N-Nitrosoatrazine: Synthesis, Kinetics of Formation, and Nuclear Magnetic Resonance Spectra and Other Properties

Sidney S. Mirvish,*,† Peter Gannett,†,‡ David M. Babcook,† Dan Williamson,‡ Sheng C. Chen,† and Dennis D. Weisenburger§

Eppley Institute for Research in Cancer and Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198

N-Nitrosoatrazine (NNAT) was reported to be produced by the nitrosation of atrazine but was not fully characterized. We report here an improved synthesis of NNAT by nitrosation of atrazine in glacial acetic acid followed by high-performance liquid chromatography. The mass and infrared spectra confirmed that NNAT is a mononitroso derivative. The ^{1}H and ^{13}C nuclear magnetic resonance spectra of atrazine and NNAT were measured, including the use of selective decoupling, two-dimensional and homonuclear and heteronuclear correlation spectra, and variable-temperature experiments. These indicated that four syn–anti conformers of atrazine are present, attributed to restricted rotation of the two exocylic bonds (interconversion energy, 16.5 kcal/mol) and that NNAT contains the nitroso group attached to the *N*-ethyl nitrogen and exists as two conformers, attributed to restricted rotation about the exocylic bond to the *N*-isopropyl nitrogen (interconversion energy, 18.3 kcal/mol). NNAT was relatively stable in alkali and was photolabile. Nitrosation of atrazine in 1:1 ethanol–water followed third-order kinetics (first order for atrazine, HNO_2, and H^+), with a relatively low rate constant of 4.6 $\times 10^{-3}$ M^{-2} s^{-1} at 25 °C.

INTRODUCTION

People are exposed to atrazine when they apply the herbicide or consume contaminated food or water. Therefore, nitrosation of atrazine (Figure 1) in the environment or in vivo to yield *N*-nitrosoatrazine (NNAT) (Figure 2) could present a hazard if this reaction occurred readily and NNAT was mutagenic and/or carcinogenic. NNAT was first synthesized by aqueous nitrosation of atrazine, but no details were supplied (Eisenbrand et al., 1975). The same group reported that nitrogen oxides react with solid atrazine to produce NNAT (Janzowski et al., 1979). Krull et al. (1980) described a method for determining NNAT in vivo using high-performance liquid chromatography (HPLC) with detection by thermal energy analysis. Kearney et al. (1977) studied the fate of ^{14}C-labeled NNAT in soil and its accumulation by fish. They reported that atrazine nitrosation by N_2O_4 at −70 °C yielded dinitrosoatrazine, which decomposed on warming in acetic acid to give the mononitroso derivative, NNAT, which was purified by column chromatography.

Wolfe et al. (1976) stated that the proposed structure of NNAT, with the nitroso group on the *N*-ethyl nitrogen, had been confirmed by its infrared (IR), nuclear magnetic resonance (NMR), ultraviolet (UV), and mass spectra, but none of these data were presented in this or the other reports. Wolfe et al. (1976) also studied the rate of atrazine nitrosation, which, under given conditions at pH 2, was stated to proceed 200 times faster than that of dimethylamine, and reported that NNAT was unusually photolabile.

In a re-examination of these questions, we describe here a synthesis of 50-g amounts of NNAT, which was needed for toxicologic tests. In these tests, NNAT produced mutation frequencies that were 4.7 times background level in the Ames test using *Salmonella typhimurium* TA-100 with activation by hamster liver S-9 fraction and were 3.4 times higher than those produced by the same dose (100 μg/mL) of dimethylnitrosamine in the Chinese hamster V-79 test in the presence of hamster hepatocytes (Weisenburger et al., 1988). However, NNAT was not carcinogenic in mice and rats treated by twice weekly gavage of 65 (mice) or 175 (rats) mg of NNAT/kg of body weight for up to 67 weeks (Weisenburger et al., 1990). In the current paper, we provide data about the isomerism of atrazine and NNAT and describe some properties of NNAT, including the kinetics of its formation from atrazine.

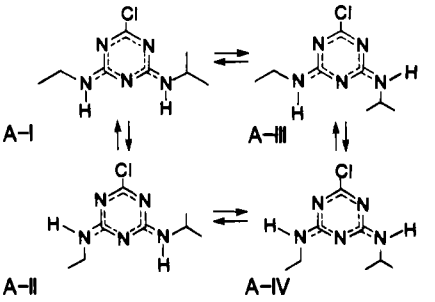

Figure 1. Four probable structures of atrazine.

MATERIALS AND METHODS

NMR spectra were measured on a Varian XL-300 (300 MHz) instrument with the temperature control on and (unless noted otherwise) set at 22 °C, using samples dissolved in dimethyl-d_6 sulfoxide (DMSO-d_6). Chemical shifts are reported relative to tetramethylsilane as internal standard. Two-dimensional (2-D) NMR experiments [homonuclear and heteronuclear correlation (HOMCOR and HETCOR)] were performed as described by Freeman and Hill (1971) and Bodenhausen et al. (1976). Where needed, ^{13}C NMR spectra were recorded with sufficient delays to obtain accurate integration values. Mass spectra were measured with a modified AEI-MS902 instrument, using a probe to introduce samples. Atrazine was supplied by Ciba-Geigy Co. (Greensboro, NC).

* To whom correspondence should be addressed.
† Eppley Institute for Research in Cancer.
‡ Present address: School of Pharmacy, West Virginia University, Morgantown, WV 26506.
§ Department of Pathology and Microbiology.

0021-8561/91/1439-1205$02.50/0

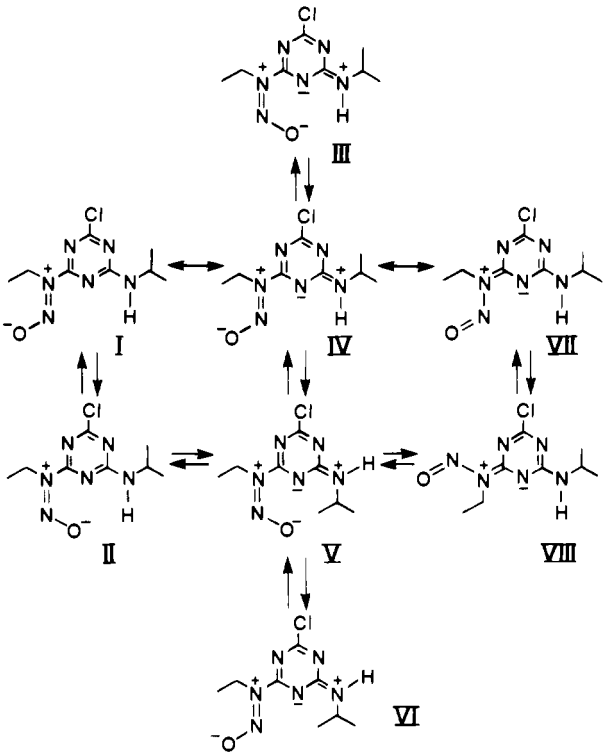

Figure 2. Eight possible structures of NNAT. The correct structures are probably IV and VI.

Synthesis and Properties of NNAT. The synthesis was modified from a method supplied by G. Boka (Ciba-Geigy). $NaNO_2$ (64 g, 0.93 mol) was added over 2 h to 50 g (0.23 mol) of atrazine in 500 mL of glacial acetic acid while the mixture was stirred at room temperature. The mixture was heated for 2 h at 50 °C without stirring, cooled, and filtered to remove sodium acetate. The filtrate was mixed with 500 mL of water and 750 mL of ethyl acetate to give a single phase. Aqueous 10 N NaOH was added until two phases separated. The upper organic phase was extracted with 10 N NaOH until the aqueous phase remained basic, washed once with 50 mL of water, dried over Na_2SO_4, and evaporated to give 49 g (86%) of crude NNAT: mp 90 °C; UV (CH_2Cl_2) 413 (ϵ 101) and 430 (96) nm. The solubility of NNAT was 560 mg/mL DMSO, 510 mg/mL CH_2Cl_2, and 320 mg/mL acetone at 22–25 °C and 0.29 mg/mL water at 25 °C, as determined by UV absorbance compared to that of standard solutions in each solvent. The same method was used to show that solid NNAT was stable for 5 weeks at −15 °C in the dark.

Some NNAT was purified by semipreparative HPLC of solutions in hexane–acetone 95:5 on a 20 × 250 mm column of 5-μm LiChrosorb-SI60 (Alltech Associates Inc., Deerfield, IL), eluted with the same solvent at 5 mL/min with detection at 234 nm. The combined NNAT fraction from several HPLC runs yielded 170 mg of purified NNAT: mp 93–94 °C; UV (CH_2Cl_2) 413 (125) and 431 (118) nm; UV (CH_3CN) 245 (21 200), 410 (114) and 427 (105) nm; UV (1:1 ethanol–water) 249 (16 200), 412 (98) and 430 (shoulder, 86) nm; IR (KBr pellet) 3317 (NH), 2979 (CH), 1601, 1433, and 1137 cm^{-1}; MS [m/z, formula and relative intensity (^{35}Cl only)] 244, M ($C_8H_{13}N_6OCl$), 50%; 229, M − CH_3, 20%; 214, M − NO, 100%; 199, M − NO − CH_3, 64%; 198, M − NO − CH_4, 38%; 184, M − NO − C_2H_5, 61%; 172, $C_6H_7N_5Cl$, 69%; and 58, C_3H_8N, 67%. Exact masses for molecular ion $C_8H_{13}N_6OCl$: calcd, 244.0839; obsd, 244.0847. Masses of quoted fragment ions agreed within 3 millimass units with those of the observed peaks.

Variable-Temperature NMR. Samples of atrazine or NNAT (0.18 M in 100% DMSO-d_6) were made up in oven-dried (110 °C) 5-mm NMR tubes, degassed by subjecting them to three freeze–thaw cycles under vacuum, and sealed. The ^1H and ^{13}C NMR spectra were recorded before and after acquisition to ensure sample integrity. Room temperature data were used for measuring υ_1 and υ_2. The ^1H NMR spectra of atrazine and NNAT were examined over the range 22–100 °C. Rates of conformational interconversion at the coalescence temperature were calculated from

$$k_{obs} = \pi/\sqrt{2}|\upsilon_1 - \upsilon_2| \quad (1)$$

where k_{obs} is the calculated rate constant at temperature of maximum broadening and υ_1 and υ_2 are frequencies (in hertz) of the two resonances in the frozen spectrum that were observed to coalesce (Gutowsky and Holm, 1956). Free energy of activation was calculated from eqs 2 (the Eyring equation) and 3

$$k_{obs} = Kk_BT/\hbar e^{-G/RT} \quad (2)$$

$$G = -RT \ln (\hbar k_{obs}/Kk_BT) \quad (3)$$

where K is the transmission coefficient (taken as 1), K_B is Boltzmann's constant, T is temperature (K), $\hbar$ is Planck's constant, k_{obs} is the rate constant for the interconversion, R is the gas constant, and G is the energy of activation.

Analysis of NNAT–Atrazine Mixtures. This was achieved by high-performance liquid chromatography (HPLC) of solutions in hexane–acetone 95:5 on a 4.6 × 250 mm column of 5-μm Lichrosorb-SI60 developed with the same solvent at 1.5 mL/min and detected at 234 nm (ϵ in the HPLC solvent, 20 000 for NNAT and 16 000 for atrazine). Quantitation was based on HPLC of standards. Retention times were 6.5 min for NNAT and 14 min for atrazine.

Kinetics of Atrazine Nitrosation. Solutions of atrazine in 95% ethanol (100 mL) and $NaNO_2$ in water (100 mL) were mixed, brought to the desired pH with H_2SO_4, and kept at 25 °C without stirring. Under "standard" conditions, 1 mM atrazine was reacted for 1–6 h with 25 mM nitrite at pH 2.0. Ten-milliliter samples of reaction mixture were made just basic with NaOH (to stop the nitrosation) and immediately extracted with 3 × 10 mL of CH_2Cl_2. The combined extracts were dried with Na_2SO_4, evaporated, and redissolved in 10 mL of hexane–acetone 95:5. Samples (25 μL) were analyzed for NNAT by HPLC. The recovery of 49.3 μg of NNAT from 10 mL of ethanol–water 1:1 was 99 ± 4% (mean ± SD for four tests). In some kinetic runs, samples were withdrawn for analysis hourly for 6 h. Since nitrosation proceeded at almost linear rates for the first 4 h, subsequent runs were analyzed at 4 h only. Nitrite stability over 4 h was tested under the standard conditions but at several pH values. Samples of reaction solution were made basic with NaOH and analyzed for nitrite as follows: diluted solution (1 mL) was mixed with 2 mL of reagent [5.5 g of sulfanilic acid, 20 mg of *N*-(α-naphthyl)ethylenediamine-2HCl, and 1.0 L of 10% aqueous acetic acid] and kept in the dark for 20 min. Absorbance was measured at 540 nm.

RESULTS

HPLC did not reveal NNAT in the supplied sample of atrazine, with a detection limit of 10 ppm. Nitrosation of atrazine in acetic acid yielded NNAT, which was shown by analytical HPLC and 2-D ^1H NMR spectroscopy to contain 20% atrazine. NNAT free of atrazine was obtained by semipreparative HPLC and used for characterization. The mass spectrum of NNAT, especially the parent ion peak, indicated that NNAT was the mononitroso derivative but did not disclose whether the nitroso group was on the *N*-ethyl or *N*-isopropyl nitrogen. The NH absorption in the IR spectrum also indicated that the product was not dinitrosoatrazine.

NMR Spectroscopy of Atrazine. At room temperature the ^1H NMR spectrum of atrazine (Table I) displayed a multiplet centered at 1.08 ppm, which integrated to nine protons and was assigned to the three methyl groups. The HOMCOR spectrum showed that the upfield region of this multiplet was due to methyl protons of the ethyl group (the "ethyl–methyl" protons) and the downfield portion to the isopropyl–methyl protons. A multiplet at 7.7–7.9 ppm, which integrated to two protons and disappeared when D_2O was added, was due to the two NH protons. According to the HOMCOR spectrum, the NH region

Table I. ^{1}H NMR Spectra of Atrazine and NNAT

assignment	chemical shifts, ppm: atrazine	NNAT[a]	diff[b]
$\underline{CH_3CH_2}$	1.08 (m)[c,d]	0.98,[c] 1.00 (t)	0.10, 0.08
$\underline{(CH_3)_2CH}$	1.08 (m)	1.20[c,e]	0.12
$\underline{CH_3CH_2}$	3.21 (q)	3.94, 3.97 (q)	0.73, 0.76
$(CH_3)_2\underline{CH}$	4.00 (m)	4.11, 4.12 (m)	0.11, 0.12
$CH_3\underline{CH_2NH}$	7.72, 7.74 (t)		
$(CH_3)_2\underline{CHNH}$	7.82, 7.86 (d)	8.90, 8.92 (d)	1.20, 1.22

[a] Chemical shifts are not assigned to the individual isomers. [b] Downfield shift upon nitrosation. [c] Letters in parentheses refer to proton multiplicities. [d] Proton chemical shifts for the four atrazine isomers were not resolved and individual assignments were not possible. The reported shifts represent the centers of the multiplets. The HETCOR data confirmed these assignments. [e] Not resolved.

showed four NH protons at 7.72, 7.74, 7.82, and 7.86 ppm, of which the first two correlated to the methylene protons and were therefore on the ethylamino nitrogen and the last two correlated to the methine proton and were due to the isopropylamino nitrogen proton.

When atrazine solutions in DMSO-d_6 were heated, all four sets of proton multiplets began to broaden at 50 °C, with maximum broadening at 55 °C, and then sharpened until at 80 °C all except the NH protons were sharp multiplets, as follows: CH_3CH_2, 1.10 (triplet); $(CH_3)_2CH$, 1.14 (doublet); CH_3CH_2, 3.27 (doublet of quartets), and $(CH_3)_2CH$, >4.60 (multiplet) ppm. The corresponding ^{13}C NMR peaks at 80 °C were at 12.77, 23.47, 34.45, and 41.42 ppm. These data suggest restricted rotation at room temperature (Figure 1). An interconversion barrier of 16.5 kcal/mol was calculated from eq 2 by using a coalescence temperature of 55 °C, an average difference of 15.6 Hz between pairs of resonances in the room temperature spectrum, a k_{obs} (derived from eq 1) of 69 s^{-1}, and a maximum irradiation temperature (T) of 345 K. This value is similar to inversion barriers for simple amides (Robin et al., 1970).

The ^{13}C NMR spectrum of atrazine (Table II) was assigned from HETCOR data and indicated that all four conformers in Figure 1 were present, because all carbon resonances, except for the methylene carbon (see Table II, footnote c) appeared as four peaks. The relative abundance ratios of the four isomers were about 46:23:23:8 (Table II). Consideration of the expected dipole moments suggests that A-1 (Figure 1) is predominant, A-II and A-III occur in intermediate amounts, and A-IV is the minor isomer. The exocyclic amino groups can be regarded as protonated amidines (Figure 1). The dipole moment in amidines should be directed toward the nitrogen lone pairs. Protonation should reduce the dipole moment but not reverse its direction. Therefore, orienting both exocyclic N–H bonds antiperiplaner (leading to 180° torsion) to the C–Cl bond (conformer A-I) should minimize the dipole moment of the molecule and hence its energy. A-II and A-III, which have only one N–H oriented antiperiplaner to the C–Cl, should have a greater dipole moment than A-I and therefore be less stable. Finally, A-IV, with neither N–H antiperiplaner to the C–Cl, should have the greatest dipole moment and be the least stable conformer.

NMR Spectroscopy of NNAT. The proton spectrum of NNAT (Table I) was tentatively assigned from the integrated signal intensities. The methylene resonances were shifted 0.73–0.76 ppm downfield relative to those of atrazine, whereas the methine proton was shifted only 0.11–0.12 ppm downfield. This indicates that the ethyl-bearing nitrogen was nitrosated, because nitrosation strongly deshields α-protons (Karabatsos and Taller, 1964).

Table II. ^{13}C NMR Spectra of Atrazine and NNAT at 22 °C

assignment	chemical shifts, ppm: atrazine value (%)[a]	atrazine weighted av[b]	NNAT	diff between atrazine and NNAT[c]	diff between NNAT isomers
$\underline{CH_3CH_2}$	14.24 (47)	14.39	11.53	−2.86	–[d]
	14.36 (22)				
	14.59 (23)				
	14.81 (9)				
$(\underline{CH_3)_2CH}$	21.89 (44)	22.05	21.66	−0.39	0.14
	22.06 (24)		21.80	−0.25	
	22.23 (23)				
	22.33 (9)				
$CH_3\underline{CH_2}$	34.83[e]	34.45	35.87	1.42	0.05
	34.93[e]		35.92	1.47	
	34.98[e]				
$(CH_3)_2\underline{CH}$	41.62 (22)	41.83	42.76	0.93	0.18
	41.70 (8)		42.94	1.11	
	41.79 (24)				
	41.97 (46)				
$\underline{C}NHCH$	164.06 (22)	165.12	164.57	−0.55	0.52
	164.39 (9)		165.05	−0.07	
	164.46 (48)				
	146.69 (21)				
$\underline{C}NHCH_2$	164.81 (22)	164.25	164.57	0.32	0.09
	165.06 (8)		164.48	0.23	
	165.14 (46)				
	165.48 (24)				
$\underline{C}Cl$	167.49 (46)	167.88	168.98	1.10	0.89
	168.09 (21)		169.80	1.92	
	169.13 (24)				
	168.86 (8)				

[a] Parentheses show the percentage of each peak obtained by integration under conditions where total acquisition time would permit complete relaxation of all carbons. [b] Average positions were weighted according to percentage of each peak. [c] Chemical shift for NNAT minus that for atrazine. [d] Isomers were not resolved. [e] This carbon showed only three peaks but with relative intensities suggesting an unresolved fourth peak.

If the isopropyl nitrogen had been nitrosated, a downfield shift for the methine proton of about 1.5 ppm would have been expected. This was confirmed by examining the ^{1}H NMR spectra of *N*-ethyl-*N*-iso-propylamine and the corresponding nitrosamine. In decoupling experiments, the broad triplet for the NH proton was unaffected by selective irradiation of the methylene protons but was collapsed by irradiation of the methine proton. This proves that the NH and isopropyl groups are attached, i.e., that the nitroso group is on the ethyl-bearing nitrogen.

Heating of NNAT solutions in DMSO-d_6 caused broadening of all sets of proton multiplets starting at about 65 °C. Broadening was most pronounced at 72 °C. The resonances sharpened on further heating until at 100 °C all except the NH protons were sharp multiplets, as follows: CH_3CH_2, 0.990 (triplet); $(CH_3)_2CH$, 1.200 (doublet); CH_3CH_2, 3.955 (quartet); and $(CH_3)_2CH$, 4.115 (multiplet) ppm. These data suggest the presence of at least two conformers at room temperature. Given a coalescence temperature of 72 °C and the observed chemical shift difference of 42.6 Hz between pairs of CH_3CH_2 resonances in the room temperature spectrum, an interconversion barrier of 18.3 kcal/mol was calculated from eqs 2 and 3 by using a k_{obs} calculated from eq 1 of 18.9 s^{-1} and T = 345 K.

Figure 2 shows the possible NNAT structures I–VIII. When the first sets of protons of the following pairs were selectively irradiated, the ^{1}H NMR spectra of the second set collapsed to the indicated signals: isopropyl–methyl, methine, doublet of doublets; methine, isopropyl–methyl, broadened singlet; methine, NH, two singlets; methylene, ethyl–methyl, two singlets; and ethyl–methyl, methylene, two singlets. This doubling of all resonances indicates restricted rotation about some bond. From the integrations, the two isomers were present in similar amounts.

Syn–anti isomerism about the N–NO bond (as in I and II) would produce 0.5–1.0 ppm differences in the ethyl–methyl and methylene protons (Karabatsos and Taller, 1964) and is unlikely because the observed differences were only 0.013 (ethyl–methyl) and 0.023 (methylene) ppm.

The remaining six structures for NNAT show restricted rotation either about the exocyclic bond to the nitrosated nitrogen (VII and VIII) or about that to the amino nitrogen (III–VI). VII and VIII can be eliminated for two reasons. First, the ethyl–methylene protons of VII and VIII should show a greater chemical shift difference than those of the isopropyl–methine protons because they are closest to the C=N bond, whereas the opposite was observed. Second, this type of resonance is unknown for related compounds such as nitrosoguanidines, nitrosocarbamates, and nitrosamides (Rice et al., 1984).

Of the remaining four conformers (III–VI), only two were present because there was only a doubling of resonances. The chemical shift difference for each pair of protons was small, eliminating the possibility of NNAT existing as the pairs III and IV or V and VI, where isomerism occurs within the nitrosamine group (Karabatsos and Taller, 1964). Therefore, the isomers were either III and V or IV and VI. Although the distinction is not unambiguous, we believe that IV and VI were present for three reasons: (a) Upon nitrosation of an amine, syn and anti α-methylene protons shift downfield by about 1.0 and 1.5 ppm, respectively, relative to the starting amine. Therefore, the observed 0.81 ppm downfield shift of the methylene protons upon nitrosation is consistent with IV and VI. (b) In III and V there is an unfavorable interaction between the electrons on the nitroso oxygen and those on the ring nitrogen, which is disfavored in related systems (Hutchins et al., 1968; Nelson et al., 1980). (c) While hydrogen bonding between the N=O oxygen and the N—H hydrogen might stabilize III, the resulting eight-membered ring would be energetically unfavorable (Galil et al., 1977). Therefore, the ^{1}H NMR spectra support structures IV and VI, with exocyclic double bonds to the *N*-isopropyl group.

The ^{13}C NMR spectrum of atrazine was only slightly affected by nitrosation (Table II) but does support the ^{1}H NMR data. Specifically, (a) the ^{13}C spectra suggest that the two NNAT isomers are not syn–anti isomers of the nitrosamine function, because the observed chemical shift differences of the methylene and ethyl–methyl carbons are only <0.06 ppm, (b) for the same reason as given for the ^{1}H NMR spectrum, the observed pair of isomers must be either III and V or IV and VI, and (c) the isomers are probably not VII and VIII because there was a larger chemical shift difference for the carbons of the isopropyl than for those of the ethyl group.

Stability of NNAT. The stability of NNAT solutions in ethanol–water 1:1 was determined by HPLC. (Monitoring at 410–430 mn was not practical because NNAT at the required concentration of 1 mg/mL precipitated when made basic.) Solutions of 5 μg/mL kept at pH 12 showed a first-order disappearance with a half-life of 25 min at 25 °C. Similar solutions were stable at pH 1 for 5 h. For comparison, a 1 mg/mL solution of 1-methyl-1-nitroso-3-nitroguanidine in the same solvent showed a half-life of 10–20 s at pH 12 and 25 °C, as determined from its absorption at 404 nm. The stability was compared of NNAT and *N*-nitrosomorpholine (NMOR) solutions in water and CH_2Cl_2, stored under laboratory lighting or in the dark. Table III shows that NNAT was photolabile, especially in CH_2Cl_2 solution, and was more so than the typical nitrosamine, NMOR.

Table III. Decomposition of 1.0 mM NNAT and NMOR in Water and Dichloromethane, in the Presence and Absence of Light[a]

compd	solvent	light/dark	max, nm	% initial absorbance after 24 h	% initial absorbance after 41 h
NNAT	water	light	408	75	63
NNAT	water	dark	408	100	93
NNAT	CH_2Cl_2	light	413	44	32
NNAT	CH_2Cl_2	dark	413	100	102
NMOR	water	light	344	97	87
NMOR	water	dark	344	97	89
NMOR	CH_2Cl_2	light	360	97	96
NMOR	CH_2Cl_2	dark	360	100	100

[a] Ten milliliters of NNAT or NMOR solutions with absorbances of about 0.10 at the indicated wavelength was stored at 21–23 °C in 20-mL Pyrex test tubes 3 m from four standard mercury fluorescent lights (lit 8 h/day) or in the dark. Absorbance was measured after 24 and 41 h.

Kinetics of Atrazine Nitrosation. This was studied by using ethanol–water 1:1 as the solvent rather than water, in which atrazine was almost insoluble. The nitrosation showed an optimum pH of 1.00–1.25 (Table IV). In the standard run (Table IV, row 3), NNAT yield was 30.5 μM (0.89% yield from atrazine) after reaction for 4 h. Because nitrite disappearance over the 4-h reaction was at least 10 times NNAT formation, this disappearance was not mainly due to NNAT formation and is attributed to acid-catalyzed decomposition of the nitrite (Mirvish et al., 1975). To correct approximately for this decomposition, the mean of initial and final nitrite (Table IV, column 5) was used to determine the rate constants.

Rate constants k_2, k_3, and k_4 (Table IV) were calculated from eqs 5–7 (Mirvish, 1975). Equations 4 and 6 are stoichiometric equations based on atrazine and nitrite concentrations irrespective of their state of ionization. Equations 5 and 7 are based on concentrations of the nonionized species, calculated by using pK_a values of 1.68 for atrazine (Wolfe et al., 1976) and 3.36 for nitrous acid (Mirvish, 1975). In eqs 4 and 5, the [nitrite]^2 and [HNO_2]^2 terms occur because the nitrosating species is N_2O_3, formed by a rapid prereaction equilibrium from two molecules of nitrous acid. In eqs 6 and 7, the [nitrite] (or [HNO_2]) and [H^+] terms occur because the nitrosating species is $H_2NO_2^+$, formed by a prereaction equilibrium from HNO_2 and H^+. The reaction followed eq 7 more closely than eq 5, as shown by the smaller variability of k_4 compared to that of k_2 (Table IV). The mean k_4 was 4.6×10^{-3} M^{-2} s^{-1}. However, the high k_4 at 41.8 mM nitrite indicated some contribution by eq 5, which is favored at high nitrite levels.

$$\text{rate} = k_1[\text{total atrazine}][\text{total nitrite}]^2 \quad (4)$$

$$\text{rate} = k_2[\text{nonionized atrazine}][HNO_2]^2 \quad (5)$$

$$\text{rate} = k_3[\text{total atrazine}][\text{total nitrite}][H^+] \quad (6)$$

$$\text{rate} = k_4[\text{nonionized atrazine}][HNO_2][H^+] \quad (7)$$

DISCUSSION

The synthesis of NNAT was convenient but yielded NNAT containing 20% atrazine, which could be removed by HPLC. In previous syntheses, NNAT was purified by column chromatography (Kearney et al., 1977) or its purity was not described. The greater stability in base of NNAT (a cyclic guanidine) compared to 1-methyl-1-nitroso-3-nitroguanidine is probably due to charge delocalization in

Table IV. Kinetic Study of Atrazine Nitrosation in Ethanol–Water 1:1 at 25 °C

parameter varied	initial conditions: pH	atrazine, mM	nitrite, mM: initial	nitrite, mM: mean[a]	no. of tests	NNAT yield at 4 h, μM	rate constants, M^{-2} s^{-1} × 10^3: k_2	k_3	k_4
standard	2.00	1.0	25	22.1	7	8.9	2.2	3.0	4.6
pH	1.00	1.0	25	20.6	1	30.4	2.9	1.0	5.9
	1.25	1.0	25	20.6	1	30.6	1.9	1.8	6.8
	1.50	1.0	25	22.1	2	18.9	0.7	1.9	4.8
	1.75	1.0	25	22.1	2	13.7	3.8	2.4	4.6
	2.00	1.0	25	22.1	1	8.6	2.0	2.7	4.2
	2.25	1.0	25	22.1	1	4.4	0.9	2.5	3.4
	3.00	1.0	25	21.3	2	1.3	0.5	0.5	7.2
atrazine	2.00	2.0	25	22.1	3	21.4	2.5	3.4	5.2
	2.00	4.0	25	22.1	3	27.5	1.8	2.4	3.7
nitrite	2.00	1.0	12.5	12.0	3	5.8	3.5	2.6	4.0
	2.00	1.0	50	43.6	3	26.3	1.5	4.0	6.2

[a] Mean of nitrite concentrations at zero time and after reaction for 4 h.

the *s*-triazine ring, reducing the ease of nucleophilic attack by OH^- at the central carbon (Hecht and Kozarich, 1973). The greater photolability of NNAT compared to that of NMOR (Table III) is attributed to UV absorption by the triazine ring and confirms the report by Wolfe et al. (1976). The finding that atrazine is nitrosated mainly according to eqs 6 and 7 indicates that nitrosation is performed by protonated nitrous acid ($H_2NO_2^+$) or NO^+, as is typical for N-substituted amides and guanidines. Nitrosation kinetics according to eq 7 showed k_4 values for 21 amides of 0.0014–400 M^{-2} s^{-1} at 25 °C (Mirvish, 1975). The mean k_4 for atrazine of 0.0046 M^{-2} s^{-1} was similar to the fourth most slowly nitrosated amide of this series, demonstrating that atrazine is nitrosated relatively slowly. Therefore, we would not expect large amounts of NNAT to be produced in the environment or in vivo. In contrast, Wolfe et al. (1976) stated that atrazine nitrosation followed eqs 4 and 5. Although rate constants were not given, the half-life of atrazine was stated to be 17 h when it was reacted with 10 mM nitrite at pH 2 and 25 °C. Under these conditions, our mean k_4 value corresponded to a half-life of 640 h. The reason for this discrepancy is unknown.

Our NMR studies confirmed the view of Wolfe et al. (1976) that NNAT is the *N*-ethyl-*N*-nitroso isomer. Because weakly basic amines are nitrosated more readily than strongly basic ones (Mirvish, 1975), preferential nitrosation of the *N*-ethyl group is attributed to the presumed weaker basicity (lower pK_a) of the *N*-ethyl nitrogen compared to the *N*-isopropyl nitrogen, due to the smaller inductive effect of the ethyl group.

The partial double bond character of the exocyclic bond to the isopropyl nitrogen is attributed to resonance with the triazine ring. This bond may show more double bond character than the exocyclic bond to the *N*-ethyl-*N*-nitroso nitrogen because of the longer linear resonance systems in IV and VI (extending from the isopropyl exocyclic bond to the nitrosamine group) compared to those of VII and VIII. From eq 2, the interconversion energies of 16.5 kcal/mol for the four isomers of atrazine and of 18.3 kcal/mol for the two isomers of NNAT indicate that these isomers would interconvert 15 (for atrazine) or 0.8 (for NNAT) times/s at 37 °C. These isomerisms might play roles in the herbicidal properties of atrazine and the toxicology of NNAT.

ACKNOWLEDGMENT

We thank Dr. G. Boka (Ciba-Geigy Co., Greensboro, NC) for supplying atrazine and his method for synthesizing NNAT. The research was supported by Grant 87-45 from the Nebraska Department of Health, Core Grant CA 36727 from the National Institutes of Health, and Core Grant SIG-16 from the American Cancer Society.

LITERATURE CITED

Bodenhausen, G.; Freeman, R.; Turner, D. L. Two-Dimensional J Spectroscopy: Proton-Coupled Carbon-13 NMR. *J. Chem. Phys.* **1976**, *65*, 839–840.

Eisenbrand, G.; Ungerer, O.; Preussmann, R. Formation of N-Nitroso Compounds from Agricultural Chemicals and Nitrite. In *N-Nitroso Compounds in the Environment*; Bogovski, P., Walker, E. A., Davis, W., Eds.; Scientific Publication 9; International Agency for Research in Cancer: Lyon, 1975; pp 71–74.

Freeman, R.; Hill, H. D. W. High-Resolution Study of NMR Spin Echoes: J Spectra. *J. Chem. Phys.* **1971**, *54*, 301–313.

Galli, C.; Illuminati, G.; Mandolini, L.; Tamborra, P. Ring-Closure Reactions. 7. Kinetics and Activation Parameters of Lactone Formation in the Range of 3- to 23-Membered Rings. *J. Am. Chem. Soc.* **1977**, *99*, 2591–2597.

Gutowsky, H. S.; Holm, C. H. Rate Processes and Nuclear Magnetic Resonance Spectra. II. Hindered Internal Rotation of Amides. *J. Chem. Phys.* **1956**, *25*, 1228–1234.

Hecht, S. M.; Kozarich, J. W. Mechanism of the Base-Induced Decomposition of N-Nitroso-N-methylurea. *J. Org. Chem.* **1973**, *38*, 1821–1824.

Hutchins, R. O.; Kopp, L. D.; Eliel, E. L. Repulsion of syn-Axial Electron Pairs. The Rabbit-Ear Effect. *J. Am. Chem. Soc.* **1968**, *90*, 7174–7175.

Janzowski, C.; Klein, R.; Preussmann, R. Formation of N-Nitroso Compounds of the Pesticides Atrazine, Simazine and Carbaryl with Nitrogen Oxides. In *N-Nitroso Compounds: Analysis, Formation and Occurrence*; Walker, E. A., Castegnaro, M. S., Griciute, L., Borzsonyi, M., Eds.; Scientific Publication 31; International Agency for Research in Cancer: Lyon, 1980; pp 329–331.

Karabatsos, G. J.; Taller, R. A. Structural Studies by Nuclear Magnetic Resonance. *J. Am. Chem. Soc.* **1964**, *86*, 4373–4378.

Kearney, P. C.; Oliver, J. E.; Helling, C. S.; Isensee, A. R.; Konston, A. Distribution, Movement, Persistence, and Metabolism of N-Nitrosoatrazine in Soils and a Model Aquatic Ecosystem. *J. Agric. Food. Chem.* **1977**, *25*, 1177–1181.

Krull, I. S.; Mills, K.; Hoffman, G.; Fine, D. H. The Analysis of N-Nitrosoatrazine and N-Nitrosocarbaryl in Whole Mice. *J. Anal. Toxicol.* **1980**, *4*, 260–262.

Mirvish, S. S. Formation of N-Nitroso Compounds: Chemistry, Kinetics, and *In Vivo* Occurrence. *Toxicol. Appl. Pharmacol.* **1975**, *31*, 325–351.

Mirvish, S. S.; Patil, K.; Ghadirian, P.; Kommineni, V. R. C. Disappearance of Nitrite from the Rat Stomach: Contribution of Emptying and Other Factors. *J. Natl. Cancer Inst.* **1975**, *54*, 869–875.

Nelsen, S. F.; Gannett, P. M.; Steffek, D. J. Nitrosohydrazine Conformations. The Effect of Replacing C(1)-H of 2-Nitroso-

2-azabicyclo[2.2.2]octane Derivatives by Nitrogen. *J. Org. Chem.* **1980**, *45*, 3857–3860.

Rice, S.; Cheng, M. Y.; Cramer, R. E.; Mandel, M.; Mower, H. F.; Seff, K. Structure of N-Methyl-N′-nitro-N-nitrosoguanidine. *J. Am. Chem. Soc.* **1984**, *106*, 239–243.

Robin, M. B.; Bovey, F. A.; Basch, H. Molecular and Electronic Structure of the Amide Group. In *The Chemistry of Amides*; Zabicky, J., Ed.; Wiley-Interscience: New York, 1970; pp 1–72.

Weisenburger, D. D.; Joshi, S. S.; Hickman, T. I.; Walker, B. A.; Lawson, T. A. N-Nitrosoatrazine (NNAT). Synthesis, Chemical Properties, and Mutagenicity. *Proc. Am. Assoc. Cancer Res.* **1988**, *29*, 106.

Weisenburger, D. D.; Hickman, T. I.; Patil, K. D.; Lawson, T. A.; Mirvish, S. S. Carcinogenesis Tests of Atrazine and N-Nitrosoatrazine: Compounds of Special Interest to the Midwest. *Proc. Am. Assoc. Cancer Res.* **1990**, *31*, 102.

Wolfe, N. L.; Zebb, R. G.; Gordon, J. A.; Fincher, R. C. N-Nitrosamine Formation from Atrazine. *Bull. Environ. Contam. Toxicol.* **1976**, *15*, 342–347.

Received for review January 14, 1991. Accepted February 19, 1991.

Registry No. NNAT, 56525-09-8; atrazine, 1912-24-9.