Identification of γ-Butenyl-(β-propenyl)nitrosamine, the Principal Volatile Nitrosamine Formed in the Nitrosation of Spermidine or Spermine

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The principal volatile *N*-nitrosamine formed in the nitrosation of spermidine or spermine at pH 3.5 and 80° was γ-butenyl-(β-propenyl)nitrosamine. Its structure was confirmed by synthesizing γ-butenyl-(β-propenyl)amine and nitrosating it to form the corresponding nitrosamine. The mass, infrared, and nuclear magnetic resonance spectra of the nitrosamine from spermidine or spermine were identical with the spectra obtained from the synthesized γ-butenyl-(β-propenyl)nitrosamine.

The polyamines spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, and spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, are nonprotein, nitrogenous bases that are widely distributed in natural materials, including viruses, bacteria, plants, and animal tissue. Spermidine and spermine might therefore be present in significant amounts in numerous food systems. These polyamines have been found in edible germs such as barley and wheat (Moruzzi and Caldarera, 1964) and in soy bean flour (Wang, 1972). Of prime interest are the relatively high levels of spermidine and spermine found in some samples of pork and pork products (Lakritz *et al.*, 1973). The amounts were variable, with spermine generally present at higher levels than spermidine. The highest levels of spermidine and spermine found in putrefied pork were 1013 and 2769 mg/100 g of wet tissue, respectively. In fresh pork the highest levels of spermidine and spermine found were 125 and 56 mg/100 g of wet tissue, respectively.

Both spermidine and spermine contain secondary amine groups which can be nitrosated by nitrite. Nitrosation might occur during processing of foods containing polyamines and nitrite, or upon ingestion of foods containing these components. Formation of nitrosamines is of importance since many of these compounds have been shown to be carcinogenic.

Since spermidine and spermine might conceivably be nitrosated in foods, we wanted to characterize the nitrosamines formed upon nitrosation of these polyamines. This work describes the identification of the principal volatile nitrosamine formed when spermine or spermidine was allowed to react with nitrite.

EXPERIMENTAL PROCEDURE

Purity of Amines. The purity of spermidine·3HCl and spermine·4HCl (Nutritional Biochemicals Co.) was tested by thin-layer chromatography on cellulose using methyl Cellosolve–propionic acid–water (70:15:15, v/v/v), saturated with sodium chloride, as a developing solvent, and ninhydrin reagent to develop the plates. Both commercial samples were free of contaminating amines down to a level of 0.5%.

The purity of the free base spermidine (Sigma Chemical Co.) was tested by gas–liquid chromatography, which revealed a total of 2.2% volatile impurities.

Reaction, Extraction, and Concentration. Five millimoles of spermidine·3HCl and spermine·4HCl was dissolved in 20 ml of distilled water, and the pH of the solutions was adjusted to 3.5 by adding 0.1 *N* H_2SO_4 and 0.1 *N* KOH, respectively. The amine solutions were heated to 80° under a reflux condenser in a round-bottomed flask on a waterbath. Ninety millimoles of sodium nitrite in 20 ml of distilled water, adjusted to pH 3.5 with 2 *N* H_2SO_4, was added dropwise to the amine solution through the condenser. A vigorous reaction took place, and the color of the solution turned yellow and brown, for spermidine and spermine, respectively.

After reacting for 1 hr, the condenser was rinsed with a small amount of water and the flask cooled in ice. One milliliter of internal standard solution (5 mg of methyl myristate/ml) was added, and the solution was saturated with anhydrous sodium sulfate (Mallinckrodt Chemical Works) and extracted with redistilled dichloromethane (3 × 50 ml) (Mallinckrodt Chemical Works). The combined extracts were dried overnight at 4° over sodium sulfate and concentrated to 4 ml in a Kuderna-Danish apparatus (Kontes). Two chips of calcium sulfate (Drierite, W. A. Hammond) were added to the concentrator tube. Further concentration to 1 ml was achieved on a micro Kuderna-Danish apparatus under a stream of N_2 at 20°.

Gas–Liquid Chromatographic Conditions. A Varian Aerograph series 1400 gas chromatograph (gc) equipped with a flame ionization detector was used in most analyses. A stainless steel capillary column (0.03 in. i.d. × 500 ft) coated with 8% Carbowax 20M and 1% Versamid 900 according to Mon (1971) was used. Temperatures of injector, column, and detector were 210, 170, and 270°, respectively, and the flow rate of carrier gas (N_2) was 11.5 ml/min.

In order to collect samples for infrared (ir) and nuclear magnetic resonance spectrometry (nmr), the compound was trapped as it eluted from the gc column. A stainless steel column (0.13 in. o.d. × 10 ft) packed with 5% Carbowax 20M on Chromosorb G was employed. The gc was fitted with an effluent splitter which directed 7% of the effluent to the flame ionization detector and the remainder to the trap. The trapping for nmr was done as described by Parliment (1973), using the principle of solvent cocondensation with spectrograde carbon disulfide (Matheson Coleman and Bell) as the solvent. Glass tubes (0.05 in. o.d. × 1 ft), cooled in Dry Ice, were used to collect material for ir.

Free amines were separated on a 0.13 in. o.d. × 1 ft stainless steel column packed with 28% Pennwalt 223 plus 4% KOH on 80–100 Gas-Chrom R (Applied Science Lab.). The injector, column, and detector temperatures were 210, 160, and 270°, respectively. The carrier gas flow rate was 30 ml/min, N_2.

The peak areas were quantified using a Hewlett Packard Model 3373 B integrator. Methyl myristate (Eastman Chemical Co.), diallylnitrosamine, and dibutylnitrosamine (Eastman Chemical Co.) were used as internal standards.

Spectrometric Analyses. A Finnigan Model 1015 C tandem gas chromatograph mass spectrometer (gc–ms) system which included a Varian Aerograph (Series 1400) gc was used with the capillary column previously de-

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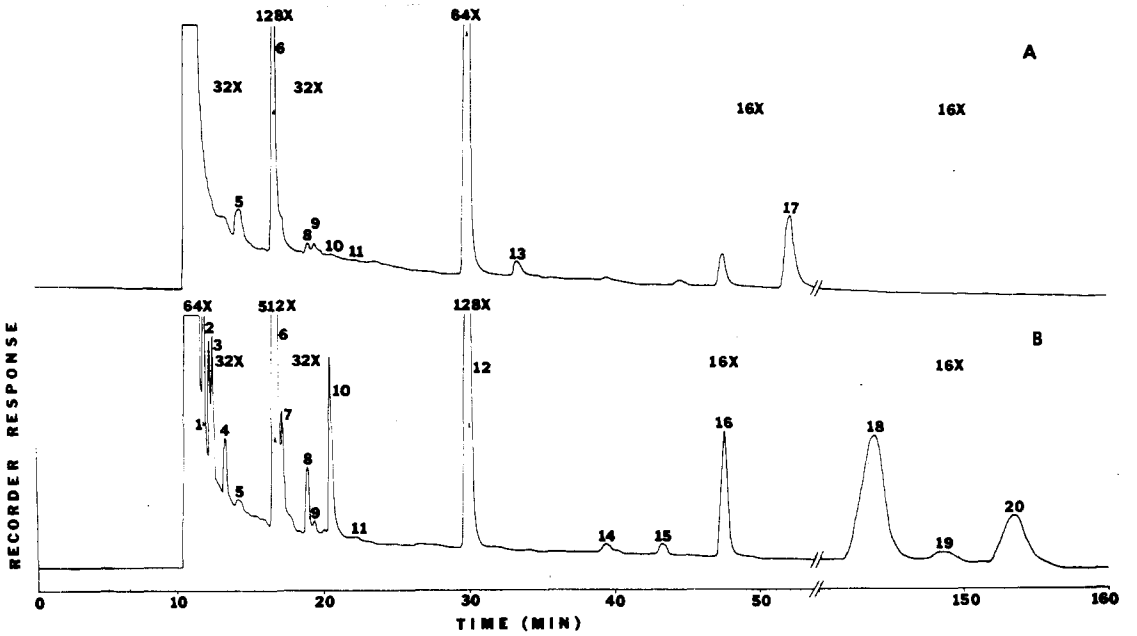

Figure 1. Gas chromatograms on 8% Carbowax 20M and 1% Versamid 900 capillary column of the volatile products from the nitrosation of (A) spermine·4HCl; (B) spermidine·3HCl.

scribed to separate and obtain mass spectra of components in the dichloromethane concentrate. The gc–ms interface was a Gohlke all-glass, jet orifice separator which allowed optimal amounts of sample components to pass into the ion source. A total ion current monitor provided a chromatographic trace. The carrier gas was helium. The operating conditions were: filament current, 300 μA; electron voltage, 70 eV; analyzer pressure, 5×10^{-7} Torr; and multiplier voltage, 1.50 kV. Spectra were scanned from *m/e* 12 to 270 in 1 sec.

Compounds subjected to infrared analysis were trapped from the Carbowax 20M column and analyzed as a thin film between two sodium chloride plates on a Beckman IR-18A infrared spectrophotometer equipped with a 5× beam condenser.

The nmr spectra were determined at 100 MHz on a Varian Model HA-100 spectrometer. Tetramethylsilane was used as internal reference.

Synthesis of γ-Butenyl-(β-propenyl)amine [BPA]. A modification of the method of Falbe *et al.* (1965) for synthesizing unsymmetrical amines was used. Allylamine (76.4 g) (Aldrich Chemical Co.) was refluxed in an oil bath at 53°. 4-Bromo-1-butene (26.0 g) (Aldrich Chemical Co.) was added dropwise through the condenser, and the reaction mixture heated under reflux for 30 min. Fifteen grams of BaO (Baker Chemical Co.) was added as a drying agent, and the mixture was subjected to a fractional distillation. The fractions were analyzed by gc and the fractions containing BPA were combined, dried over 2 g of BaO, and subjected to further purification by distilling over a 20-cm Vigreux column (Kontes). The yield of BPA was 73%, and its boiling point was 135–138°. The purity of BPA was 98%, and the identity was confirmed by gc–ms. Nitrosation of BPA was done according to the method of Dutton and Heath (1956). The nitrosamine formed was identified by gc–ms. The compound was trapped as described above and the ir and nmr spectra were obtained.

Safety Precautions. Precautions were taken in the handling of samples containing nitrosamines to prevent inhalation and skin exposure. All handling of nitrosamines was done under hoods. In gc work with these compounds, the outlet of the flame ionization detector was vented to a hood. All trappings from the column were done under a hood. Gloves were used whenever nitrosamines were handled. All glassware exposed to concentrated samples were decontaminated with 5% HBr in acetic acid.

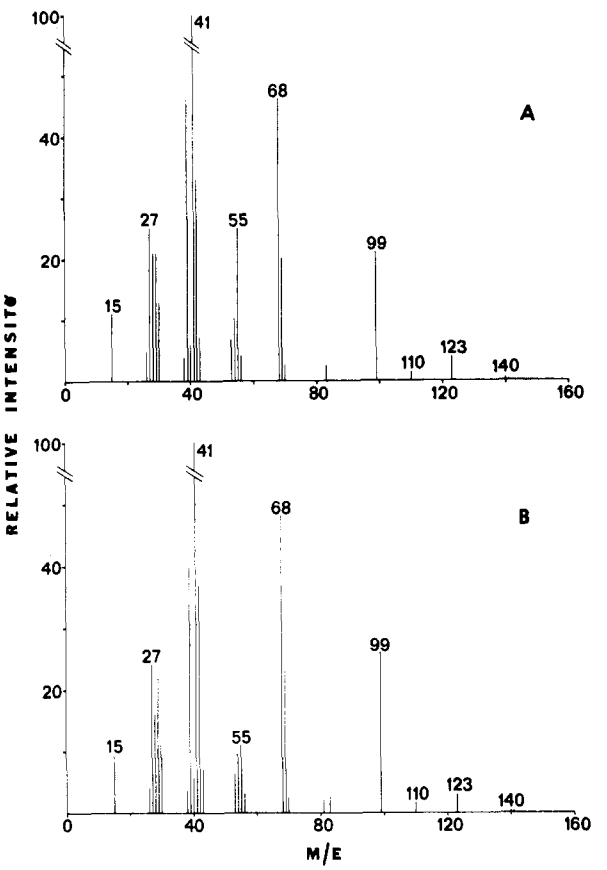

Figure 2. Mass spectra of γ-butenyl-(β-propenyl)nitrosamine from (A) spermidine·3HCl; (B) γ-butenyl-(β-propenyl)amine.

RESULTS AND DISCUSSION

A number of peaks appeared in the gas chromatograms of the concentrated dichloromethane extracts from reaction mixtures following nitrosation of spermidine and spermine (Figure 1).

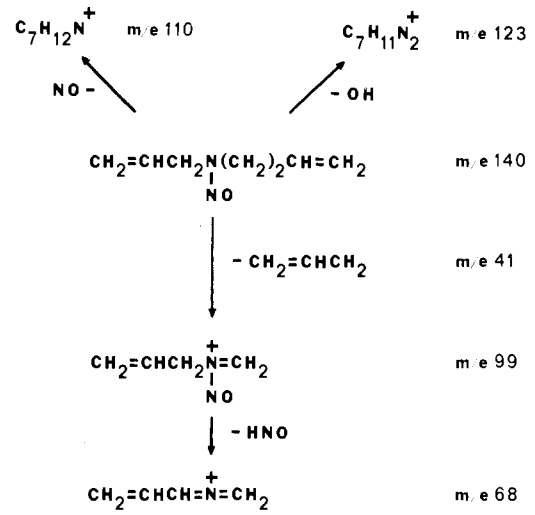

Figure 3. Mass spectrometric fragmentation scheme of γ-butenyl-(β-propenyl)nitrosamine.

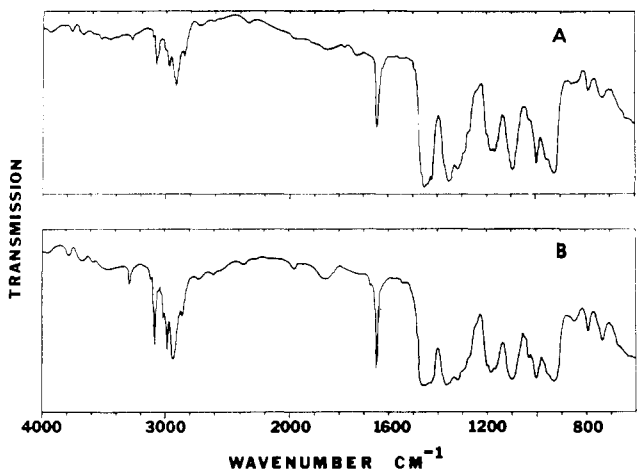

Figure 4. Infrared spectra of γ-butenyl-(β-propenyl)nitrosamine from (A) spermidine·3HCl; (B) γ-butenyl-(β-propenyl)amine.

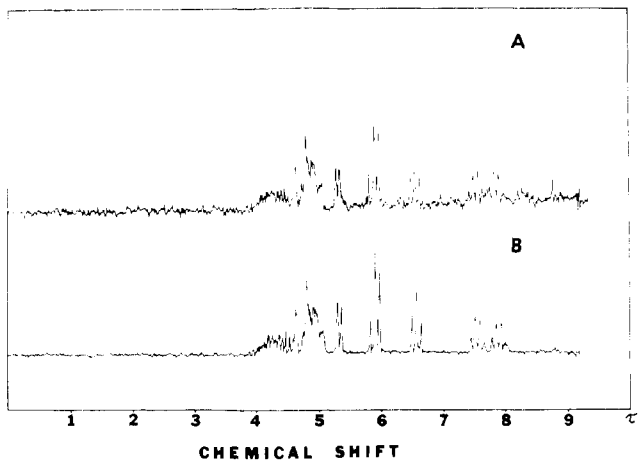

Figure 5. Nuclear magnetic resonance spectra of γ-butenyl-(β-propenyl)nitrosamine from (A) spermidine·3HCl, (B) γ-butenyl-(β-propenyl)amine.

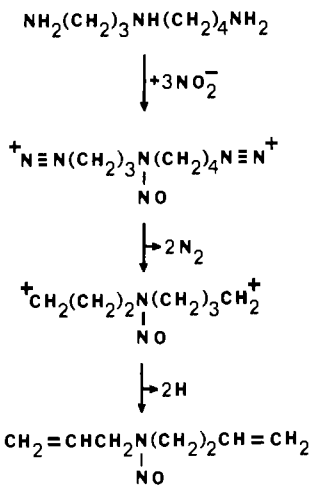

Figure 6. Proposed mechanism of formation of γ-butenyl-(β-propenyl)nitrosamine from spermidine and nitrite.

Peak 10 was nitrosopyrrolidine and had been previously identified as a nitrosation product of spermidine at 170° by Bills *et al.* (1973). In this work nitrosopyrrolidine was found to be formed at 80° from both spermidine and spermine. Nitrosopyrrolidine was identified by gc–ms and by comparison of retention times.

Peak 6, the largest peak in the chromatograms, was trapped as it eluted from the column and was found to be Griess positive (Walters *et al.*, 1970). Interpretation of the mass spectra obtained for this compound prompted us to assign a structure consistent with γ-butenyl-(β-propenyl)-nitrosamine (BPN). The mass spectrum of peak 6 was essentially identical with the mass spectrum obtained for BPN which was synthesized from the corresponding secondary amine (Figure 2). Peak 6 also had the same retention time on the capillary column as synthesized BPN.

A mass spectral fragmentation scheme for BPN is shown in Figure 3. The molecular weight of BPN is 140, and although the parent ion at m/e 140 is rather small, it is clearly present in the spectrum. The fragmentation is consistent with the scheme recently proposed by Saxby (1972), for the fragmentation of dialkyl-*N*-nitrosamines. Loss of hydroxyl radical produces the m/e 123 ion. Fragmentation at the α carbon of the longest alkyl chain produces an ion at m/e 99 which further loses HNO to produce an ion at m/e 68. Allylic cleavage at both ends of the molecule with the charge remaining with the group containing the double bond produces the m/e 41 ion which is the base peak in the spectrum.

To confirm the identity of BPN (peak 6), ir and nmr spectra were obtained, and compared with the corresponding spectra of synthesized BPN. The information from the infrared spectra bears primarily on the vinyl unsaturation in the molecule (Figure 4). A band at 3082 cm^{-1} is assigned to vinylic assymmetric stretch. A double bond stretch occurred at 1637 cm^{-1}. The bands at 985 and 910 cm^{-1} are typical of the C–H bend bands found in compounds containing vinyl unsaturation (Colthup *et al.*, 1964).

The nuclear magnetic resonance spectra offer further confirmation of the identity of the nitrosamine (Figure 5). Identical spectra were obtained for BPN from spermidine (peak 6) and from synthesized BPA. Because of the partial double bond character of the N–N linkage, the nitrosamino group assumes an essentially planar conformation, in which the O atom is syn to one substituent and anti to the other. The substituents are, in general, magnetically nonequivalent, and when the substituents are different, two isomeric conformations are possible, which are generally distinguishable by nmr spectrometry (Karabatsos and Taller, 1964). The protons generally resonate at higher fields when syn than when anti to the O atom. The nmr spectrum of BPN is complex. By comparing it with the

simpler spectrum of diallylnitrosamine, it is possible to assign most of the resonances.

The two quartets centered at τ 7.60 and 7.95 correspond to syn and anti forms of the β-methylene group in the butenyl side chain of BPN. Two triplets centered at τ 6.62 and 5.95 are assigned to the syn and anti conformations of the α-methylene group in the butenyl side chain. The latter triplet is overlapping with the resonance of the *syn*-α-methylene of the propylene side chain. The resonance of the corresponding *anti*-α-methylene occurs at lower fields, giving a doublet centering at τ 5.40. The vinylic hydrogens in both groups resonate between τ 3.9 and 5.2.

The yields of BPN from the hydrochlorides of spermidine and spermine were estimated to be 1.7 and 1.4%, respectively, using diallylnitrosamine as an internal standard. The yield of BPN from free spermidine was 2.4%.

In order to substantiate the formation of BPN from spermidine and not from a γ-butenyl-(β-propenyl)amine (BPA) impurity in the spermidine, the following experiment was performed. The purity of free spermidine was examined by gc, with special attention to the possible occurrence of BPA. Since one of the impurities in spermidine cochromatographed with BPA, the effect of the concentration of this impurity on the yield of BPN was determined. Spermidine was purified by vacuum distillation (bp 94° (2 mm)), and the concentration of the impurity was reduced from 370 to 40 ppm. The yield of BPN from the purified spermidine, however, remained the same as before the vacuum distillation, confirming that BPN was a genuine product formed from spermidine.

The mechanism we propose for the formation of BPN from spermidine and nitrite assumes the reaction at the secondary amine function will stop at the nitrosamine stage (Figure 6). Nitrosation of the primary amines produces unstable diazonium ions, which degrade to carbonium ions (Ridd, 1961). Elimination of protons from both carbonium ions produces BPN. The observation that BPN was formed from the reaction between spermine and nitrite was puzzling. As tested by thin-layer chromatography, no spermidine was found in the spermidine·4HCl. Heat stability tests of spermine showed no significant conversion to spermidine under the reaction conditions. Due to lack of information, no mechanism can be suggested for the formation of BPN from spermine at this time.

Besides elimination, nucleophilic additions or rearrangements also may occur at the carbonium ion with the possibility of forming a multiplicity of different nitrosamines upon nitrosation of spermidine and spermine. Experiments are currently underway to determine other nitrosation products of spermidine and spermine.

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Reduced Binding of Nitric Oxide in Irradiated Horse Heart Myoglobin

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The ligand binding of nitric oxide with a 0.05 m*M* concentration of horse heart myoglobin has been studied as a function of radiation dose. At 100 krads, the nitrosylmyoglobin (NOMb) peaks at 575 and 545 nm decreased by about 50% as against 20% at 410 nm. Formation of NOMb could not be detected at higher radiation doses. Evidence for conformational changes in the apoglobin moiety caused by irradiation has been presented in terms of increased susceptibility to tryptic hydrolysis and changes in polyacrylamide gel electrophoretic pattern.

The kinetics of ligand binding of nitric oxide with myoglobin has been extensively studied to explain the reaction mechanisms associated with the formation of nitrosylmyoglobin (NOMb), the major pigment of cured meats (Fox, 1966; Fox and Thomson, 1963; Watts and Lehmann, 1952). Some of the processing treatments (Brown and Mebine, 1969; Kraft and Ayres, 1954; Brown and Dolev, 1963) including γ-radiation (Ginger *et al.*, 1955; Tappel, 1958) are known to cause discoloration of meat. Most of the studies on radiation damage to myoglobin relate to the mechanisms of interconversions of met and oxy forms (Tappel, 1950; Satterlee *et al.*, 1971; Madhavan *et al.*, 1973) and loss in spectral characteristics (Ginger *et al.*, 1955; Ginger and Schweigert, 1956; Brown and Akoyunoglou, 1964; Madhavan and Kumta, 1971; Paul *et al.*, 1973). Besides oxidative changes caused by γ-radiation in the protoporphyrin nucleus, in an aqueous system, the apoglobin moiety undergoes initial unfolding followed by aggregation (Brown and Akoyunoglou, 1964; Satterlee *et*

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