Synthesis and Occurrence of Nitrosated Cyanazine in Soil

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In the course of our studies on the contamination of maize-cultured fields treated with a commercial herbicide formulation containing cyanazine, a more lipophilic species besides the *s*-triazine itself was observed in HPLC chromatograms of soil extracts. GC–MS analysis suggested it to be a nitrosated cyanazine species. Nitrosation of cyanazine in aqueous media yielded three products. The main product is the mononitroso derivative containing the nitroso group attached to the propanenitrile group. This nitroso derivative coeluted with the unknown compound extracted from soil samples. It was detected in all cyanazine-contaminated soils. The nitrosation of two other well-known *s*-triazines, atrazine and simazine, yielded three and two products, respectively, which were clearly separated in HPLC from each other as well as from the nitroso derivatives of cyanazine.

Keywords: *s-Triazines; cyanazine; nitrosation; N-nitrosocyanazine; occurrence in soil*

INTRODUCTION

Triazine herbicides belong to the most widely used pesticides all over the world. Cyanazine [2-[(4-chloro-6-(ethylamino)-*s*-triazin-2-yl)amino]-2-methylpropionitrile] is used alone or in combination with other herbicides to control broad-leafed weeds in corn and maize especially. Much interest has concentrated in exploring the persistence, properties, and degradation of cyanazine in soil species (Sirons et al., 1973; Yu et al., 1975; Blumhorst and Weber, 1992). In field experiments as well as in model ecosystems the propanenitrile side chain was the preferred site for the microbial degradation. Using ^{14}C-labeled cyanazine, the main route of degradation in soil was hydrolysis of the nitrile group, giving cyanazine amide and cyanazine acid. Minor degradation products result from *N*-dealkylation at both *N*-alkyl side chains and substitution of a hydroxyl group for chlorine in the 2-position. In a single oral dose metabolism study with ^{14}C-labeled cyanazine in rats, the major metabolites found in urine and feces were identified as the deethylated species and its mercapturic acid derivative (Hutson et al., 1970).

Another possible reaction of *s*-triazine herbicides containing secondary *N*-alkyl substituents in the environment is *N*-nitrosation. This reaction was extensively studied only with atrazine, and many data about kinetics, spectroscopy, and chemical reactions under different conditions were collected (Wolfe et al., 1976; Kearney et al., 1977; Mirvish et al., 1991). However, none of the three nitrosation derivatives of atrazine has been so far detected in atrazine-treated soil. No information was available on the nitrosation of cyanazine. This paper is concerned with the proof of cyanazine and its nitrosated derivatives in soil samples of cultured maize fields.

MATERIALS AND METHODS

Extraction of Soil Samples. One hundred grams of moderately moist sample and 100 mL of methanol p.a. were shaken for 24 h. The solution was decanted, centrifuged at 6000 rpm for 10 min, and filtered through two filters with increasing capacity, consecutively (No. 604 and 594, Schleicher & Schüll). The filtrate was concentrated to dryness and redissolved in 1 mL of methanol for analysis by GC–MS or HPLC.

Instrumentation. HPLC analysis was performed on a Hewlett-Packard 1050 series system, consisting of a gradient pump, sample valve, and spectrophotometer, combined with an integrator. The separation was achieved on a 125 × 4.6 mm column, filled with LiChrospher 100 RP-18, 5 μm (Merck). The standard and calibration solutions were applied directly with a 20 μL sample loop. For purification the methanolic soil extracts were first applied on a 50 × 4.6 mm precolumn, packed with Lichrosorb 60 RP-select B, 20–30 μm (Merck), rinsed with 1 mL of water or 5% methanol in water, and subsequently eluted onto the analytical column. The mobile phase consisted of 50/50 or 60/40 (v/v) 0.05 M ammonium acetate buffer (pH 6.5) and methanol. The detection wavelength was 245 nm, and the flow rate of the pump was 0.7 mL/min.

GC–MS measurements were performed in the electron impact mode at 70 eV on a Hewlett-Packard 5988 A system. The methanolic samples were separated on a SE-54 capillary column (30 m × 0.25 mm i.d., 0.205 μm film thickness) from Supelco, Inc. The temperature program was started at 90 °C, held for 5 min, and then raised at 8 °C/min to 250 °C, where it was held for 7 min. The temperatures of the injection port and the source of the mass spectrometer were set at 125 and 200 °C, respectively. The gas flow rate was adjusted to 0.8 mL/min.

Thin-layer chromatography was carried out on analytical or preparative Kieselgel F254 plates (Merck) being eluted with toluene–ethanol [95/5 (v/v)]. The spots were visualized by UV fluorescence emission, scrapped off, and extracted with ethyl acetate. After evaporation, the residue was taken up in methanol for analysis by GC–MS or HPLC.

NMR spectrometry was executed on a JEOL GSX 270,1674 MHz instrument (Kontron) in $CDCl_3$; chemical shifts (in δ) are relative to $(CH_3)_4Si$.

Nitrosation. One millimole of cyanazine was dissolved in 20–30 mL of 50% HCl to which aqueous solutions of 15 mmol of $NaNO_2$ in 5 mL and 2.5 mmol of NaSCN in 3 mL of water were carefully added simultaneously. The colorless solution changed to an intense red-brown, covered by a dense yellow foam. After gentle shaking for ~3 h at room temperature, the mixture was extracted with CH_2Cl_2 (3 × 20 mL) and with ethyl acetate (2 × 20 mL). The combined organic phases were dried (Na_2SO_4) and evaporated to dryness. The yellow-green residue was dissolved in 3–4 mL of methanol and subjected together with cyanazine as standard to preparative TLC as described. The zones containing the nitrosated species were subjected to

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Table 1. Concentration of Cyanazine in Samples from Maize Cultivated Soil

field no.	wt of sample (g)	cyanazine content: area × 10^{-3} (mm^2)	cyanazine content: ppb	structure II[a] area × 10^{-3} (mm^2)
1	101	39	210	11
2	101	90	480	24
3	106	65	350	20
4	104	190	1035	45
5	107	95	510	27
6	105	128	690	34

[a] The nitroso derivative of cyanazine was clearly detectable but not quantitatively evaluated because no calibration curve and recovery values were available.

further purification and enrichment by HPLC. Atrazine and simazine were nitrosated according to the same procedure and the products separated by HPLC as described above.

Determination of the Recovery Rate and the Blank Value. Soil samples from a region free of maize cultures which were supposedly not treated with *s*-triazines were used for determination of recovery rate and the blank value. Three soil samples of 100 ± 3 g each were treated either with 50 μL of a methanolic solution of cyanazine (200 ng/μL) or with 50 μL of methanol only. All samples were extracted in the same manner as described before. The rates of recovery and the blank values were determined using HPLC as mentioned above.

RESULTS

Analysis of Soil Samples. All samples were drawn from a typical agricultural landscape, mainly used for cattle breeding and milk production. The cultivation of maize was done in a region of morainic soil structure (typic alluvial, sandy loams, thermic). The tillage of the maize cultivating was effected in April–May 1991. One month after sowing, the fields were treated with a cyanazine-containing herbicide formulation. Five months after herbicide application, the fields were harvested. Thereafter, samples were collected to 10 cm under the surface of a field area.

Detection of Cyanazine. The extraction of the material was executed as described above. The calibration curve of cyanazine measurements in the HPLC mode was linear over a range from 1.6 to 200 ng with a correlation coefficient of 0.998. The detection limit of the analytical HPLC system was 7 ± 2 ng, the rate of recovery 57 ± 3%. All HPLC measurements were performed in duplicate samples. The quantity of cyanazine was calculated in accordance to the calibration curve and the recovery rate. In the chromatograms from extracts of control soil samples (blanks), no peaks at the retention time of cyanazine were detected. This was confirmed by reanalyzing these extracts after spiking with cyanazine.

Cyanazine was found in all six soil samples from maize cultured fields, with an average contamination of 546 ppb (Table 1). The presence of cyanazine in soil extracts was confirmed by GC–MS. Acquisition in the SIM mode showed two significant ions of M^+ (amu = 240) and $M - CH_3^+$ (amu = 225) with identical retention times as compared to the cyanazine standard (Figure 1).

Nitrosation. The nitrosation of atrazine and cyanazine, according to Eisenbrand et al. (1975), yielded only 10% of nitrosated products. Addition of NaSCN in aqueous media increased the yield up to 40–50%. Under both conditions cyanazine nitrosation produced three *N*-nitroso compounds represented by the large

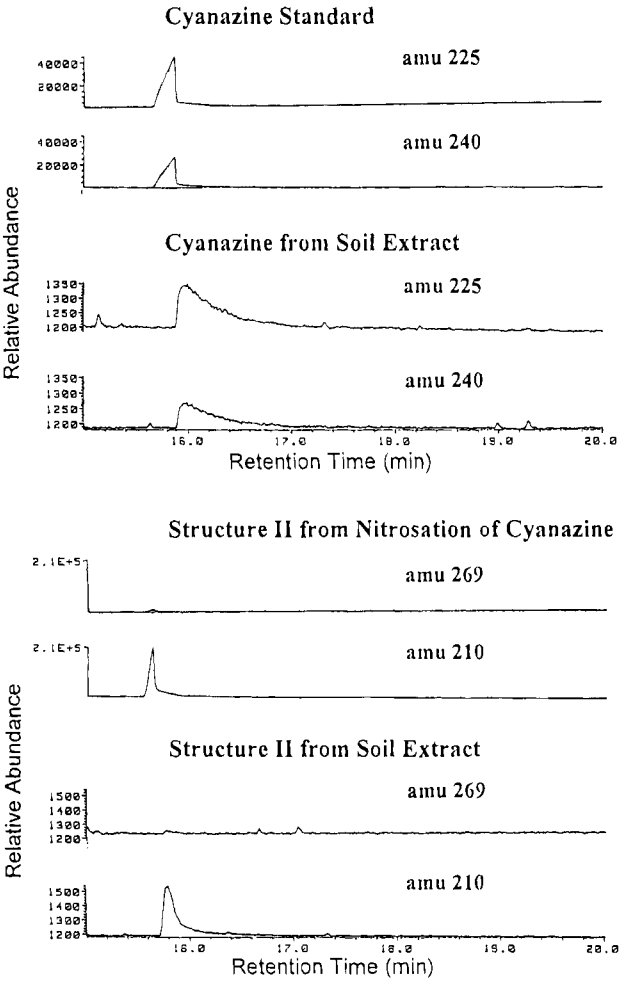

Figure 1. GC–MS identification of cyanazine and its main nitrosation product (structure II from Figure 3) in soil samples. Subsequent to HPLC purification, chromatograms of standards and soil extracts were recorded in the SIM mode at the ions indicated.

peak in the HPLC chromatogram at 9.8 min (estimated 80%), a smaller one at 7.5 min (estimated 15%), and a very small one at 14 min (estimated 5%) (Figure 2).

Nitrosation of atrazine in the presence of NaSCN also produced three nitrosated derivatives with retention times in HPLC mode of 29.7 min for the main peak and 24.7 and 38.3 min for the two minor peaks. After nitrosation of simazine, only two peaks with retention times of 28 (main peak) and 43 min were observed (data not shown).

Detection and Characterization of a Nitrosated Cyanazine Species in Soil Samples. In Figure 2 the existence of a nitrosated cyanazine species in soil is demonstrated by comparative HPLC measurement of a sample extract (C) with the solution of the nitrosation reaction (B) and a cyanazine standard (A). Cyanazine, eluting at 5.9 min, was found in all three chromatograms. At 9.8 min the supposed nitrosated species was detected in the chromatogram of the nitrosation synthesis as well as in the soil extract.

For further characterization the main peak of the nitrosation reaction, appearing also in soil extracts, was fractionated from 10 HPLC runs, extracted with ethyl acetate, and subjected to GC–MS analysis. The MS data of the *N*-nitroso compound eluting from the capillary column at a retention time of 14.3 min are presented in Table 2. The same HPLC fractionation was done with extracts of soil samples. GC–MS analy-

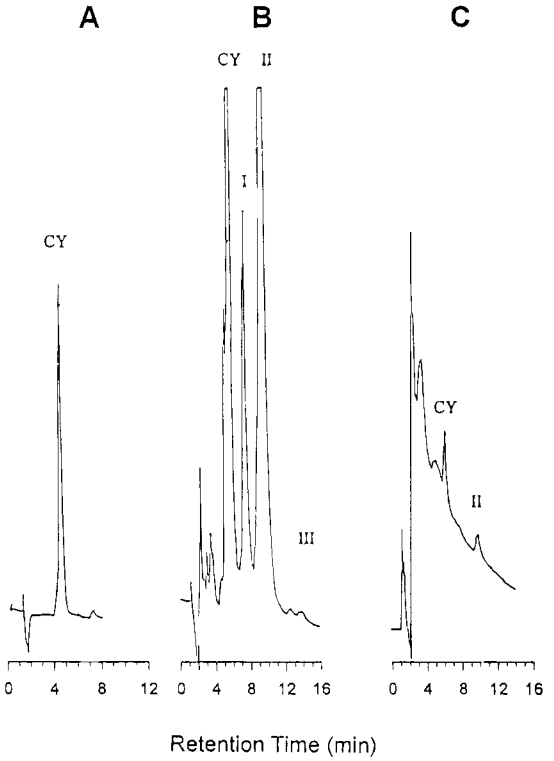

Figure 2. HPLC chromatograms on a 125 × 4.6 mm column filled with 5 μm of LiChrospher 100 RP-18 eluted with 0.7 mL/min 60/40 (v/v) 0.05 M ammonium acetate buffer (pH 6.5) and methanol; the detection wavelength was 245 nm. CY, cyanazine; I, structure I; II, structure II; III, structure III. Chromatograms: A, cyanazine standard solution; B, nitrosated reaction solution; C, extracted soil sample.

Table 2. MS Fragmentation of the Major Nitrosation Product of Cyanazine, Structure II in Figure 3

mass found (*m/e*)	% abundancy	structure
269	11	M^+
254	3	$M - CH_3^+$
238	3	$M - HNO^+$
210	100	$[M - NO - C_2H_5]^+$
194	20	$[M - NO - C_2H_5 - CH_3]^+$

sis of the two characteristic ions (amu = 269 and 210) in the SIM mode revealed a peak at the same retention time as the main peak of the nitrosation reaction (Figure 1).

The nitrosated species of cyanazine were detected in every soil sample with roughly 4 times lower peak areas as compared to the parent compound (Table 1). The concentrations could not be determined exactly because a calibration curve has not yet been established and no data on recovery of the *N*-nitroso compound are available. No peaks were found at the retention times of the nitrosated species of atrazine and simazine.

To obtain more information about the real structure of the nitrosated cyanazine compounds, we tried denitrosation with diluted hydrobromic acid to reduce the nitroso to the amine function (Eisenbrand and Preussmann, 1970). However, cyanazine itself as well as the collected nitrosated fractions unfortunately decomposed under this condition.

All of our attempts to adapt the HPLC separation of the nitrosated products of cyanazine to an adequate TLC separation system failed. The three nitrosated species were separated neither from each other nor from unreacted cyanazine. However, a purification from hydrophilic byproducts was successfully performed by

Table 3. ^{1}H NMR Spectra of Cyanazine and Its Main Nitrosation Product, Structure II in Figure 3

	chemical shifts (ppm)	
assignment	cyanazine	structure II
$\underline{CH_3}CH_2NH$	1.23 (t)[a]	1.23 (t)
$\overline{(CH_3)_2}CCN$	1.77 (s)	1.78 (s)
NH		
$CH_3\underline{CH_2}NH$	3.48 (m)	3.53 (m)
$CH_3\overline{CH_2}\underline{NH}$	5.43	nd[b]
$\overline{(CH_3)_2}CCN$	5.85	nd
$\underline{NH}$		

[a] Letters in parentheses refer to proton multiplicities. [b] nd, not detected.

TLC. This prompted us to install a precolumn in our HPLC separation system.

The attempts to perform a separation with the commonly used nitroso-specific detection system, the TEA analyzer, are still in preparation. The NMR spectra listed in Table 3, give no further information about the position of the nitroso group because of the lack of exact information on the N−H proton signals stemming from either the *N*-ethyl or the *N*-propanenitrile moiety. The alkyl signals from the ethyl and isopropyl protons were found in the same regions as reported by Mirvish et al. (1991).

DISCUSSION

In only two papers on the nitrosation of *s*-triazines in aqueous media are quantitative data on the reaction given. Eisenbrand et al. (1975) obtained *N*-nitroso derivatives of simazine and atrazine with yields of 1−7%. Mirvish et al. (1991) reported about 20% containment of unreacted atrazine in the crude nitrosation product without giving quantitative data on the *N*-nitroso compounds formed. The application of the nitrosation procedure of Eisenbrand et al. (1975) to cyanazine yielded about 10% of *N*-nitroso species. However, the addition of NaSCN to the reaction solutions enhanced the yield up to 40−50%. The use of NaSCN to raise the rate of nitrosation is well-known and documented for other secondary amines (Boyland and Walker, 1974; Fan and Tannenbaum, 1973).

No reports on the nitrosation of cyanazine have been found in the literature. HPLC separation of the crude nitrosation reaction of cyanazine showed the formation of three possible *N*-nitroso derivatives of cyanazine (Figure 3). The observed retention times by HPLC are in accordance with the expected structure of the three possible nitrosated species, which are more lipophilic than the *s*-triazine itself. From the sequence of elution on C_{18} material we suppose structure I to have the shortest retention time of 7.5 min, followed by structure II at 9.8 min and structure III at 14 min. As expected, the greatest amount of nitrosated species was represented by structure II. This compound was detected also in soil extracts and characterized as a nitrosation species of cyanazine by HPLC, MS, and NMR.

Whereas with atrazine the *N*-ethyl nitrogen is primarily attacked, cyanazine nitrosation took place preferably at the *N*-propanenitrile nitrogen. The difference in nitrosation between the two *s*-triazines may be due to the existence of the cyano group in cyanazine, which is lacking in atrazine. The negative inductive effect of that substituent may favor the attachment of the nitroso group on the propanenitrile moiety as opposed to the *N*-ethyl group. This is in accord with the results of

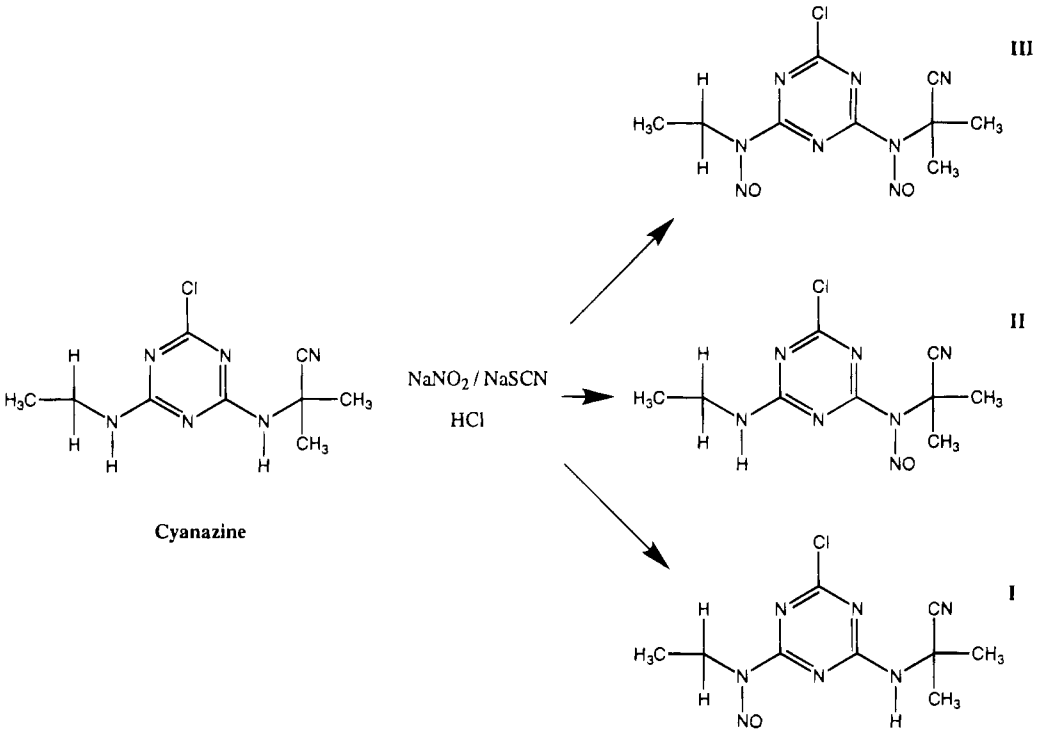

Figure 3. Three possible nitrosation products of cyanazine.

Mirvish et al. (1975), who showed that weakly basic amines are nitrosated more readily than strongly basic ones. Through the negative inductive effect caused by the cyano group, the nitrogen at the propanenitrile group is supposed to be weakly basic as compared to the nitrogen at *N*-ethyl group. Structures I and III were only determined by their HPLC retention behavior in comparison to cyanazine standard and structure II.

Upon nitrosation of atrazine three nitrosated species were obtained as postulated by Wolfe et al. (1976) and Kearney et al. (1977). Because of their retention behavior in reversed-phase HPLC and the yield of the three nitrosated species, we assume that the most lipophilic compound at 38.3 min represents the twofold nitrosated atrazine, the *N*-ethyl-*N*-nitroso-*N'*-isopropyl-*N'*-nitroso species, yielding the smallest reaction amount. The *N*-isopropyl-*N*-nitroso derivative presumably elutes at 29.7 min, whereas the major nitrosation product of atrazine, the *N*-ethyl-*N*-nitroso species (Mirvish et al., 1991), is the most hydrophilic compound eluting after 24.7 min. In the case of the symmetric *s*-triazine, simazine, nitrosation yielded one mono- and the disubstituted product eluting at 28 and 43 min, respectively.

The soil extracts did not contain any of the three *N*-nitrosoatrazine species or the two minor nitrosation products of cyanazine (structures I and III). The reason for this could be either inefficient extraction or insufficient sensitivity of the detection system. No further attempts were made using more extensive enrichment to verify the possible existence of these structures in soil samples. The influence of possible stability criteria on the recovery of all nitrosated species during the analysis of soil samples was not further investigated.

Measurement of reaction mixtures of cyanazine and atrazine at daylight and room temperature over a period of several days showed a greater stability of the nitrosated products of cyanazine as compared to atrazine (data not shown). Our investigations confirm the results concerning the stability of *N*-nitrosoatrazine reported by Wolfe et al. (1976) and Kearney et al. (1977).

The presence of a nitrosated cyanazine derivative in the soil samples leads to a wide range of speculations upon its origin. One reason could be the use of fertilizers resulting in a high nitrate dissipation, giving rise to nitrite release which may be a deciding factor in nitrosamine formation. However, Kearney et al. (1977) were not successful in nitrosating atrazine in soil experiments even under a 100-fold surplus of NH_4NO_3 to the *s*-triazine. The influence of soil properties such as bacteria, other microorganisms, or soil-specific enzymatic systems, as has been used previously to elucidate the fate of this class of compounds (Blumhorst and Weber, 1992), should be taken into consideration in experiments upon nitrosation of *s*-triazines under natural conditions.

Nothing is known about the toxicologic consequences of cyanazine nitrosation. Conceivably, the nitrosated cyanazine derivative could be the precursor of a possible metabolic intermediate with electrophilic properties undergoing interaction with biological materials such as proteins or DNA. Further investigations are indicated to elucidate the fate of nitrosated *s*-triazines in soil and biological material.

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