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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Table I. Effect of pH on the Yields[a] of Several Nitrosamines during the Nitrosation of Spermidine[b]

Initial pH	Final pH	Yield, % BPN	BPN·Cl	BPN·OH
3.0	2.0	0.39	0.030	0.016
3.5	3.85	0.80	0.041	0.019
4.0	4.26	0.99	0.039	0.027
4.5	4.78	0.64	0.023	0.0056
5.0	5.32	0.21	0.010	0
6.0	6.02	0.035	0.0034	0

[a] Yield in percent based on spermidine added. [b] 66.7 mM spermidine, 600 mM sodium nitrite, 0.2 M acetate buffer, 0.33 M sodium chloride, 1 h, 50 °C.

followed by 5 ml of 0.6 M acetate buffer (pH 3 to 6) containing sufficient 2 N H_2SO_4 to attain the desired pH after the addition of sodium nitrite. Five milliliters of a solution being 1.8 M in sodium nitrite and 1.0 M in sodium chloride was added, the pH was measured, and the flask was capped and placed in a shaking water bath at 50 °C for 1 h.

After the reaction period of 1 h the flasks were cooled in ice for 20 min, the pH was recorded, and the reaction immediately stopped by adding 7.5 ml of 4 M ammonium sulfamate (Matheson Coleman and Bell) in 0.5 N aqueous H_2SO_4. One milliliter of internal standard solution (2.00 mg of methyl myristate/ml of acetone) was added and the solution was saturated with anhydrous sodium sulfate and extracted with redistilled dichloromethane (2 × 30 ml). The combined extracts were dried over anhydrous sodium sulfate overnight, concentrated to 0.2 ml using Kuderna-Danish concentrators (Kontes Glass Co.), and subjected to gas–liquid chromatographic (GC) analysis.

The GC separation and quantitation of the nitrosamines were carried out as outlined in a previous paper (Hildrum et al., 1975a). As 4-hydroxybutyl-(2-propenyl)nitrosamine and 3-butenyl-(3-hydroxypropyl)nitrosamine were not resolved on the column used, these monohydroxylated, unsaturated nitrosamines were quantified together (BPN·OH). The identity of the nitrosamines was confirmed by mass spectrometry of selected samples.

As many nitrosamines are potent carcinogens, precautions were taken in the handling of samples to prevent inhalation and skin exposure.

RESULTS AND DISCUSSION

The recoveries during the extraction and concentration steps for BPN, BPN·Cl, and BPN·OH were 89, 93, and 32%, respectively. The low recovery of BPN·OH may have been caused by polymerization or denitrosation of the compound during the cleanup procedure. The estimated standard deviations on the yields were 8, 5, and 14% for BPN, BPN·Cl, and BPN·OH, respectively, in percent of the mean yields. The values reported were corrected for recovery.

The pH of the reacting medium changed during the reaction period (Table I). At an initial pH of 3.0, a substantial decrease in the pH value was noted, most likely due to the decomposition of nitrous acid to the stronger nitric acid (Turney and Wright, 1959). In all samples other than the pH 3.0 sample, the pH increased moderately during the reaction. Since pH changed during the reaction, no true pH optima for the formation of nitrosamines can be reported. However, the results show that the highest yields of the nitrosamines, under these conditions, were obtained between pH 3.5 and 4.5.

The yield of all nitrosamines at both pH 3.5 and 5.0 either declined or remained essentially constant at reaction times longer than 5 h (Figure 1). This was presumably

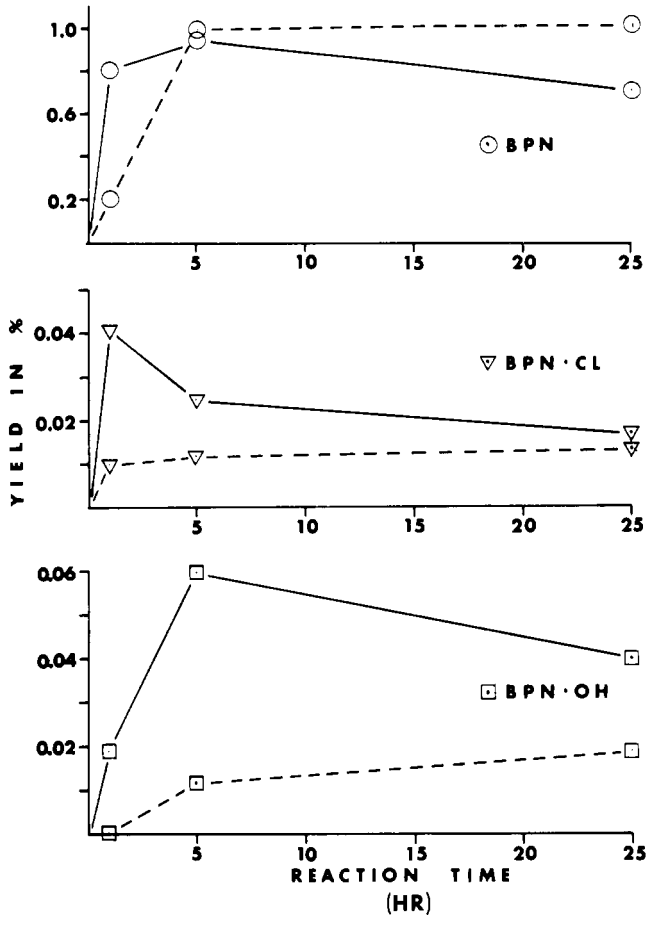

Figure 1. Effect of reaction time in hours at pH 3.5 and 5.0 on the yields of several nitrosamines during the nitrosation of spermidine: (—) pH 3.5; (---) pH 5.0. Reaction conditions: 66.7 mM spermidine, 600 mM sodium nitrite, 0.2 M acetate buffer, 0.33 M sodium chloride, 55 °C.

due both to the depletion of nitrite and to secondary reactions with loss of nitrosamines. The loss may have been caused by interactions of nitrite with the hydroxy, chloride, or vinyl groups in the nitrosamines (Norman, 1968; Ridd, 1961). Polymerization or denitrosation of the nitrosamines might also have occurred. Both the depletion of nitrite and secondary reactions apparently proceed faster at a lower pH level. Consequently, the yield of BPN after 25 h at pH 5.0 exceeded the yield at pH 3.5 by approximately 45%. The effect of reaction time on the yields of BPN·Cl and BPN·OH followed a similar pattern as for BPN, although the yields at pH 5.0 for these nitrosamines never exceeded the corresponding yields at pH 3.5.

Initial rates of formation have been suggested for predicting nitrosamine formation in foods (Fan and Tannenbaum, 1973; Mirvish et al., 1973). The highest rates of formation of nitrosamines were obtained by these workers between pH 2.5 and 3.5. As this study shows, the maximum accumulation of nitrosamines can take place at higher pH levels when the reaction time is longer. This observation should be kept in mind when utilizing initial rate data for the prediction of nitrosamine formation in mildly acidic or neutral food materials during storage.

The yields of all nitrosamines from spermidine increased with the reaction temperature between 24 and 48 °C (Table II). The effect of temperature on reaction rate is accurately described by an Arrhenius plot (Figure 2). The activation energy was approximately 19 kcal/mol for the formation of BPN from spermidine and nitrite under these

Table II. Effect of Temperature on the Yields[a] of Several Nitrosamines in the Nitrosation of Spermidine[b]

Nitrosamine	Yield, %		
	24 °C	37 °C	48.5 °C
BPN	0.024	0.089	0.27
BPN·Cl	0.0004	0.0023	0.0082
BPN·OH	0	0	0.0090

[a] Yield in percent based on spermidine added. [b] Reaction conditions: 66.7 mM spermidine, 600 mM sodium nitrite, 0.2 M acetate buffer, 0.33 M sodium chloride, pH 5.0, 1 h.

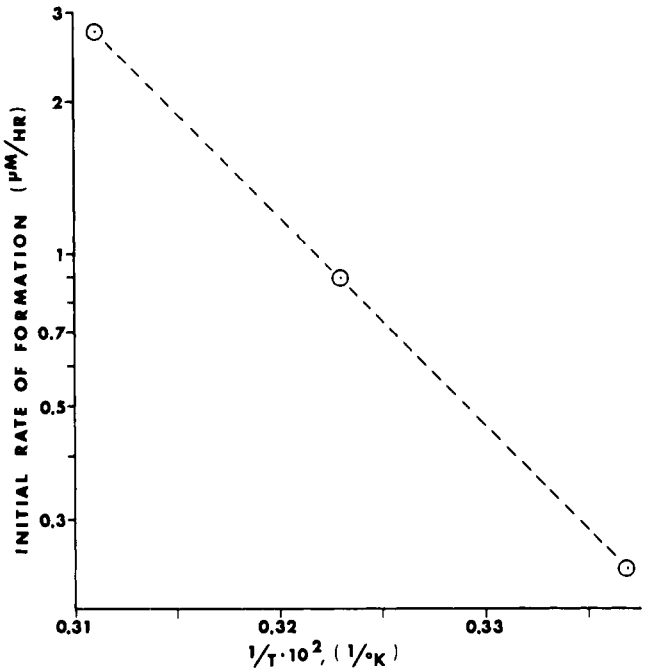

Figure 2. Effect of temperature (T) on the formation of 3-butenyl-(2-propenyl)nitrosamine (Arrhenius plot). Reaction conditions: 66.7 mM spermidine, 600 mM sodium nitrite, 0.2 M acetate buffer, 0.33 M sodium chloride, pH 5.0, 1 h.

conditions. In comparison, the activation energy for the nitrosation of morpholine is reported to be approximately 10 kcal/mol (Fan and Tannenbaum, 1973).

No significant effect of sodium chloride (0.1–1.0 M) on the yields of BPN and BPN·OH was observed in 0.1 M acetate buffer at pH 4.0 (Figure 3). The yield of BPN·Cl, however, increased markedly with increasing salt concentration. At sodium chloride concentrations higher than 1.0 M, strong inhibition of the formation of nitrosamines was observed.

Since the presence of acetate buffer might have masked the inhibiting effects of sodium chloride at the lower salt concentrations, the reaction was also carried out in a buffer-free system. A significant inhibition on the formation of BPN (16% inhibition) was found in the presence of 0.5 M sodium chloride in the buffer-free system. The inhibiting effects of both acetate buffer and sodium chloride on the nitrosation reaction were probably due to a combination of primary and secondary salt effects (Hildrum et al., 1975b).

Previous work (Hildrum et al., 1975a, 1976) demonstrated that several nitrosamines can be formed from spermidine and nitrite at 80 °C, pH 3.5. The results of this investigation show that the formation of nitrosamines from spermidine can occur over a fairly wide range of temperatures and pH. 3-Butenyl-(2-propenyl)nitrosamine and 4-chlorobutyl-(2-propenyl)nitrosamine formed over the temperature range 25–80 °C and from pH 3 to 6.0. This

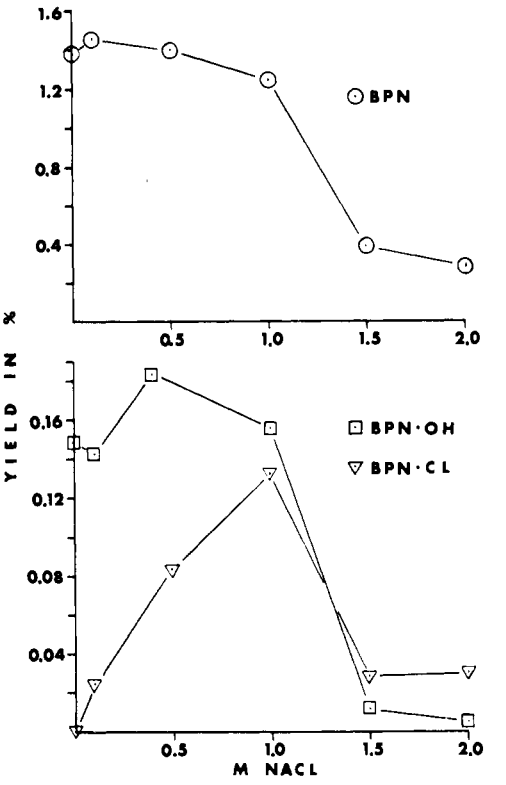

Figure 3. Effect of sodium chloride concentration on the yields of several nitrosamines in the nitrosation of spermidine. Reaction conditions: 66.7 mM spermidine, 600 mM sodium nitrite, 0.1 M acetate buffer, pH 4.0, 1 h, 50 °C.

may be significant since it suggests that nitrosamine formation from spermidine might be possible over a broad range of biological and environmental conditions.

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