atrazine. The other two metabolites are required in much higher concentra-

Table II. Recovery of Atrazine, Deethylated Atrazine, Deisopropylated Atrazine, and Cyanazine from Soil Samples Fortified with Above Compounds 24 hr before Extraction

	Fortification, ppm				Recovery, %			
	Atrazine	Deethylated atrazine	Deisopropylated atrazine	Cyanazine	Atrazine	Deethylated atrazine	Deisopropylated atrazine	Cyanazine
Soil, air dry, 5% H_2O	0.10	0.10	0.10	0.10	86.3	72.6	45.5	95.7
					78.2	71.0	40.0	87.5
	0.15	0.15	0.15	0.15	86.7	65.8	41.8	100.0
					78.3	64.7	37.0	95.6
	0.20	0.20	0.20	0.20	86.3	76.4	49.1	97.9
					85.6	71.0	38.9	117.4

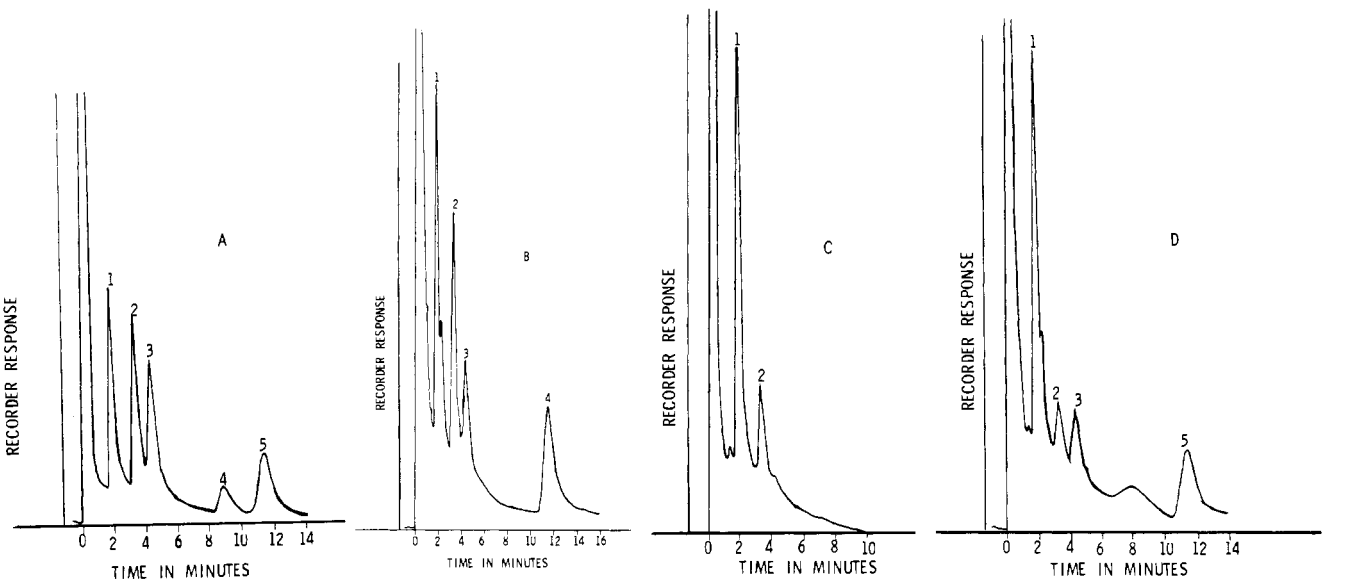

Figure 3. Coulson conductivity glc, 6% Carbowax 20M on Aeropak 30 column; 1, atrazine; 2, deethylated atrazine; 3, deisopropylated atrazine; 4, 2-Cl-4,6-diamino-*s*-atrazine; 5, cyanazine. Chromatograms: A, standards, 10 ng injected, attenuation 2×. B, mixed soil fortified at 0.2 ppm, 20 g/ml extract, 5 μl injected, attenuation 2×. C, sample 73-0164, 2.5–5 in. depth. Soil treated with 3 lb/A atrazine. Extract 20 g/ml, 10 μl injected, attenuation 2×. D, sample 73-0186, 0–2.5 in. depth. Soil treated with 3 lb/A cyanazine. Extract, 20 g/ml, 10 μl injected, attenuation 2×.

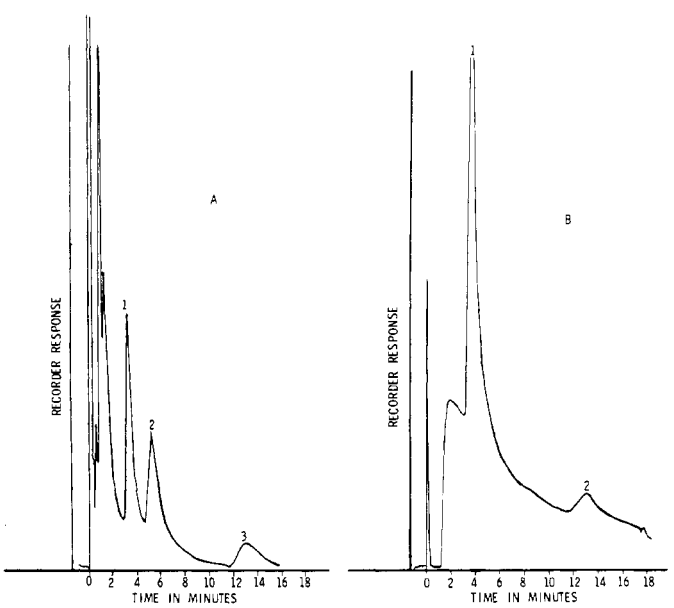

Figure 4. Coulson conductivity glc, 1.5% CHDMS on Gas Chrom Q column. 1, cyanazine; 2, deethylated cyanazine; 3, cyanazine amide. Chromatograms: A, standards, 20 ng injected, attenuation 2×. B, sample 73-0186, 0–2.5 in. depth. Soil treated with 3 lb/A cyanazine. Extract 20 g/ml, 50 μl injected, attenuation 2×.

tions to produce biological activity (Benyon *et al.*, 1970; Kaufman and Kearney, 1970).

In this investigation data were gathered on the residues of atrazine, cyanazine, and their phytotoxic metabolites under field conditions. These metabolites were not detected in five formulations available in Canada. The analytical results indicate an exponential decline of the residues of atrazine and cyanazine in soil for a period of 12 months (Figures 5 and 6). There were differences between residual levels for pre- and postemergence treatments due to the soil and weather conditions at and following the time of application. The higher residue levels following postemergence spraying are probably the result of bonding with the soil particles activated by the reduced water content in the first few inches of the soil. The increased soil temperatures which facilitate greater microbial activity probably gave rise to correspondingly increased residues of the dealkylated metabolites. In particular, the residual levels of deethylated atrazine increased rapidly, reaching a plateau in midseason. Thereafter it remained steady or declined slightly as if the presence of the crop affected further accumulation. By the end of the growing season a further increase of residue occurred (Figure 5B). The deisopropylated metabolite, which could originate from either of the two herbicides, appears to be terminated with the exhaustion of cyanazine (Figure 6B). It was interesting to note that the deisopropylated metabolite resi-

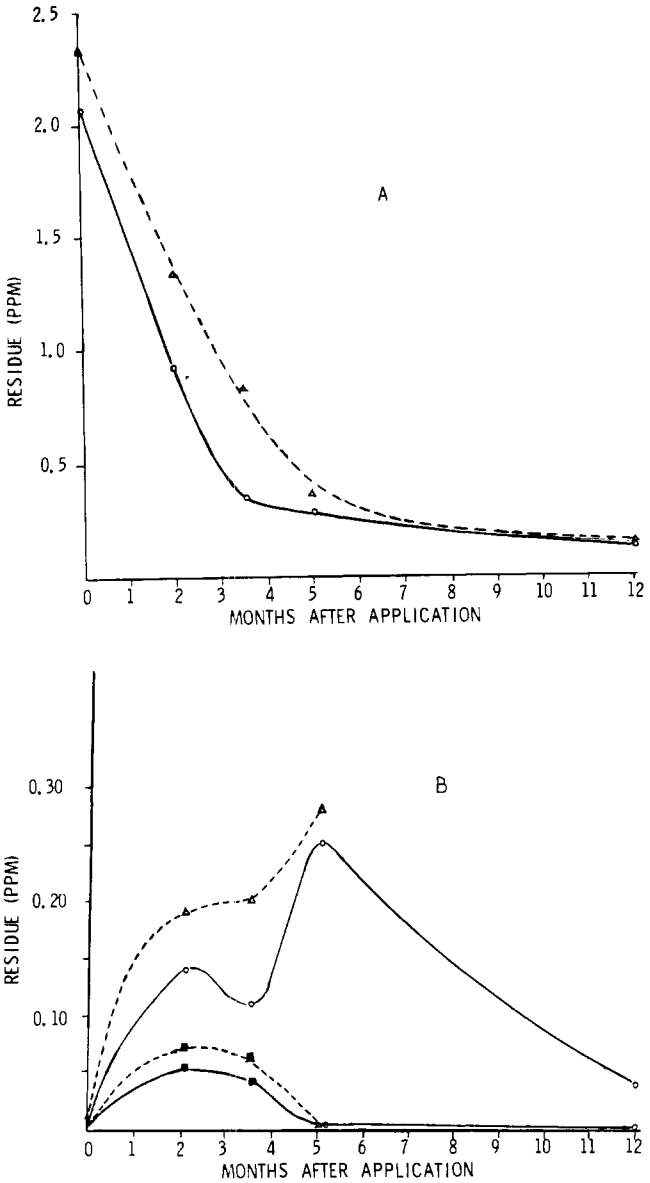

Figure 5. Residues of atrazine and phytotoxic metabolites in soil treated with 3 lb/A ai atrazine, 0–2.5 in. depth. Graph A: O—O, atrazine preemergence; Δ- - -Δ, atrazine postemergence. Graph B: O—O, deethylated atrazine; ■—■, deisopropylated atrazine preemergence; Δ- - -Δ, deethylated atrazine; and ■- - -■, deisopropylated atrazine postemergence.

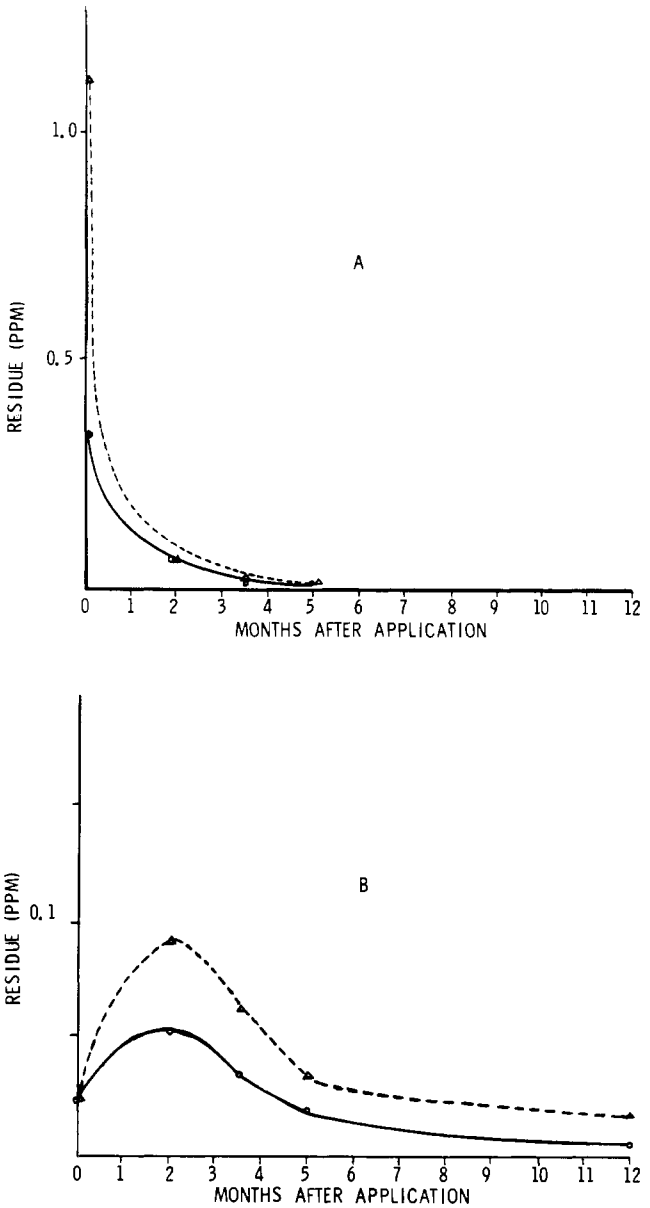

Figure 6. Residues of cyanazine and phytotoxic metabolites in soil treated with 3 lb/A ai cyanazine; 0–2.5 in. depth. Graph A: O—O, cyanazine preemergence; Δ- - -Δ, cyanazine postemergence. Graph B: O—O, deisopropylated atrazine preemergence; Δ- - -Δ, deisopropylated atrazine postemergence.

dues increased with the addition of cyanazine. This indicates that the cyanomethylethyl group is the preferred site for the microbial degradation of cyanazine. The presence of cyanazine amide in the same samples suggests that the hydrolysis of nitrile group precedes the microbial attack because the modified, either carbamoyl or carboxy methylethyl, group is more attractive than the isopropyl moiety (Figure 4B). This observation is supported by the fact that in case of atrazine the deisopropylated metabolite is formed only in trace quantities (Figure 3C). According to Beynon *et al.* (1970) the hydrolysis of the nitrile group into carboxylic acid *via* amide is fast and could not be followed after 2 months. This accelerated chemical conversion and the less phytotoxic metabolite produced by the microbial degradation reduces the biological activity of cyanazine faster than that of atrazine. The phytotoxicities of the cyanazine amide and acid are either low or nil, respectively; hence only qualitative studies were made on these compounds. By uv spectra we were not able to detect the hydroxylated metabolites. Similar results were obtained by Corke and his associates (1972). The failure to find any signs of hydroxy-*s*-triazines, together with the fact that the analytical procedure used by Beynon (1972) and his associates (1970, 1972a,b) and Harris (1967) tends to induce hydrolysis of chlorine in the 2 position, leads us to believe that hydroxylation may not be involved in the decomposition of cyanazine. The dealkylation process would appear to be a more important pathway associated with the persistence of atrazine and cyanazine herbicidal activity in the soils.

The presented data show that a certain amount of atrazine persists in soil in the parent form and as the deethylated metabolite. It is possible that, following high application rates, the residues may reach levels where the subsequent year's crop is affected (Table I). The microbial attack on atrazine differs from that on cyanazine. In the former, the ethyl group is removed, producing the major phytotoxic metabolite, while the removal of the isopropyl group appears to be insignificant. In the case of cyanazine, the cyanomethylethyl group with its hydrolysis products in the 4 position becomes the primary target. Hydrolysis of the nitrile group precedes the microbial degrada-

tion. Further investigation of the dealkylation products, namely the chlorinated and hydroxylated 4,6-diamino-s-triazines, was hindered by extraction and partition difficulties but is in progress.

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Atrazine Metabolism in Sorghum: Catabolism of the Glutathione Conjugate of Atrazine

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The major pathway of atrazine metabolism in intact sorghum was shown to involve the following steps: atrazine → *S*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)glutathione (III) → γ-glutamyl-*S*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cysteine (IV) → *S*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cysteine (V) → *N*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cysteine (VI) → *N*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)lanthionine (VII). From 40 to 87% of the atrazine entering sorghum through the roots was estimated to be metabolized *via* this pathway. Evidence indicated that atrazine can also be metabolized *via* this route after first undergoing N-dealkylation. The conversion of V to VI was shown to be a nonenzymatic rearrangement. This is the first reported occurrence of V, VII, 2-hydroxy-4-amino-6-isopropylamino-*s*-triazine (VIII), and 2-hydroxy-4,6-diamino-*s*-triazine (ammeline, XII) in sorghum.

Although three reaction types have been demonstrated in the metabolism of atrazine in higher plants, N-dealkylation, hydrolysis, and conjugation with glutathione, a clear picture has not yet emerged on the complete metabolism of atrazine in any tolerant plant species (Shimabukuro *et al.*, 1971). Dealkylation of the alkylamino side chains is a general pathway that occurs in many plants, but in tolerant species this pathway is generally in competition with faster reactions and simple dealkylated products such as 2-chloro-4-amino-6-isopropylamino-*s*-triazine (I) do not accumulate in high concentrations. Because of this, N-dealkylation may not appear to be of significance in short-term metabolic studies, even though terminal metabolites may have undergone N-dealkylation. Hydrolysis of the 2-chloro group is an important metabolic reaction in certain species such as corn, and in this species hydrolysis and dealkylation result in metabolites such as 2-hydroxy-4-amino-6-isopropylamino-*s*-triazine (VIII). However, even in corn, where two of the predominant reactions result in simple products, the chemical nature of the majority of the terminal residue is still uncertain. Most atrazine-tolerant species differ from corn in that displacement of the 2-chloro substituent by glutathione, rather than hydrolysis, is the first step in the primary route of metabolism (Lamoureux *et al.*, 1970; Lamoureux *et al.*, 1972; Thompson, 1972). Preliminary studies with atrazine metabolism in sorghum indicated that the glutathione and γ-glutamylcysteine conjugates of atrazine are transitory intermediates in this metabolic pathway. This report is primarily concerned with the further elucidation of the steps involved in the metabolism of atrazine *via* conjugation with glutathione. The structures of the metabolites discussed in this report are shown in Figure 8.

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EXPERIMENTAL SECTION

General Methods. Electron impact mass spectra were measured on a Varian MAT CH-5DF or on a Varian M-66 with a V-5550 console. Samples were introduced with a solid-sample probe which was heated from 25° to 250°, depending on the sample. The source temperature on both instruments was 180°. The methods used for monitoring ^{14}C, preparation of *S*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)glutathione (III) and γ-glutamyl-*S*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cysteine (IV), and the preparation of thin-layer plates have been described previously (Lamoureux *et al.*, 1972). Silica gel thin-layer plates used for purification of samples for mass spectral analyses were washed with the solvent used for chromatography or with absolute methanol before chromatography. The following solvent systems were used (solvent ratios are expressed on a volume:volume basis): (A) 1-butanol–acetic acid–water (12:3:5); (B) ethanol–water (7:3); (C) benzene–acetic acid–water (60:40:3); (D) 88% phenol–water–28% ammonium hydroxide (100:20:0.3); (E) benzene–ethanol (7:3); (F) ethyl acetate–acetic acid–water (23:1:1); (G) ethanol–28% ammonium