Formation of Volatile, Hydroxylated, and Chlorinated *N*-Nitrosamines during the Nitrosation of Spermidine 3-Hydrochloride

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Two hydroxylated dialkyl-*N*-nitrosamines, 3-butenyl-(3-hydroxypropyl)nitrosamine and 4-hydroxybutyl-(2-propenyl)nitrosamine, were identified as products in the reaction between sodium nitrite and spermidine·3HCl at pH 3.5 and 80 °C. The compounds were identified by mass and infrared spectrometry and by characterization of their trifluoroacetate derivatives. In the presence of chloride ions, chlorinated, dialkylnitrosamines were identified as nitrosation products from spermidine. The identification was based on mass spectrometry. Although other isomers may have been formed, 4-chlorobutyl-(2-propenyl)nitrosamine was the major chlorinated product.

In previous investigations (Bills et al., 1973; Hildrum et al., 1975a), the presence and identification of nitrosopyrrolidine and 3-butenyl-(2-propenyl)nitrosamine as products in the nitrosation of spermidine and spermine were demonstrated. This report describes the identification of other volatile nitrosation products from spermidine.

EXPERIMENTAL SECTION

The purity of the free spermidine (Sigma Chemical Co.) and spermidine·3HCl (Nutritional Biochemicals Co.), the procedures for reaction, extraction, and concentration, as well as the conditions for GC and spectrometric analyses, were as previously described (Hildrum et al., 1975a).

To facilitate separation of isomers of nitrosamines containing hydroxy groups, trifluoroacetate (TFA) derivatives were made. Three milligrams of hydroxylated nitrosamines were trapped during elution from the GC column, dissolved in 0.1 ml of pyridine, and reacted with 0.1 ml of *N*-methylbis(trifluoroacetamide) (Pierce Chemical Co.) at 75 °C for 1 h. For the separation of the TFA derivatives, a 610 cm × 1.6 mm o.d. stainless steel column packed with 5% OV 17 on 100/120 Varaport No. 30 was used. Temperatures of injector, column, and detector were 220, 150, and 270 °C, respectively, and the flow rate of carrier gas (N_2) was 6.3 ml/min. As many nitrosamines are potent carcinogens, precautions were taken in the handling of samples containing nitrosamines to prevent inhalation and skin exposure.

RESULTS AND DISCUSSION

A number of volatile compounds were formed in the nitrosation of spermidine. The tandem gas chromatograph–mass spectrometer was used to screen the components of the reaction mixture for nitroso groups. The ions m/e 30 and m/e 41 were monitored during the chromatographic runs as they are important ions in the fragmentation process of aliphatic nitrosamines (Budzikiewicz et al., 1967). Figure 1 shows the gas chromatograms of the dichloromethane extracts of the reaction mixtures from spermidine and spermidine·3HCl reacted at pH 3.5 for 1 h at 80 °C. Peak 12 was the internal standard methyl myristate. Peaks 6 and 10 were 3-butenyl-(2-propenyl)nitrosamine and nitrosopyrrolidine, respectively (Hildrum et al., 1975a).

The mass spectrum of peak 18 in the gas chromatogram of the nitrosation products from spermidine suggested the presence of hydroxylated nitrosamines (Figure 2A). The parent peak in the mass spectrum appeared to be m/e 158, which is the molecular weight of a compound with a structure similar to 3-butenyl-(2-propenyl)nitrosamine, except that instead of a double elimination, substitution with a hydroxy group has taken place at one of the carbonium ions. The hydroxy group could conceivably be located on either side chain. Furthermore, it could either be a primary alcohol formed by direct substitution, or a secondary alcohol formed after rearrangement of a carbonium ion. Four structural isomers of this monohydroxylated nitrosamine are possible.

In the mass spectrometry of alcohols, cleavage of the bond β to the oxygen atom is frequent (Silverstein and Bassler, 1964). Primary alcohols consequently produce a large m/e 31, while secondary alcohols with an adjacent methyl residue give a prominent m/e 45, which is often the base peak. As the mass spectrum of peak 18 showed a large m/e 31, but no m/e 45, it was concluded that the compound probably was a primary alcohol.

The mass fragmentation pattern for the monohydroxylated nitrosamine was consistent with the scheme for dialkylnitrosamines proposed by Saxby (1972). Loss of a hydroxy radical from the parent molecule produced the m/e 141 ion, while loss of NO gave the m/e 128 ion. Assuming the hydroxy group was located on the terminal carbon of the propyl side chain, fragmentation at the α carbon of the longest alkyl chain will produce the m/e 117 ion, which subsequently will lose HNO to produce an ion at m/e 86. Both these ions were clearly present in the spectrum, the latter being one of the major ions. If the hydroxy group is located on the terminal carbon of the butyl side chain, the corresponding fragmentation pathway would yield the m/e 99 and m/e 68 ions, which were also significant ions in the mass spectrum. The mass spectrum of peak 18 thus offered evidence for the presence of primary monohydroxylated nitrosamines, and suggested the presence of two structural isomers, one with the hydroxy group in the butyl side chain, and one with the hydroxy group in the propyl side chain.

The presence of monohydroxylated nitrosamines was confirmed by trapping peak 18 from the Carbowax 20M column and obtaining its infrared spectrum. Evidence for primary alcohol and vinyl unsaturation was present in the infrared spectrum.

To obtain additional information on the isomeric monohydroxylated nitrosamines, TFA derivatives were made from material trapped from peak 18. Gas chromatography of the derivatives on the OV 17 column gave two well-resolved peaks. The mass spectra of the peaks revealed molecular ions of m/e 254 for both compounds (Figure 3), which correspond to the molecular weight of the TFA derivative of the monohydroxylated nitrosamine. The base peak in the mass spectra of both compounds was m/e 41,

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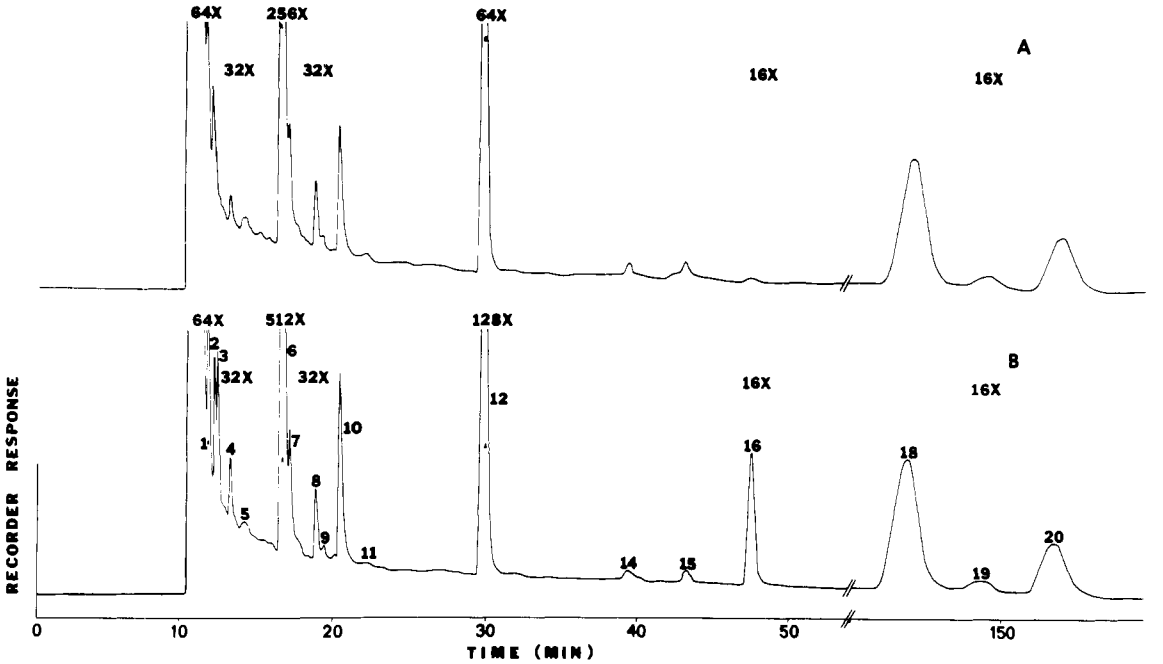

Figure 1. Gas chromatograms of the volatile products from the nitrosation of (A) spermidine and (B) spermidine·3HCl, on a 152.4 m × 0.76 mm i.d. stainless steel capillary column coated with 8% Carbowax 20M and 1% Versamid 900.

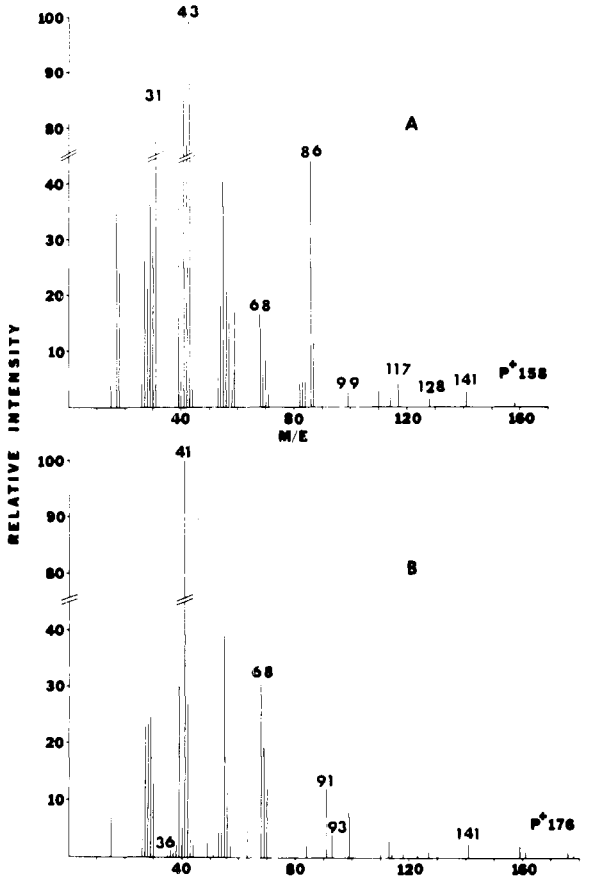

Figure 2. Mass spectra of (A) a mixture of the isomers of 3-butenyl-(3-hydroxypropyl)nitrosamine and 4-hydroxybutyl-(2-propenyl)nitrosamine (peak 18, Figure 1); (B) 4-chlorobutyl-(2-propenyl)nitrosamine (possibly a mixture of isomers) (peak 16, Figure 1).

and m/e 30 was prominent in both spectra. The m/e 31 ion was absent in both cases, indicating that the hydroxy groups were derivatized. Major fragment ions were obtained at m/e 69 for CF_3, which is an energetically favored species in the fragmentation of trifluoro compounds

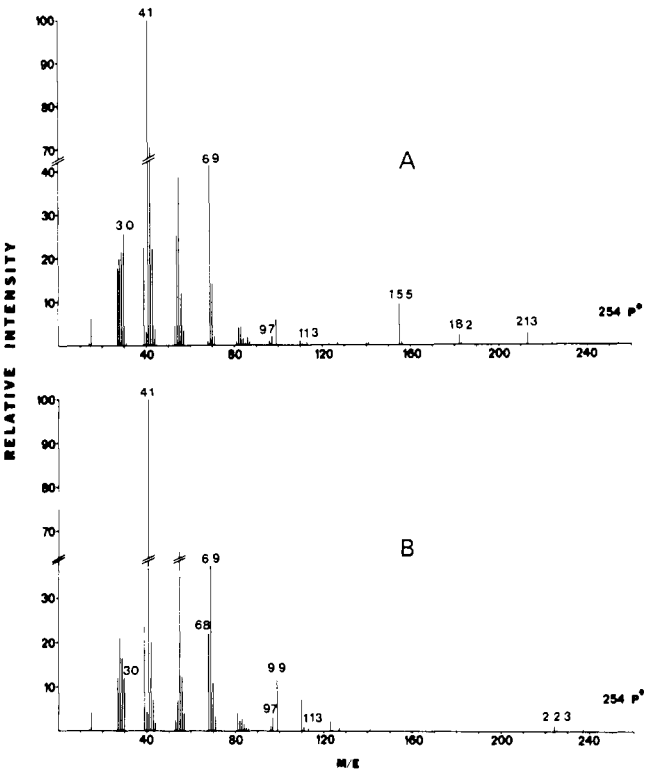

Figure 3. Mass spectra of the trifluoroacetate derivatives of (A) 3-butenyl-(3-hydroxypropyl)nitrosamine and (B) 4-hydroxybutyl-(2-propenyl)nitrosamine.

(Budzikiewicz et al., 1967). The fragments CF_3CO and CF_3CO_2 appeared in the mass spectra at m/e 97 and m/e 113, respectively.

Certain significant differences in the fragmentation patterns of the two isomers, however, enabled assignment of structures. The mass spectrum of the isomer with the shorter retention time on the OV 17 GC column (Figure 3A) had significant ions at m/e 213 and 182, which were practically absent from the spectrum of the other isomer. For the TFA derivative of 3-butenyl-(3-hydroxypropyl)-nitrosamine, fragmentation at the α carbon of the longest

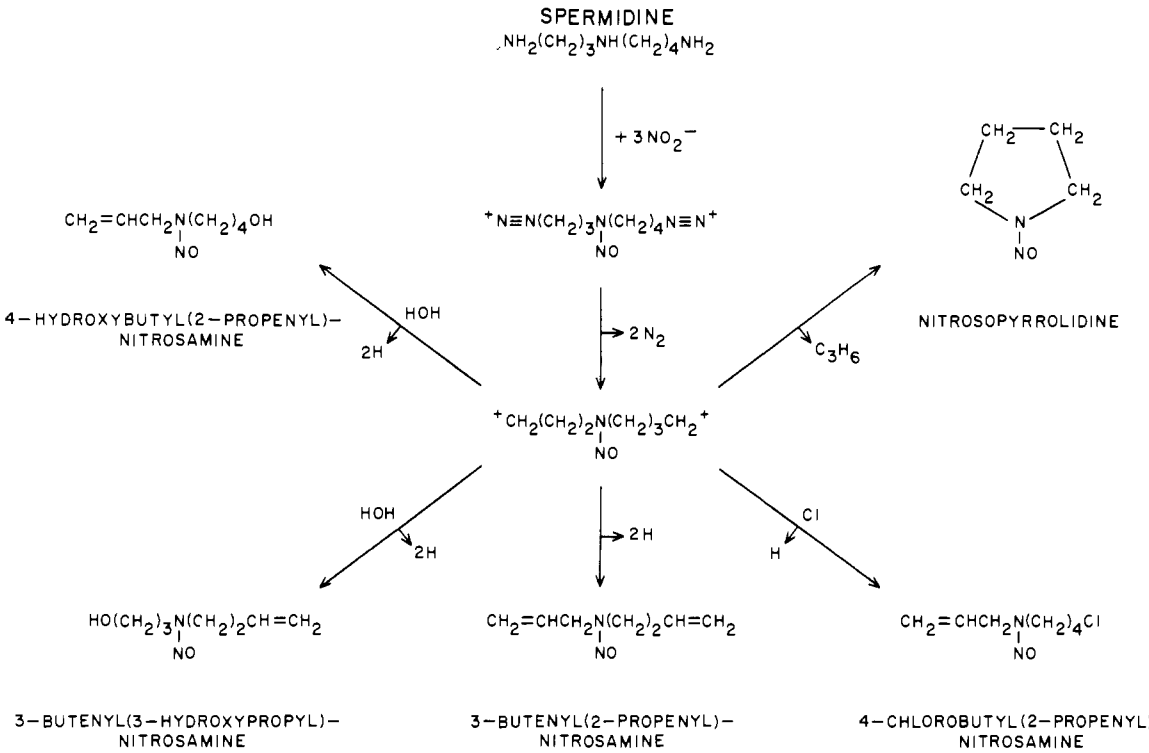

Figure 4. Pathway suggested for formation of nitrosamines from spermidine.

alkyl chain would produce an ion at m/e 213. Further loss of HNO would produce the m/e 182 ion. Although m/e 213 conceivably could be formed also from the TFA derivative of 4-hydroxybutyl-(2-propenyl)nitrosamine, the m/e 182 ion would not be produced from this isomer through known mass spectral fragmentation pathways for nitrosamines. A similar fragmentation process for the latter isomer would lead to the m/e 68 ion through the m/e 99 ion and both of these appeared as prominent ions in the mass spectrum of this isomer (Figure 3B). On the basis of the information provided by the mass spectra, it was concluded that the isomer with the shorter retention time on the OV 17 GC column was the TFA derivative of 3-butenyl-3-(hydroxypropyl)nitrosamine, while the isomer with the longer retention time was the TFA derivative of 4-hydroxybutyl-(2-propenyl)nitrosamine. From the gas chromatographic peak areas, the relative amounts of 3-butenyl-(3-hydroxypropyl)nitrosamine and 4-hydroxybutyl-(2-propenyl)nitrosamines were 61 and 39%, respectively.

The intensity of peak 16 increased in the presence of chloride ions and the mass spectrum of peak 16 suggested the presence of a monochlorinated nitrosamine (Figure 2B). The parent ion appeared to be m/e 176, which is the molecular weight of a nitrosamine where elimination and substitution with chloride ion have occurred at opposite ends of the molecule (Figure 4).

The mass spectrum of a compound containing one chlorine atom will have a P + 2 peak approximately one-third the intensity of the parent peak due to the presence of molecular ions containing the ^{37}Cl isotope (Silverstein and Bassler, 1964). Such isotope peaks were clearly present in the mass spectrum of peak 16. The P + 2/P intensity ratio was estimated to be 0.39, matching well with the theoretical value. The presence of the m/e 36 ion (HCl) in the mass spectrum gave additional proof of chlorine in the molecule.

The mass spectral fragmentation pattern of the chlorinated compound showed many similarities to the patterns of the unsaturated and hydroxylated nitrosamines.

Table I. Yields of Several Nitrosamines Formed in the Nitrosation of Spermidine[a]

Nitrosamine	% yield[b]	
	Spermidine	Spermidine·3HCl
3-Butenyl-(2-propenyl)-nitrosamine	2.4	1.7
4-Chlorobutyl-(2-propenyl)-nitrosamine[c]	0	0.12
Monohydroxylated nitrosamines[d]	0.47	
Nitrosopyrrolidine	0.60	0.62

[a] 5 mM amine, 90 mM sodium nitrite, pH 3.5, reactants adjusted with H_2SO_4, 80 °C, 1 h. [b] Yields on the basis of amine precursor added. [c] Possibly a mixture of isomers. [d] 3-Butenyl-(3-hydroxypropyl)nitrosamine and 4-hydroxybutyl-(2-propenyl)nitrosamine.

Loss of a hydroxy radical (nitroso oxygen + H) produced the m/e 159 ion, which still maintained a chlorine isotope peak at m/e 161. Loss of the chloride ion produced the m/e 141, which showed no indication of the P + 2 isotope ion.

Four structural isomers (at least) of monochlorinated nitrosamines derived from spermidine can be formed—two primary and two secondary chlorides. The fragmentation pattern of peak 16 indicated that 4-chlorobutyl-(2-propenyl)nitrosamine was an important isomer (Figure 4). Following loss of the chloride ion, fragmentation at the α carbon of the longest alkyl chain produced the m/e 99 for this isomer. Further loss of HNO gave the m/e 68 ion. The m/e 91 ion with an apparent chlorine isotope peak at m/e 93 suggested the presence of a butyl chloride fragment. However, the existence of more than one of the monochlorinated isomers in peak 16 is possible.

The yields of volatile nitrosamines from the nitrosation of spermidine were all relatively low (Table I). On the basis of the amount of amine precursor added, the yields of the individual nitrosamines ranged from 0 to 2.4%. At pH 3.5, the yield of 3-butenyl-(2-propenyl)nitrosamine from spermidine·3HCl was approximately 30% lower than

from spermidine, which is consistent with the previously observed effect of chloride ions on the nitrosation of proline (Hildrum et al., 1975b).

In the pathway proposed for the formation of the volatile nitrosamines from spermidine and nitrite shown in Figure 4 it is assumed that the reaction at the secondary amine group will stop at the nitrosamine stage, while nitrosation of the primary amines produces unstable diazonium ions, which degrade to intermediate carbonium ions (Ridd, 1961). Elimination of protons from both carbonium ions produces 3-butenyl-(2-propenyl)nitrosamine. Solvolysis with water at one carbonium ion and elimination of a proton at the other carbonium ion yield monohydroxylated nitrosamines. Correspondingly, in the presence of chloride ions, nucleophilic addition of the chloride ion to the carbonium ion would produce monochlorinated nitrosamines. Nitrosopyrrolidine may be formed by nucleophilic attack by the secondary amine nitrogen on the carbonium ion on the butyl side chain to form a tertiary amine. Nitrosative dealkylation of the tertiary amine could produce nitrosopyrrolidine (Smith and Loeppky, 1967). An alternate route is the formation of pyrrolidine from spermidine followed by nitrosation.

In the reaction between sodium nitrite and a secondary amine which contains no additional functional groups in the molecule, one nitrosamine is usually formed. This study appears to be the first where a number of different nitrosamines have been identified as products of a single, secondary amine. These nitrosamines may have very different carcinogenic potencies. The assessment of the public health hazard from the occurrence in the environment of a secondary amine which yields a single nitrosamine is a difficult task. The fact that a single secondary amine can form a range of different nitrosamines adds complexity to the toxicological and analytical aspects of the nitrosamine problem.

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Factors Influencing the Rate of Formation of Volatile *N*-Nitrosamines during the Nitrosation of Spermidine

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The influence of time, temperature, pH, and sodium chloride concentration on the formation of *N*-nitrosamines during the nitrosation of spermidine was investigated. Nitrosamines were formed from pH 3.0 to 6.0 and from 25 to 80 °C. These observations indicate that nitrosamine formation from spermidine is possible over a wide range of biological and environmental conditions providing the reactants are present in sufficient quantity.

The presence of the polyamines spermidine and spermine in food materials such as cereal germs, soybeans, and pork has been reported (Moruzzi and Caldarera, 1964; Wang, 1972; Spinelli et al., 1974; Lakritz et al., 1975). These findings have initiated an interest in their roles as precursors of carcinogenic *N*-nitrosamines in the presence of nitrite in our food supply.

Bills et al. (1973) identified *N*-nitrosopyrrolidine, a known carcinogen in rats, as a product in the nitrosation reaction of spermidine. Later, 3-butenyl-(2-propenyl)-nitrosamine (BPN) was found to be the principal, volatile nitrosation product both from spermidine and spermine (Hildrum et al., 1975a). Other volatile *N*-nitrosamines which have been identified from spermidine include 3-butenyl-(3-hydroxypropyl)nitrosamine and 4-hydroxybutyl-(2-propenyl)nitrosamine. In the presence of chloride ions a group of isomeric chlorinated compounds was formed in which 4-chlorobutyl-(2-propenyl)nitrosamine (BPN·Cl) was the major isomer (Hildrum et al., 1977).

The influence of factors such as pH, temperature, and the presence of inhibitors and promotors on the nitrosation of secondary amines has been intensely studied (Mirvish, 1970; Boyland et al., 1971; Mirvish et al., 1972; Fan and Tannenbaum, 1973). In this report, the influence of time, temperature, pH, and sodium chloride concentration on the nitrosation of a mixed polyamine, spermidine, will be described.

EXPERIMENTAL PROCEDURE

In all experiments, unless otherwise stated, 66.7 mM spermidine base (Sigma Chemical Co.) was reacted with 600 mM sodium nitrite (Mallinckrodt Chemical Works) at 50 °C for 1 h in 0.2 M acetate buffer, pH 4.0, in the presence of 0.33 M sodium chloride. All samples were run in duplicate. The experiment where pH was the variable will be outlined in detail below.

Five milliliters of an aqueous solution of 0.2 M spermidine was pipetted into a 250-ml Erlenmeyer flask,

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