AN ABSTRACT OF THE THESIS OF

KJELL IVAR HILDRUM for the degree DOCTOR OF PHILOSOPHY
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Title: FORMATION OF N-NITROSAMINES FROM THE NITROSATION OF SPERMIDINE AND SPERMINE

Abstract approved: R. A. Scanlan

The reactions between sodium nitrite and the polyamines spermidine and spermine were investigated. When reacted at 80°C for 1 hr at pH 3.5 with a 1:3 molar ratio of amine groups to sodium nitrite, five volatile nitrosamines were identified as products in the nitrosation of spermidine. 3HCl, and two volatile nitrosamines were identified from spermine. 4HCl.

The principal volatile nitrosation product both from spermidine and spermine was γ-butenyl(β-propenyl)nitrosamine (BPN). The identification of this compound was based on the spectral characteristics of the compound using mass, infrared, and nuclear magnetic resonance spectrometry. To confirm the identity, BPN was synthesized from γ-butenyl(β-propenyl)amine and sodium nitrite, and the spectra obtained for this substance and the unknown compound were compared. The amine precursor was synthesized from allylamine

and 1-Bromo-4-butene.

Two hydroxylated, dialkyl nitrosamines were identified as products from spermidine·3HCl: γ-butenyl(γ-propanol)nitrosamine and δ-butanol(β-propenyl)nitrosamine. The compounds were characterized by mass and infrared spectrometry, and by the Griess test for nitrosamines. The structures were confirmed by making the trifluoroacetate derivatives of the compounds, and obtaining their mass spectra.

In the presence of chloride ions, chlorinated dialkyl nitrosamines were tentatively identified as nitrosation products from spermidine. The identification was based on mass spectrometry, particularly the isotope effects by chlorine, and by the Griess test. It appeared that δ-butylchloride(β-propenyl)nitrosamine probably was a major isomer, but other structural isomers may also be formed.

The nitrosamines described above have previously not been synthesized or characterized. Their individual carcinogenic potency therefore is not known. Nitrosopyrrolidine, which was a nitrosation product both from spermidine and spermine, is a potent carcinogen. The yields of the individual nitrosamines from spermidine·3HCl and spermine·4HCl were, respectively: BPN, 1.7%, 1.4%; γ-butenyl(γ-propanol)nitrosamine, 0.29%, 0%; δ-butanol(β-propenyl)nitrosamine, 0.18%, 0%; δ-butylchloride(β-propenyl)nitrosamine (and its isomers), 0.12%, 0%; nitrosopyrrolidine, 0.60% and trace amounts. The yields were estimated on the basis of the amount of polyamine precursor.

Maximum accumulation of all nitrosamines from spermidine was observed between pH 3.0 and 4.5, when reacting for 1 hr at 50°C. Increasing the reaction time to 25 hrs at 50°C, the yield of BPN at pH 5.0 exceeded the yield at pH 3.5. From the temperature effect on the nitrosation rate, activation energy for the formation of BPN from spermidine and nitrite was estimated to be 19 kcal/mol. In the presence of 0.1-1 M sodium chloride at pH 4.0, no significant effects on the yields of BPN, γ-butenyl(γ-propanol)nitrosamine and δ-butanol (β-propenyl)nitrosamine were observed, while the yield of δ-butyl-chloride(β-propenyl)nitrosamine (and its isomers) was strongly enhanced with increased sodium chloride concentration. The yields of all nitrosamines were drastically reduced in the presence of 1.5 M or 2.0 M sodium chloride in the system.

In the nitrosation of proline, sodium chloride in concentrations up to 1 M strongly activated the reaction at pH 0.5. Small inhibiting effects were observed at pH 2.5, however, and moderate inhibition by sodium chloride was seen at pH's 4.0 and 5.5. Multiple regression analysis showed the best fitted model was of the form, log (initial rate of nitrosation) = $a + b[NaCl] + c[NaCl]^2$ at all pH levels tested.

Formation of N-nitrosamines from the Nitrosation of Spermidine and Spermine

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Kjell Ivar Hildrum

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Dean of Graduate School

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INTRODUCTION

The carcinogenic properties of N-nitrosamines in test animals have been known to the scientific community for almost 20 years. That human cancer might result from the presence of N-nitrosamines in the environment is a serious concern from a public health standpoint.

N-nitrosamines are formed by the interaction of nitrite with amines, particularly secondary amines. Recent surveys have demonstrated the presence of N-nitrosamines in very low concentrations in various types of cured meat and fish products. Nitrite is used as a preservative in these foods. Information about the occurrence of amines in foods is scarce. To gain a better understanding of the occurrence of N-nitrosamines in foods, more information on the precursors of N-nitrosamines and the conditions of N-nitrosamine formation is needed.

The polyamines spermidine and spermine are widely distributed in natural materials, including viruses, bacteria, plants, and animal tissue. Recent reports show appreciable amounts of spermidine and spermine in edible germs such as barley and wheat, soy bean flour, and notably in pork and pork products.

Since pork products are often cured with sodium nitrite, it seemed important to identify the main N-nitrosamines formed in the nitrosation of spermidine or spermine, and to investigate what conditions are favorable to these reactions. Since the polyamines contain both primary and secondary amine groups, a range of nitrosation products could be expected from each compound.

Sodium chloride is an important food additive in cured meat and fish products. The combined effects of sodium chloride concentration and acidity on the nitrosation of amines have not been fully elucidated, and it was warranted to initiate a study to clarify this aspect.

REVIEW OF LITERATURE

Since the toxic and carcinogenic action of dimethylnitrosamine in rats was first described (Barnes and Magee, 1954; Magee and Barnes, 1956), the results of numerous investigations concerning the tumor-promoting effects of nitroso compounds have been published. The subject has been reviewed by Druckrey et al. (1967), and Magee and Barnes (1967). The N-nitroso compounds are very potent carcinogens, which are active in a wide range of species. Tumors have been induced in almost every organ of the rat, and in some cases N-nitrosamines have been shown to have the capability of inducing cancer after a single dose. Several N-nitroso compounds are also mutagenic or teratogenic. For these reasons, the occurrence of N-nitroso compounds in food or other environmental situations is a matter of grave concern. Although cancer in man has not yet been traced to nitrosamines as the causative agents, the experimental results in animals suggest that these compounds also would be carcinogenic to man (Wolff and Wasserman, 1972). In the following, N-nitrosamines and N-nitroso compounds will be termed nitrosamines and nitroso compounds, respectively.

Chemical Properties and Biological Effects

Nitroso compounds are generally divided into two groups, nitrosamines and nitrosamides. These groups differ both in chemical

properties and in biological activities. Nitrosamines are relatively stable over a wide pH range, but degrade on exposure to ultraviolet light. Nitrosamides break down quickly under alkaline conditions, and some are even unstable at neutrality (Magee and Barnes, 1967).

The main biological effect of nitroso compounds is their potent carcinogenicity. More than 100 substances of this type have been tested, and more than 80 of these acted as carcinogens in experimental animals (Preussmann, 1973).

The organspecificity of nitrosamines is a striking property. When nitrosamines are administered orally to experimental animals, there is a relationship between the structure of the compound and the organ attacked (Magee and Barnes, 1967). In contrast to nitrosamines, nitrosamides show both organspecificity and local cytophatic action, capable of producing tumors at the site of administration.

No correlation seems to exist between carcinogenic activity and acute toxic effects of nitrosamines (Magee and Barnes, 1967). As an example, while dimethylnitrosamine is much more toxic than diethylnitrosamine, diethylnitrosamine is the stronger carcinogen. The ability to induce tumors in progeny of animals which have been treated with nitroso compounds, is one of the most sobering facts about nitroso compounds. N-nitrosomethylurea, which is a nitrosamide, was given to rats on the 15th day of pregnancy and produced nervous system tumors in the mothers (Ivankovic and Druckrey, 1968). It was further

observed that nervous system tumors were produced in the offspring. The dosages were well below that which showed a toxic effect on the mother. Nitrosamines appear to be less able to induce transplacental carcinogenesis.

The dose-response relationship has been examined for some nitrosamines. Druckrey et al. (1967), working with diethylnitrosamine, found that the relationship between the medium induction time (t) and the daily dosage (d), followed the formula, $d \cdot t^n = \text{constant}$. The value of n for diethylnitrosamines was 2.3, which indicated that the carcinogenic action is an accelerated process ($n > 1$). When rats were fed a fixed total amount of diethylnitrosamine, the carcinogenic effect of many small doses was greater than that of a few larger doses. This phenomenon was explained by the assumption that the cell could better "utilize" small doses of carcinogen in exerting the effect on the target molecules.

Occurrence of Nitrosamines in Foods

The potential sources of nitroso compounds in the environment are many. Nitrosamines can be formed in the food prior to ingestion. (Lijinsky and Epstein, 1970; Wolff and Wasserman, 1972). Claims have been made that nitroso compounds can be formed in the body, particularly in the stomach, from the intake of appropriate precursors, (Sander, 1967; Sander and Burkle, 1969). Other possible sources for

nitrosamines are tobacco smoke or polluted air. In the following, focus will be on the formation of nitrosamines in food prior to ingestion.

The possibility that nitrosamines might be formed in food for humans was raised in the early 1960's, after dimethylnitrosamine was reported to occur in nitrite-treated herring meal (Ender et al., 1964; Sakshaug et al., 1965) which had caused a liver disease when fed to farm animals. Many meat and fish products for human consumption are preserved with nitrite, and programs of analysis for nitrosamines in food were launched in several countries. From the mid-1960's to approximately 1970 there were a number of claims for the occurrence of N-nitrosamines in food (Sebranek and Cassens, 1973). In reviewing these claims (Marquardt and Hedler, 1966; Devik, 1967; Freimuth and Gläser, 1970), it is now recognized that many of the methods used, such as spectrophotometry, polarography or thin layer chromatography, lack the specificity necessary for an unambiguous identification. Analytical methods for nitrosamines have greatly improved, and it is recognized today that gas chromatography and mass spectrometry (gc-ms) offer a reliable means for positive identification of individual nitrosamines (Crosby et al., 1972). More recent reports, many of which used gc-ms, have confirmed the presence of dimethylnitrosamine, diethylnitrosamine and nitrosopyrrolidine in extremely low concentrations (in the ppb range) in various foods

including bacon, sausages, fish, and cheese (Crosby et al., 1972). In smoke-processed sable, salmon and shad, Fazio et al. (1972a) reported 4-46 ppb dimethylnitrosamine. Sen (1972) reported evidence for 10-80 ppb of dimethylnitrosamine in fermented sausages. Fazio et al. (1971b) found 5 ppb dimethylnitrosamine in nitrite-processed ham. When analyzing frankfurters from eight large producers, 11-48 ppb dimethylnitrosamine was found in 3 out of 34 samples (Wasserman et al., 1972). No correlation was found between nitrite and nitrosamine contents in the frankfurters.

The findings of nitrosamines in some meat products such as frankfurters and hams have been sporadic, and might indicate that there are localized areas in a product where the conditions are favorable to nitrosamine formation (Sebranek and Cassens, 1973). In certain meat products the occurrence of nitrosamines has been more consistent. Analysis of mettwurst sausages in Canada showed nitrosopyrrolidine and nitrosopiperidine, up to 105 and 60 ppb, respectively, in most of the samples analyzed (Sen et al., 1973a). The source of the nitrosamines in this case was found to be a meat curing mixture, which contained sodium nitrite and spices. The carcinogens had apparently been formed in the premix through the reaction between sodium nitrite and amines in black pepper and paprika. Recently several investigators have consistently found nitrosopyrrolidine in fried bacon in levels up to 108 ppb (Fazio et al., 1973; Sen et al.,

1973b; Crosby et al., 1972; Pensabene et al., 1974). No nitrosamines were found in raw bacon.

Several studies have been conducted to identify the possible precursors of nitrosopyrrolidine in bacon (Bills et al., 1973; Huxel, 1973). In a system designed to simulate the frying of fatty foods, nitrosopyrrolidine was produced from N-nitrosoproline, pyrrolidine, spermidine, proline, and putrescine in yields of 2.6, 1.0, 1.0, 0.4, and 0.04% of theoretical yield, respectively. Nitrosopyrrolidine was not formed from glutamine, glutamic acid or hydroxyproline. In a dry system at high temperature, nitrosopyrrolidine was formed from proline, dipeptides containing proline, and collagen.

The levels of nitrosamines found so far in foods are extremely low. The hazard they might constitute to man is presently very difficult to evaluate since dose-response relationships and pharmacological data for experimental animals at these low levels have not been documented (Sebranek and Cassens, 1973). Extension of dose-response studies with nitrosamines and other carcinogens to lower dose-levels requires the use of increasingly large numbers of animals and poses formidable problems.

Precursors of Nitrosamines

Nitrite and Nitrate

The principal source of nitrite in the human diet is processed (cured) meat or fish (Wolff and Wasserman, 1972). Preservation of these products with nitrite is considered necessary to prevent the growth of Clostridium botulinum, which produces a dangerous toxin under suitable conditions. It has been reported that more than 100 ppm nitrite in the product is necessary to insure this protection under commercial conditions (Ingram, 1973). In addition to the preservation effect, some of the added nitrite is converted into nitrosomyoglobin to give the characteristic pink color of cured meat, and is essential for the development of the characteristic flavor of cured meat (Cho and Bratzler, 1970; Wasserman and Talley, 1972; Simon et al., 1973). Smaller amounts than 100 ppm of nitrite are necessary to achieve the latter two effects (Ingram, 1973). Government regulations allow cured food to contain 500 ppm nitrate and 200 ppm nitrite in the finished product (Code of Federal Regulations). A significant source of nitrite, when considering nitrosation in vivo, is the presence of nitrite (1-10 ppm) in human saliva (Tannenbaum, 1972). This nitrite is presumably formed from nitrate by bacterial reduction in the mouth.

The largest sources of nitrate in our food are vegetables, water supplies, and nitrate used as an additive in the curing of meat. Nitrate

is present in large quantities in spinach, beets, celery, and lettuce (Wolff and Wasserman, 1972). In the storing of raw spinach, bacterial reduction of substantial amounts of nitrate to nitrite occurred (Phillips, 1968).

Nitrite is more toxic than nitrate, interacting with blood pigments to produce methemoglobinemia. The intake required for serious toxic effects is usually large. However, accidental addition of excessive amounts of nitrite to food has lead to instances of poisoning of both adults and children (Wolff and Wasserman, 1972).

Recent epidemiological studies have indicated a relationship between gastric cancer death rates in Chilean farmers and their exposure to nitrate used as fertilizer (Zaldivar and Robinson, 1973). In Mexico, Boom (1969) found a high proportion of farmers among male patients with stomach carcinoma.

Nitrite and nitrate have been controversial food additives in recent years. It might appear desirable to reduce the use of these food additives where possible. Opponents of such restrictions point to the value claimed for nitrite in controlling the botulism hazard. By removing nitrite one hazard might be replaced by another. Intensive research is presently under way to seek solutions to these problems.

Polyamines

The polyamines, spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, and spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, are widely distributed in natural materials, including bacteria, plants, and animal tissues (Cohen, 1971; Smith, 1971). Although spermine has been known for nearly 300 years (Lewenhoeck, 1678), little research has been directed towards the polyamines until recent years. The renewed interest in these compounds is primarily due to the fact that they may have a wide range of biological significance not recognized earlier. Polyamines have been implicated as bacterial growth factors (Herbst and Snell, 1949), and have been reported to have a stabilizing effect in vitro on various cellular and subcellular components including nucleic acids (Tabor and Tabor, 1964). In addition, the polyamines might play a role in the regulation of cellular RNA synthesis (Bachrach, 1970; Tabor and Tabor, 1972; Smith, 1972). Many of the unique properties of spermidine and spermine are due to their polybasic nature. They have a high affinity for cellular polyamines, and have pronounced effects even at low concentrations.

The increased polyamine concentrations in the urine of cancer patients recently reported deserve attention, since this might provide a diagnostic tool to evaluate tumor activity in humans (Russell, 1971; Russell, 1973; Gehrke et al., 1974).

Since spermidine and spermine have secondary amine functions, they could conceivably be precursors in the formation of nitrosamines in foods. In Table 1 the contents of polyamines in various foods are given, as reported in the literature. Significant amounts of spermidine and spermine were found in germs of higher plants such as barley, oats, and wheat (Moruzzi and Caldarera, 1964). Under potassium-deficient conditions, large increases in spermidine and spermine were observed in higher plants (Smith, 1970).

Of prime interest are the high levels of spermidine and spermine found in samples of pork and pork products (Lakritz et al., 1973). However, large variations were found from sample to sample. This was not unexpected, since the meat was purchased from retail consumer stores, and the history of the individual sample was not known. Significant increases in spermine and spermidine occurred during putrefaction. No appreciable changes in the amounts of amines were detected in the smoked and cooked hams, as compared to fresh pork.

Analysis of Nitrosamines

The total analytical procedure for determining nitrosamines in food usually involves an extraction step, followed by distillation, partitioning with solvents, a clean-up step with columns or thin layer chromatography, and finally separation, detection, and confirmation (Sebranek and Cassens, 1973).

Table 1. Contents of spermidine and spermine in various foods.

Food	Spermidine	Spermine	References
Barley (germ)	29.1[a]	12.8	Moruzzi and Caldarera (1964)
Rice (germ)	15.3	14.1	"
Oats (germ)	30.7	10.9	"
Corn (germ)	12.4	9.0	"
Wheat (germ)	25.4	4.1	"
Sorghum (germ)	8.3	2.1	"
Soybean (flour)	1.64	0.57	Wang (1972)
Apple (fruit)	0.99	0.22	Smith (1970)
Spinach (leaves)	3.45	0.77	"
Pork (fresh)	13.4-125.0	1.2-55.7	Lakritz et al. (1973)
Pork (cooked)	9.7-70.0	2.2- 9.7	"
Pork (putrified)	20.1-1013.0	5.4-2769.0	"
Ham (smoked and cured)	15.1-127.3	0.6-79.6	"

[a]mg amine/100 g of wet tissue

The most satisfactory procedure for the latter steps include separation by gas chromatography followed by detection and confirmation by mass spectrometry (Pensabene et al., 1972; Heyns and Koch, 1971; Fazio et al., 1971a; Telling et al., 1971). Recently, the use of specific detectors for nitrogen compounds have increased the selectivity for nitrosamines at the gas liquid chromatographic step (Palframan et al., 1973; Adams and Pigliucci, 1974; Riedman, 1974).

To accommodate the analysis of nonvolatile nitrosamines, separation has been achieved by high pressure, liquid chromatography (Cox, 1973), but specific detection methods have not been available. The recent development of the thermo luminous detector which, apparently, is specific for nitrosamines, might solve that problem (Fine et al., 1974).

Nitrosation of Amines

Nitrosation of Primary and Secondary Amines

Several reviews have dealt with the mechanism of nitrosation of amines in aqueous solution (Challis and Butler, 1968; Ingold, 1969; Ridd, 1961). The reaction between amines and nitrous acid follows the common reaction path,

$$R \cdot NH_2 \rightarrow RNHNO \rightarrow RNNOH \rightarrow RN_2^+ \rightarrow R^+ \rightarrow \text{Deamination products}$$

With secondary amines, the reaction stops at the nitrosamine stage. With primary aromatic amines, the reaction stops at the diazonium ion stage, while with primary aliphatic amines the diazonium ion is unstable, and converts readily to a carbonium ion, which reacts with the available reactants in the system to form a multitude of products. For the nitrosation or deamination of all three groups of amines the reaction sequence starts with a N-nitrosation, which usually is the rate-determining step (Ridd, 1961).

In aqueous solution, several nitrous species, many of which are effective nitrosating agents, exist in equilibrium with molecular nitrous acid. Molecular nitrous acid does not react directly with amines. Listed in order of increasing activity, some of the covalent nitrosating agents formed in aqueous solution are (Challis and Butler, 1968):

$ON - NO_2$	nitrous anhydride
ON - Hal	nitrosyl halide
$ON - OH_2^+$	nitrous acidium ion
ON^+	nitrosonium ion

The nitrosation reaction can occur simultaneously via more than one nitrosation reagent.

The nitrous anhydride mechanism is important both in deamination and in nitrosamine formation. Under acidic conditions the nitrosating species nitrous anhydride is formed in a rapid pre-equilibrium

step, and subsequently slowly attacks the unprotonated amine.

$$2\ HNO_2 \xrightarrow{fast} N_2O_3 + H_2O$$

$$RNH_2 + N_2O_3 \xrightarrow{slow} R\overset{+}{N}H_2NO + NO_2^-$$

$$R\overset{+}{N}H_2NO \xrightarrow{fast} RN_2^+ \xrightarrow{fast} \text{Deamination products.}$$

The deamination of methylamine has been shown to give third order kinetics under acidic conditions (Taylor, 1928):

$$\text{Rate} = k[R \cdot NH_2] \cdot [HNO_2]^2$$

However, on decreasing the acidity, the reaction rate progressively became independent of the amine concentration, and eventually followed the second-order rate equation given below (Hughes et al., 1958):

$$\text{Rate} = k[HNO_2]^2$$

Under the latter conditions the formation of nitrous anhydride is rate controlling because the increased concentration of nucleophilic free amine at lower acidities is sufficient to react with the nitrous anhydride as soon as it forms.

In the presence of halide ions, the nitrosyl halide mechanism becomes important. Nitrosation by nitrosyl chloride is the predominant mechanism for the diazotisation of aniline in concentrations of hydrochloric acid exceeding about 0.1 M (Schmid and Muhr, 1937),

but the mechanism is also important in the nitrosation of secondary amines. The rate increases rapidly with acidicity since the concentration of nitrosyl chloride is proportional to the square of the hydrochloric acid concentration (Challis and Butler, 1968). In a mode similar to the nitrous anhydride mechanism, the nitrosyl halide mechanism involves a rapid pre-equilibrium step where nitrosyl halide is formed. This species further reacts with unprotonated amine. The effect of sodium chloride on the nitrosation of amines will be discussed specifically in a later section.

The nitrous acidium ion and the nitrosonium ion mechanisms, which are important in strongly acidic solutions in the absence of halides, are of less importance in the present context, as food systems rarely or never reach a pH of less than 1.0.

Various studies have been made on systems containing nitrite and amines to evaluate the potential of nitrosamine formation under various reaction conditions. Most nitrosamines have a pH optimum of formation between pH 2 and 4, depending on the basicity of the amine (Mirvish, 1970; Fan and Tannenbaum, 1973). Ender and Ceh (1971) investigated the nitrosation of amines at low temperatures and long reaction times. They found that significant nitrosation could even occur at -18°C.

Inhibitors and accelerators of the nitrosation reaction have been studied. Several anions accelerate the nitrosation reaction,

particularly thiocyanate (Boyland, 1972). The presence of thiocyanate has been established in human saliva, which might be significant in the in vivo formation of nitrosamines. Formaldehyde has been found to increase the rate of nitrosation of amines (Keefer and Roller, 1973), particularly under neutral or basic conditions. Mirvish et al. (1972) recently found that ascorbate inhibited the reaction between amines and nitrite. The possibility of blocking nitrosamine formation in food by adding ascorbate is presently being investigated.

Products in the Nitrous Acid Deamination

The reaction of aliphatic primary amines with nitrous acid has long been known to yield a variety of products including those resulting from solvolysis, elimination, and rearrangement.

The deamination products of methylamine by nitrous acid were identified by Austin (1950). He found methyl nitrite (35-45%) to be the main product, followed by methylalcohol (6-25%). Appreciable quantities of nitromethane (6%), methylnitrolic acid (10-12%), and methyl chloride (13%) were also formed. The chlorinated derivative was formed because methylamine hydrochloride was used as a reactant.

In the deamination of higher primary aliphatic amines by nitrous acid, a wider range of reactions are open for the carbonium ion. Substitutions, eliminations or rearrangements at the carbonium ion can lead to a multitude of different products. The stereochemistry

of the direct substitution shows that the reaction usually involves racemisation, accompanied by some inversion (Brewster et al., 1950). This indicates the intermediate formation of a carbonium ion, which reacts rapidly with a nucleophile, while one side of the carbonium ion is still partly shielded by the departing nitrogen molecule. The amount of racemisation increases with the stability of the carbonium ion. The carbonium ion can also rearrange by the migration of hydrogen or methyl and subsequently undergo substitutions or eliminations. (Ingold, 1969).

In an early experiment in which n-propylamine was deaminated by nitrous acid, Meyer and Forster (1876) identified n-propyl alcohol, isopropyl alcohol, propene, and dipropylnitrosamine as products. Whitmore and Langlois (1932) identified the following products in the deamination of n-butylamine by nitrous acid: n-butylalcohol 25%, sec-butylalcohol 13.2%, n-butenes 36.5%, n-butylchloride, 5.2%, and sec-butylchloride 2.8%. Adamson and Kenner (1934) showed that on increasing the number of carbon atoms in the amine, the amount of secondary alcohol decreased rapidly. Also, under comparable conditions, ethylamine gave 60% ethyl alcohol; n-propylamine gave 7% propylalcohol, 32% isopropylalcohol, and 28% propylene; n-butylamine gave 25% n-butylalcohol, 13% sec-butylalcohol, 8% n- and sec-butyl chlorides, and 36% 1- and 2-butenes (Noller, 1965).

Nitrosation of Spermine and Spermidine

The information available on the nitrosation reaction of spermidine and spermine is scarce. Dudley, Rosenheim and Starling (1926), working on the chemical constitution of spermine, used nitrosation as a means of characterizing the polyamine. As products they found a volatile base with the suggested structure N-γ-aminopropylpyrroline, but also a substance giving a nitroso reaction. However, the latter substance or substances were not identified. Van Slyke determinations gave inconclusive results. Varying proportions of gas, measured as nitrogen, were evolved depending on the length of reaction time.

The relative reactivity of the primary and secondary amine groups in spermidine was recently studied by Ferguson et al. (1973). The total amount of nitroso compounds formed was estimated by measuring the nitrite released on ultraviolet irradiation of the sample (Fan and Tannenbaum, 1971). The reaction was observed for 17 hrs in 1 M citrate buffer, pH 3.4, at 25°C. At a nitrite to spermidine ratio of 1:1, nitrosamine formation was relatively rapid. By increasing the nitrite to spermidine ratio to 6:1, the amount of nitroso compounds formed was doubled. No structural identification of the nitrosamines formed was carried out.

The multitude of products to be expected from the nitrosation of a primary amine makes a study of the reaction between nitrous acid

and spermine or spermidine a complex problem. The spermidine contains two primary and one secondary amine groups, while spermine contains two amine groups of each type. Substitution, elimination, and rearrangement reactions can take place at each end of the molecule in the presence of nitrous acid. Some of the products formed may be labile in the presence of a surplus of nitrite. For example, hydroxylated or chlorinated compounds may conceivably be converted to new compounds in secondary reactions. In addition to this, cyclizations and polymerizations might take place, and further increase the variety of products.

Ferguson et al. (1973) made an estimation of the number of possible nitrosamines that can form in these reactions. Assuming that seven different reactions can take place at each of the primary amine groups (hydroxylation, chlorination, nitration and elimination), and that nitrosation occurs at the secondary amine group, 49 different nitrosamines could be formed from spermidine. If there was a limited amount of nitrite, some of these compounds may contain nitrosamine groups and others not, expanding the number of possible products to 98. Where a similar estimation was made with spermine, the number of theoretically possible products was 105. Possible products from cyclization and polymerization reactions were not included in these estimates.

Effect of Sodium Chloride on the Nitrosation of Amines

It has been demonstrated that several anions exert an accelerating effect on the nitrosation of amines in acidic media (Ridd, 1961; Boyland et al., 1971; Boyland, 1972). The order of the accelerating effect was $I^- > SCN^- >$ Acetate $> Br^- > Cl^-$ with the chloride ion exhibiting a very slight catalytic effect at pH 2.0 (Boyland, 1971). The effect of anions appears to result from the formation of covalent nitrosyl compounds, which become the main nitrosating species. In agreement with these findings, Fan and Tannenbaum (1973) demonstrated that chloride and bromide ions (0.1-0.3 M) promoted the formation of nitrosomorpholine at pH 0.5.

In some reports, however, an inhibitory rather than a catalytic effect of chloride was observed. Taylor and Price (1929) observed that 0.05 N potassium chloride had an inhibitory effect on the nitrosation of dimethylamine, but it was not clear at what pH the nitrosation reaction took place. Mirvish et al. (1973) investigated the effect of 0.05, 0.15, and 0.50 M sodium chloride on the nitrosation of sarcosine at pH 1.5, 2.5, and 3.0. The yield of nitrososarcosine was reduced by 34% at pH 2.5 and by 55% at pH 3.5, which was attributed by the authors to activity effects. The presence of 0.05 M sodium chloride in the reacting medium reduced the nitrosation rate of dimethylamine in phthalate buffer at pH 3.4 by 9% (Mirvish, 1970).

Fiddler et al. (1973) found no effect of sodium chloride (0.4 M) on the formation of dimethylnitrosamine in a pH 5.6 buffer solution (0.5 M KH_2PO_4 and NaOH).

EXPERIMENTAL PROCEDURES

Identification of Nitrosamines Formed

Reaction and Clean-up Procedure

Five mmol of spermidine· 3HCl and spermine· 4HCl were each dissolved in 20 ml 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°C under a reflux condenser in a round bottom flask on a waterbath. Ninety mmol sodium nitrite (Mallinckrodt Chemical Works) in 20 ml distilled water, adjusted to pH 3.5 with 2 N H_2SO_4, were 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 ml of internal standard solution (2 mg methyl myristate/ml) was added, and the solution was saturated with anhydrous sodium sulfate (Mallinckrodt Chemical Works) and extracted with redistilled dichloromethane (3 x 50 ml) (Mallinckrodt Chemical Works). The combined extracts were dried overnight at 4°C over sodium sulfate, and concentrated to 4 ml in a Kuderna-Danish apparatus (Kontes). Two boiling chips (Boileezers,

Fisher Chemical Co.) were added to the concentrator tube. Further concentration down to 1 ml was achieved on a micro Kuderna-Danish apparatus under a stream of nitrogen at 20°C.

In several instances the dichloromethane concentrate was cleaned up by column chromatography as described by Fazio et al. (1971b). A 1 ml sample was applied on a Silica gel column (E. Merck) containing 5 g of the absorbent, which had been prewashed with 50 ml pentane. The column was washed with 200 ml of 25% dichloromethane in pentane, which was discarded. The nitrosamines were eluted by passing 100 ml of 30% anhydrous ethyl ether (Mallinckrodt Chemical Works) in dichloromethane through the column. The eluate was concentrated as described above.

Gas Chromatographic Analysis

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. x 500 ft.) coated with 8% Carbowax 20 M and 1% Versamid 900 according to Mon (1971) was used. Temperatures of injector, column, and detector were 210°C, 170°C, and 270°C, respectively, and the flow rate of carrier gas (N_2) was 11.5 ml/min.

For the separation of the trifluoroacetate derivatives, a stainless steel packed column (0.062 in. o.d. x 20 ft.) coated with 5% OV

17 on 100/120 Varaport #30 was used. Temperatures of injector, column and detector were 218°C, 150°C, and 270°C, respectively, and the flow rate of carrier gas (N_2) was 6.3 ml/min.

The amine precursors were analyzed on a stainless steel column (0.13 in. o.d. x 1 ft.) 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°C, 160°C, and 270°C, respectively. The carrier gas flow rate (N_2) was 30 ml/min.

Trapping Techniques

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. x 10 ft.) packed with 5% Carbowax 20 M 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 co-condensation with spectrograde carbon disulfide (Matheson, Coleman and Bell) as solvent.

The apparatus consisted of two pear-shaped flasks of 10 ml capacity to which had been fused short 3.5 mm o.d. side arms. These were connected to a three-way valve (Type 3 MM, Hamilton Co.) by means of short teflon tubes. The third inlet of the valve was connected

to the exit port of the gc. A micro spiral condenser was placed above each flask to provide efficient reflux. Steel springs were used to tighten the system. The valve was heated to 150°C to prevent premature condensation.

In operation, 1.0 ml carbon disulfide was added to each flask and brought to reflux on a moderately hot sand bath. As undesired peaks passed through the exit port, they were collected in one of the flasks. When the component of interest eluted, the valve was switched, and the effluent collected in the other flask.

After repetitive collection of the peak of interest, the compound trapped in carbon disulfide was concentrated down to 0.05 ml, and repurified by a second trapping from the same column. Glass tubes (0.05 in. o.d. x 1 ft.), cooled in dry ice, were used to collect the material.

Quantitative Analysis by Gas Chromatography

In gc, quantitative measurements of the components in a sample can be made by estimating the peak areas in the chromatogram. The estimated peak areas can be converted into mass units by comparison with the peak area of a known amount of an internal standard. Peak areas were estimated by multiplying the peak height by retention time, or by the use of a digital integrator (Hewlett Packard model 3373B). To establish a response factor, f, γ-butenyl (β-propenyl)nitrosamine

(BPN) was trapped from the column, weighed, added to a known amount of methylmyristate in acetone, and analyzed by gc. The response factor was calculated from the following formula:

$$\frac{\text{peak area of nitrosamine}}{\text{peak area of methylmyristate}} = f \cdot \frac{\text{mass nitrosamine}}{\text{mass methylmyristate}}$$

The response factor for BPN was also used for the hydroxylated and chlorinated derivatives of BPN. In quantifying nitrosopyrrolidine, authentic nitrosopyrrolidine (Aldrich Chemical Co.) was used in determining the response factor.

The recoveries of the nitrosamines involved were estimated in the following way. A dichloromethane extract of the products from the nitrosation of spermidine was concentrated almost to dryness, dissolved in acetone, concentrated once more to near dryness, and diluted to a fixed volume with acetone. An aliquot of this solution was added to 15 ml of 0.2 M acetate buffer, pH 4.0, and subjected to the clean-up procedure outlined later (p. 32). The recoveries of the nitrosamines of interest were calculated from the losses of material during this clean-up procedure.

Trifluoroacetate Derivatives of Hydroxylated Nitrosamines

To facilitate separation of isomers of nitrosamines containing hydroxy groups, trifluoroacetate derivatives of these compounds were

made. Three mg of hydroxylated nitrosamines were trapped as outlined above, dissolved in 0.1 ml pyridine, and reacted with 0.1 ml N-methyl-bis-trifluoroacetamide (Pierce Chemical Co.) at 75°C for 1 hr. The trifluoroacetate derivatives were analyzed by gc-ms using the OV 17 column.

Spectrometric Analyses

A Finnigan Model 1015 C tandem gas chromatograph quadrapole mass spectrometer (gc-ms) system, which included a Varian Aerograph (series 1400) gc, was used with the Carbowax 20 M capillary column to separate and obtain mass spectra of the 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, 300μ A; electron voltage 70eV, analyzer pressure 5×10^{-7} torr, and multiplier voltage 1.50 KV. Spectra were scanned from m/e 12 to m/e 270 in one second. The ir spectra were obtained neat between two micro sodium chloride discs using a Beckman Model IR-18A infrared spectrophotometer equipped with a 5X beam condenser. The nmr spectra were determined at 100 MC on a Varian model HA-100 spectrometer. Tetramethylsilane was used as an 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 oilbath at 53°C. Twenty-six grams 4-Br-1-butene (Aldrich Chemical Co.) was added dropwise through the condenser, and the reaction mixture heated under reflux for 30 min. Fifteen grams BaO (Baker Chemical Co.) was used as a drying agent, and the mixture was subjected to a fractional distillation. The fractions were analyzed by gc, the fractions containing BPA combined, dried over two grams of BaO, and subjected to further purification by distilling over a 20 cm Vigreaux column (Kontes). The yield of BPA was 73%, and its boiling point was 135-138°C. The purity of BPA was 98%, and the identity was confirmed by gc-ms.

Synthesis of γ-butenyl (β-propenyl) Nitrosamine (BPN)

A method by Dutton and Heath (1956) was modified for the synthesis of BPN. Ten mmol of BPA was neutralized with 10 ml 1.0 N hydrochloric acid, cooled in ice, and 2.5 ml of glacial acetic acid was added. Twenty mmol of sodium nitrite in 5 ml cold, distilled water was added dropwise to the amine solution while stirring in the cold. After reacting for one hour in ice while stirring, the mixture was heated for one hour under a condenser at 63°C. After cooling, the

solution was saturated with sodium sulfate, and the yellow oil extracted with redistilled dichloromethane (3 x 20 ml). The extract was dried over sodium sulfate, and concentrated down to 6 ml on a Kuderna Danish concentrator. The nitrosamine formed was identified by gc-ms. The compound was trapped, and the ir and nmr spectra obtained.

Thin Layer Chromatography

Thin layer chromatography (tlc) of the concentrated dichloromethane extracts was performed on 0.25 mm thick, precoated Silica gel G plates 10 x 20 cm (Macherey, Nagel & Co.). Five μl of the dichloromethane extracts were spotted and the plate developed for 20 min with hexane:diethyl ether:dichloromethane (4:3:2 v/v/v) as the mobile phase. Five μg each of dipropylnitrosamine, dibutylnitrosamine and nitrosopyrrolidine were used as standards. The developed plate was sprayed generously with the Griess reagent, modified by Fan and Tannenbaum (1971). The plate was immediately exposed to ultraviolet light for 10 min. (Black-Raymaster, Geo. W. Gates & Co.). Nitrosamine spots appeared as red-purple spots after irradiation.

Influence of Selected Parameters on the Nitrosation of Spermidine

General Remarks

In all experiments in the following section, unless otherwise stated, 1 mmol of spermidine base was reacted with 9 mmol of sodium nitrite at 50°C in 0.2 M acetate buffer, pH 4.0, for 1 hr, in the presence of 0.33 M sodium chloride. The experiment where pH was the variable will be outlined in detail. All samples were run in duplicate. The identity of the nitrosamines was confirmed by mass spectrometry of selected samples. Quantification of the nitrosamines was carried out as outlined in the previous section.

Effect of pH

Five ml of an aqueous solution of 0.2 M spermidine (Sigma Chemical Co.) was pipetted into a 250 ml Erlenmeyer flask with screw cap (Kimax), followed by 5 ml 0.6 M acetate buffer (pH 3, 3.5, 4, 4.5, 5 or 6).included sufficient 2N H_2SO_4 to attain the desired pH after the addition of sodium nitrite. Five ml of a solution being 1.8 M in sodium nitrite and 1.0 M in sodium chloride was added, the flask capped, shaken,and placed in a shaking waterbath (Research Specialties Co.) at 50°C. After a reaction period of 1 hr the flask was cooled in ice for 20 min., pH of the solution was recorded, and the reaction immediately stopped by adding 7.5 ml 4 M ammonium sulfamate (Matheson,

Coleman & Bell) in 0.5 N aqueous H_2SO_4. One ml of internal standard solution (2.00 mg methylmyristate/ml acetone) was added, the solution saturated with anhydrous sodium sulfate, and extracted with redistilled dichloromethane (2 x 30 ml). The combined extracts were dried over sodium sulfate overnight, and concentrated down to 0.2 ml using the Kuderna Danish apparatus. The concentrated samples were analyzed using the stainless steel capillary column (0.03 in. i.d. x 500 ft.) coated with 8% Carbowax 20 M and 1% Versamid 900.

Effect of Other Variables

The effect of time on the nitrosation reaction of spermidine was followed at pH 3.5 and pH 5.0 at 50°C. Sampling was done after 1.0, 5.0 and 25.0 hours. The effect of temperature on the nitrosation of spermidine was studied by observing the yields of nitrosamines at 24°C, 37°C and 48.5°C at pH 5.0 after 1 hr. The effect of sodium chloride concentration in the reacting medium was studied at 50°C and pH 4.0 at salt concentrations of 0, 0.1, 0.5, 1.0, 1.5 and 2.0 M. The reaction time was 1 hr, and the acetate buffer concentration was reduced to 0.1 M. The effect of sodium chloride on the nitrosation rate in a buffer-free system also was examined.

Effect of Sodium Chloride Concentration on the Nitrosation of Proline at Different pH Levels

The rate of nitrosation of proline was investigated at pH 0.5, 2.5, 4.0, and 5.5, in the presence of 0 to 1.0 M sodium chloride. Triplicate runs were done on each sample. Due to the instability of sodium nitrite under acid conditions, care was taken to avoid lowering the pH of nitrite solutions until the time of reaction. Stock solutions of L(-) proline (Eastman Chemical Co.) were adjusted with 0.1 N H_2SO_4 to attain the desired pH after the addition of sodium nitrite. Stock solutions of sodium nitrite were prepared in distilled water (pH approximately 5.4). Standard nitrosoproline was prepared and purified by the method of Lijinsky et al. (1970).

Before the start of the reaction the reactants were equilibrated separately at 25°C. Five ml of the proline solution was mixed with 5 ml of sodium chloride solution in a 50 ml reagent tube with screw cap (Kimax). Five ml sodium nitrite solution was added, the tube shaken, and the recorder on the spectrophotometer started (Beckman DB-G Grating Spectrophotometer). Two ml of the reacting solution was immediately transferred to a quartz cell, and the absorption recorded for 30 min. at 260 nm. At pH 5.5 the reaction was very slow, and was followed for four days, measuring the absorption at 24 hr intervals. The temperature in the sample compartment of the spectrophotometer was maintained at 25°C throughout the reaction

period. Sodium nitrite solution was replaced with distilled water in the blank. A new blank was made up for each sample, and was treated as the sample.

The direct spectrophotometric procedure as described above was tested against a similar assay by Mirvish et al. (1973) for nitrosoproline, in which the reaction was stopped before measuring the absorption. The latter method was used at pH 2.5 at all salt concentrations. The reaction was quenched at 5 min. intervals by adding 5 ml 200 mM ammonium sulfamate in 2N H_2SO_4. After a 5 min equilibration period the absorption of the sample was measured at 260 nm. For the zero time samples, ammonium sulfamate was added before sodium nitrite. These samples were run in duplicate.

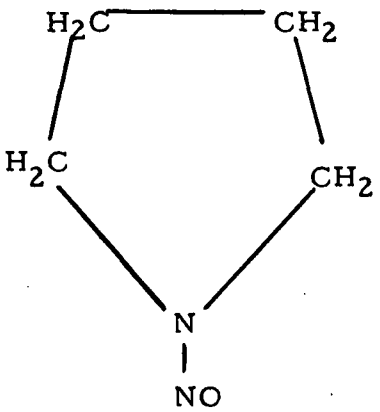
The rate of nitrosation of proline was determined from initial rates, since nitrite in open vessels is unstable upon prolonged incubation (Fan and Tannenbaum, 1973). The rate was estimated from the slope of the initial part of the recorded curve. The molar absorptivity at 260 nm for nitrosoproline was found to be 1985 at pH 0.5; 2150, pH 2.5; 2865, pH 4.0; and 2820, pH 5.5. Absorption at 260 nm instead of 238 nm was used to reduce the interference by nitrite (Mirvish et al., 1973). The stability of nitrosoproline to ultraviolet light in the spectrophotometer was checked at pH 0.5 and 5.5 by leaving a sample of standard nitrosoproline in a quartz cell in the sample compartment for 1 hr. No change in absorbance was observed in either case.

Purity of Amine Precursors

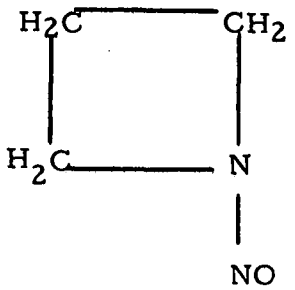
The purity of spermidine·3HCl and spermine·4HCl (Nutritional Biochemicals Corp.) was tested by thin layer chromatography on cellulose using methylcellusolve:propionic acid:water (70:15:15 v/v/v), saturated with sodium chloride, as a developing solvent, and ninhydrin reagent to develop the plates. The purity of the free base spermidine (Sigma Chemical Co.) and spermine (Nutritional Biochemicals Corp.), was tested by gas chromatography.

Safety Precautions

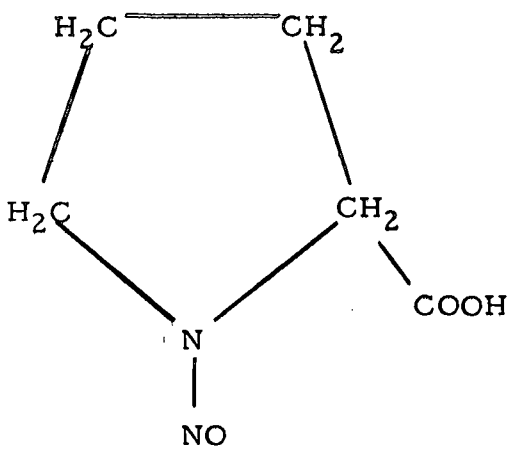
As most nitrosamines are potent carcinogens in test animals, precautions were taken in the handling of samples containing nitrosamines, to prevent inhalation and skin exposure. All handling of nitrosamines was done under fume hoods. All work surfaces were covered with absorbent paper with a plastic undercoat, which was replaced regularly. All handling of nitrosamines was done in containment trays. Gloves and laboratory coats were worn whenever nitrosamines were handled. In gc work with nitrosamines the outlet from the flame-ionization detector was vented to a hood. All trappings from the column were done under a hood. Glassware exposed to concentrated samples was decontaminated with 5% HBr in acetic acid before it was washed (Eisenbrand and Preussmann, 1970).



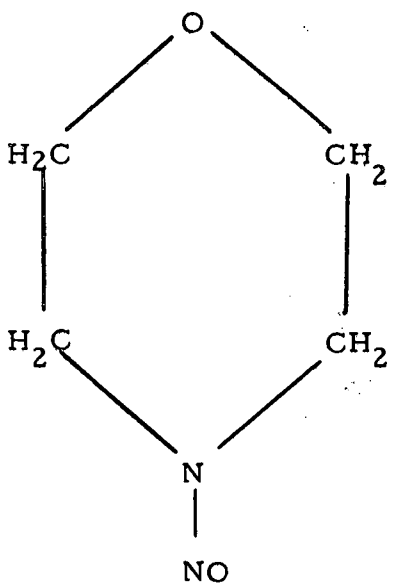
Nitrosopyrrolidine



Nitrosoazetidine

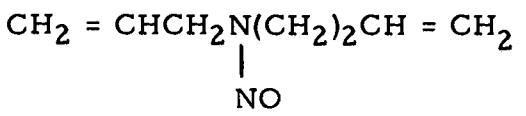


Nitrosoproline

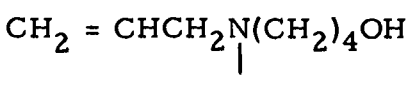


Nitrosomorpholine

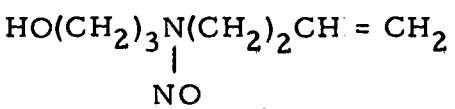
Figure 1. Structures of cyclic nitrosamines discussed in this study.



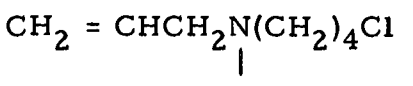
γ-butenyl(β-propenyl)nitrosamine



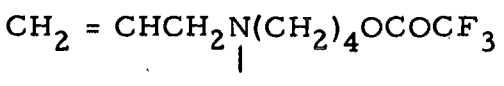
δ-butanol(β-propenyl)nitrosamine



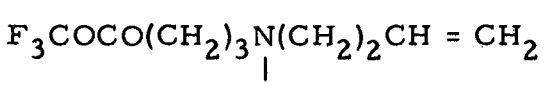
γ-butenyl(γ-propanol)nitrosamine



δ-butylchloride(β-propenyl)nitrosamine



TFA-δ-butanol(β-propenyl)nitrosamine



γ-butenyl(TFA-γ-propanol)nitrosamine

Figure 2. Structures of dialkylnitrosamines as discussed in this study.

RESULTS AND DISCUSSION

Identification of Nitrosamines Formed

Screening for Nitrosamines

A number of volatile compounds were formed by nitrosating spermidine and spermine. The tandem gas chromatograph-mass spectrometer (gc-ms) was used to screen the products 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). Thus they can serve as indicators of the presence of nitrosamines. By this technique the majority of volatile products formed in the nitrosation of spermidine were found to be compounds other than nitrosamines. Figure 3 shows the gas chromatograms of the dichloromethane extracts of the reaction mixtures from spermidine and spermine, reacted at pH 3.5 at 80°C for 1 hr. Peak 12 was the internal standard methylmyristate, and peak 9 was methylnonanate, which was an impurity in the internal standard sample.

Nitrosopyrrolidine

Peak 10 in the chromatograms (Figure 3) was nitrosopyrrolidine (NPy) (Figure 1), and has previously been identified as a nitrosation

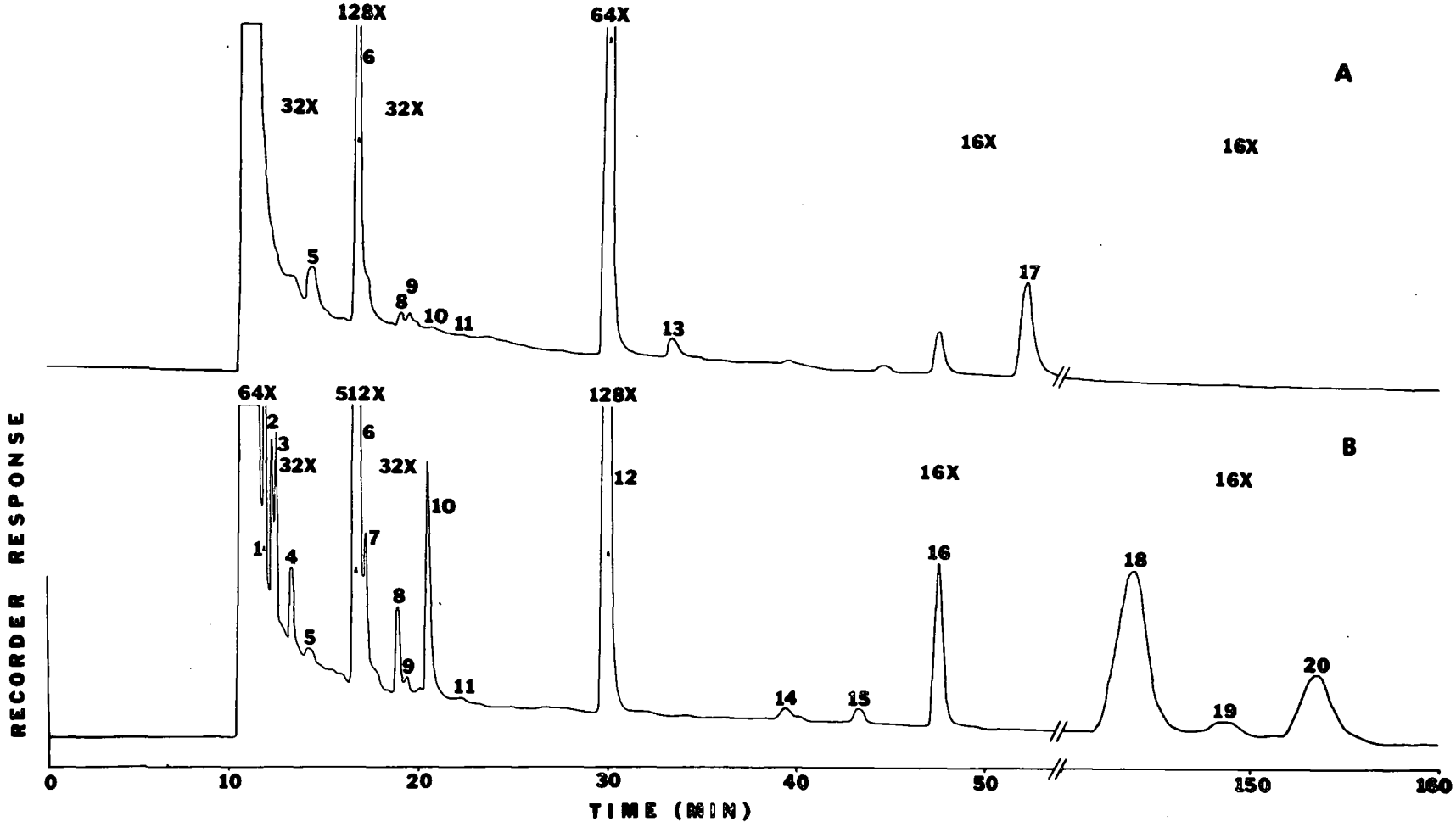


Figure 3. Gas chromatograms on 8% Carbowax 20 M and 1% Versamid 900 capillary column of the volatile products from the nitrosation of A. spermine·4HCl, B. spermidine·3HCl.

product of spermidine in an oil system at 170°C by Bills et al. (1973).

In this study NPy was formed from nitrous acid and spermidine or spermine in aqueous solutions at pH 3.5 after 1 hr at 80°C. The yields of NPy were 0.62% and 0.60% from spermidine and spermidine·3HCl, respectively, while NPy was formed from spermine·4HCl only in trace amounts (Table 3). The yields were calculated on the basis of the amount of amine precursor added. Below 80°C a sideproduct with the same retention time on gc as NPy appeared, which made quantitative assessments of NPy difficult at lower temperatures. However, the identity of NPy in the extracts of the reaction between spermidine and nitrite at lower temperatures was confirmed by gc-ms. In all instances NPy was identified by comparison of the mass spectrum of peak 10 with the spectrum obtained from authentic NPy, and by comparison of retention times on gc.

γ-butenyl-(β-propenyl)nitrosamine

Peak 6 was the largest peak in the gas chromatograms both from spermidine and spermine (Figure 3). The peak was trapped as it eluted from the column, and was found to give a red color with the Griess reagent, which indicates the presence of a nitroso group. Interpretation of the mass spectrum obtained for the compound prompted us to assign a structure consistent with γ-butenyl-(β-propenyl) nitrosamine (BPN) (Figure 2). The mass spectrum of peak 6 was

Table 2. Yields of several nitrosamines formed in the nitrosation of spermidine in preparative experiments.[a]

Nitrosamine	Percent yield[b]	
	Spermidine	Spermidine·3HCl
BPN	2.4	1.7
BPN·Cl[c]	0	0.12
BPN·OH[d]	0.47	---
N-Py	0.60	0.62

[a] 5 mM amine, 90 mM sodium nitrite, pH 3.5, reactants adjusted with H_2SO_4, 80°C, 1 hr.

[b] yields on the basis of amine precursor added, no adjustments for recovery of the nitrosamines.

[c] δ-butylchloride(β-propenyl)nitrosamines (possibly a mixture of isomers).

[d] γ-butenyl(γ-propanol)nitrosamine and δ-butanol(β-propenyl) nitrosamine.

essentially identical to the mass spectrum obtained for BPN, which was synthesized from γ-butenyl-(β-propenyl)amine (Figure 4). Peak 6 also had the same retention time as synthesized BPN on the Carbowax 20 M capillary column.

A mass spectral fragmentation scheme for BPN is shown in Figure 5. The molecular weight of BPN is 140, and although the parent ion (P.) m/e 140 was rather small, it was clearly present in the spectrum. The fragmentation was consistent with the scheme proposed by Saxby (1972) for the fragmentation of dialkyl nitrosamines. Loss of hydroxyl radical produced the m/e 123 ion. Fragmentation at the α-carbon of the longest alkyl chain produced an ion at m/e 99 which further lost 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, produced the m/e 41 ion which was the base peak in the spectrum.

The P+1/P mass ratio was in the range 35-100% for BPN, which was significantly higher than that calculated from the abundances of isotopes of the atoms in $C_7H_{12}N_2O$ (16%) by Beynon (1963). Presumably, this was due to a relatively high inlet pressure in the instrument, which caused a contribution to the P+1 peak from a bimolecular addition of hydrogen to the parent ion. This is a common occurrence with molecules containing a hetero atom (Silverstein and Bassler, 1964).

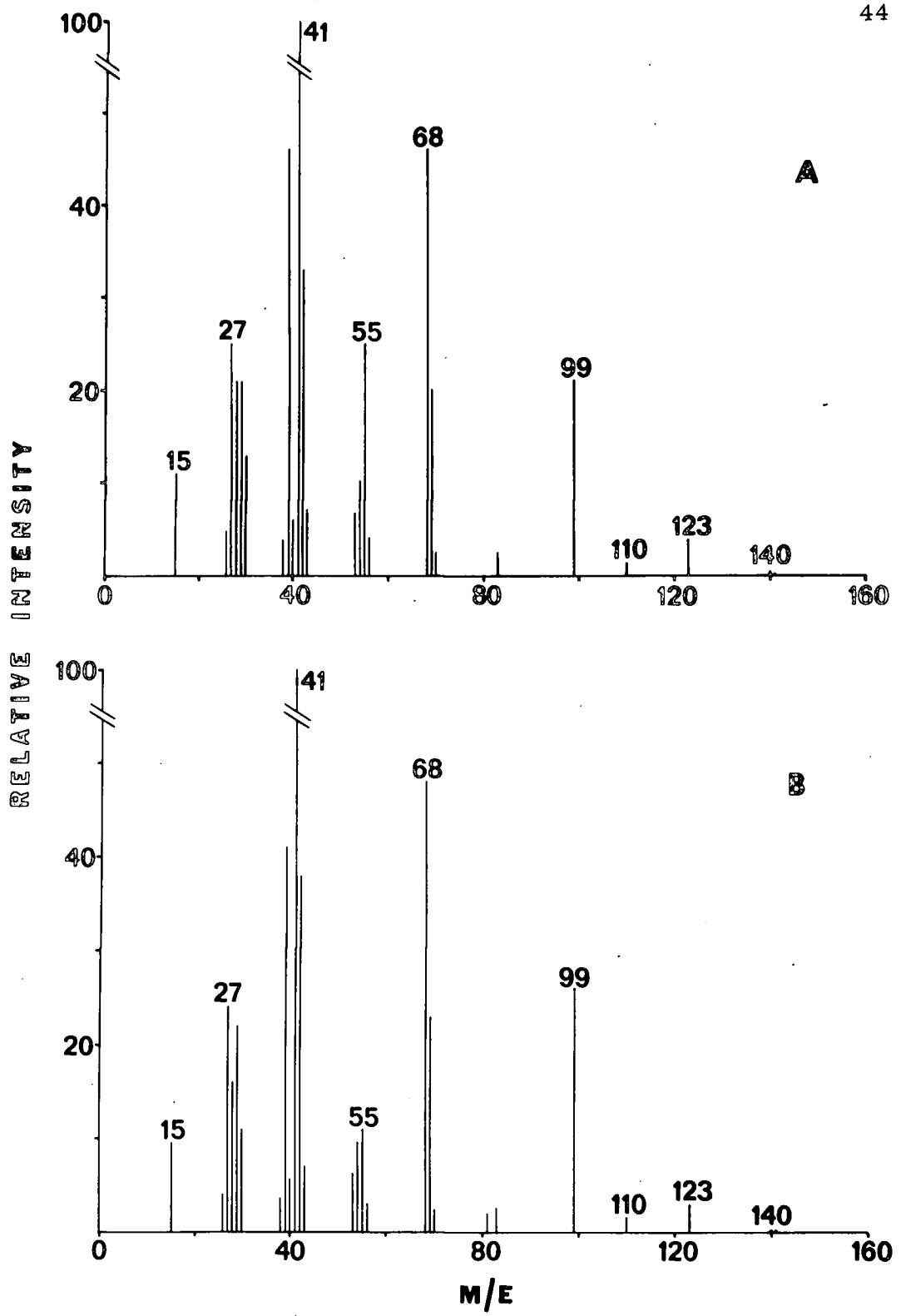


Figure 4. Mass spectra of γ-butenyl(β-propenyl)nitrosamine from A. spermidine·3HCl, B. γ-butenyl(β-propenyl)amine.

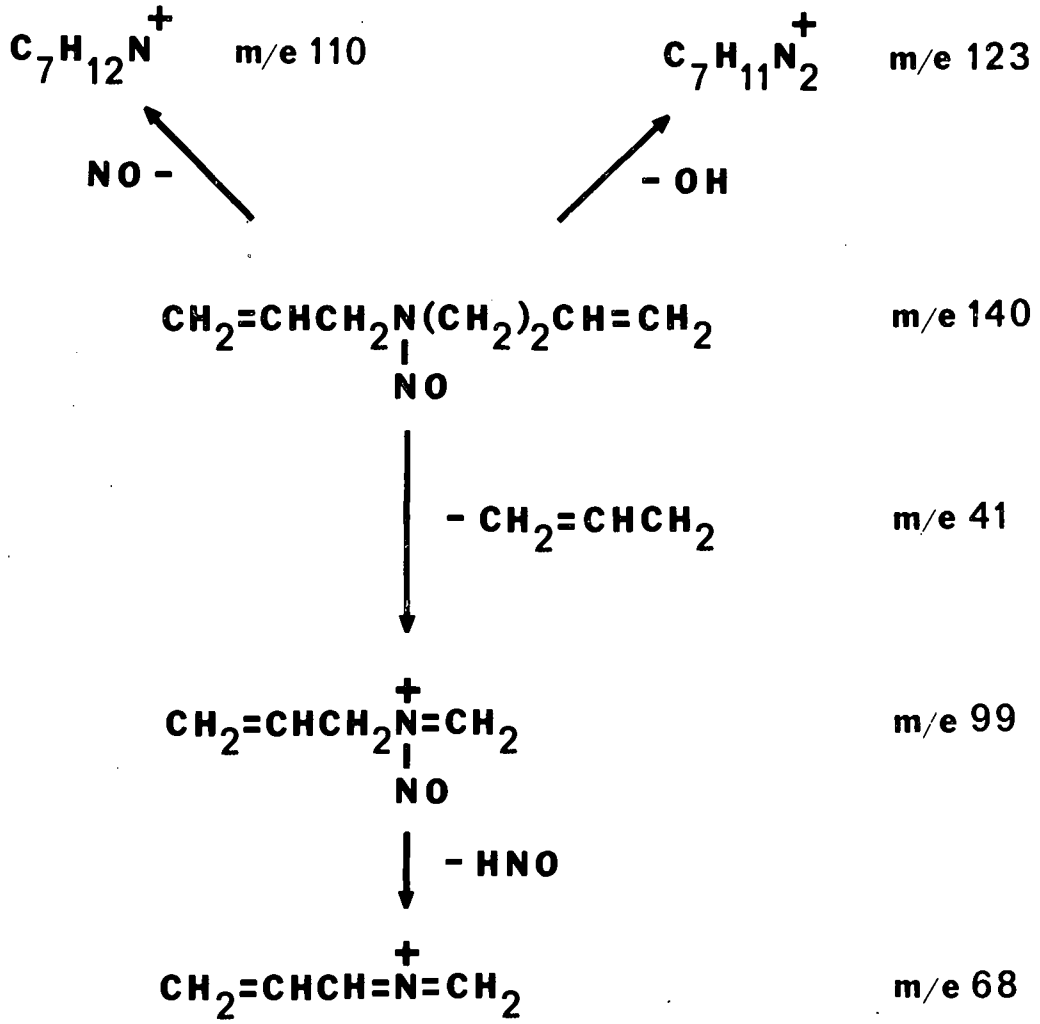


Figure 5. Mass spectrometric fragmentation scheme of γ-butenyl(β-propenyl)nitrosamine.

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 6). A band at 3082 cm^{-1} was assigned to vinylic asymmetric stretch. A double bond stretch occurred at 1637 cm^{-1}. The bands at 985 cm^{-1} and 910 cm^{-1} were typical of the C-H bend bands found in compounds containing vinyl unsaturation (Colthup et al., 1964).

The nuclear magnetic resonance spectra offered further confirmation of the identity of the nitrosamine (Figure 7). 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 nitrosamine 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 non-equivalent. When the substituents are different, two isomeric conformations are possible, which are 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 was complex. By comparing it with the simpler spectrum of diallylnitrosamine, it was possible to assign most of the resonances. The two quartets centered at 7.60 τ and 7.95 τ corresponded to syn and anti forms of the β-methylene group in the

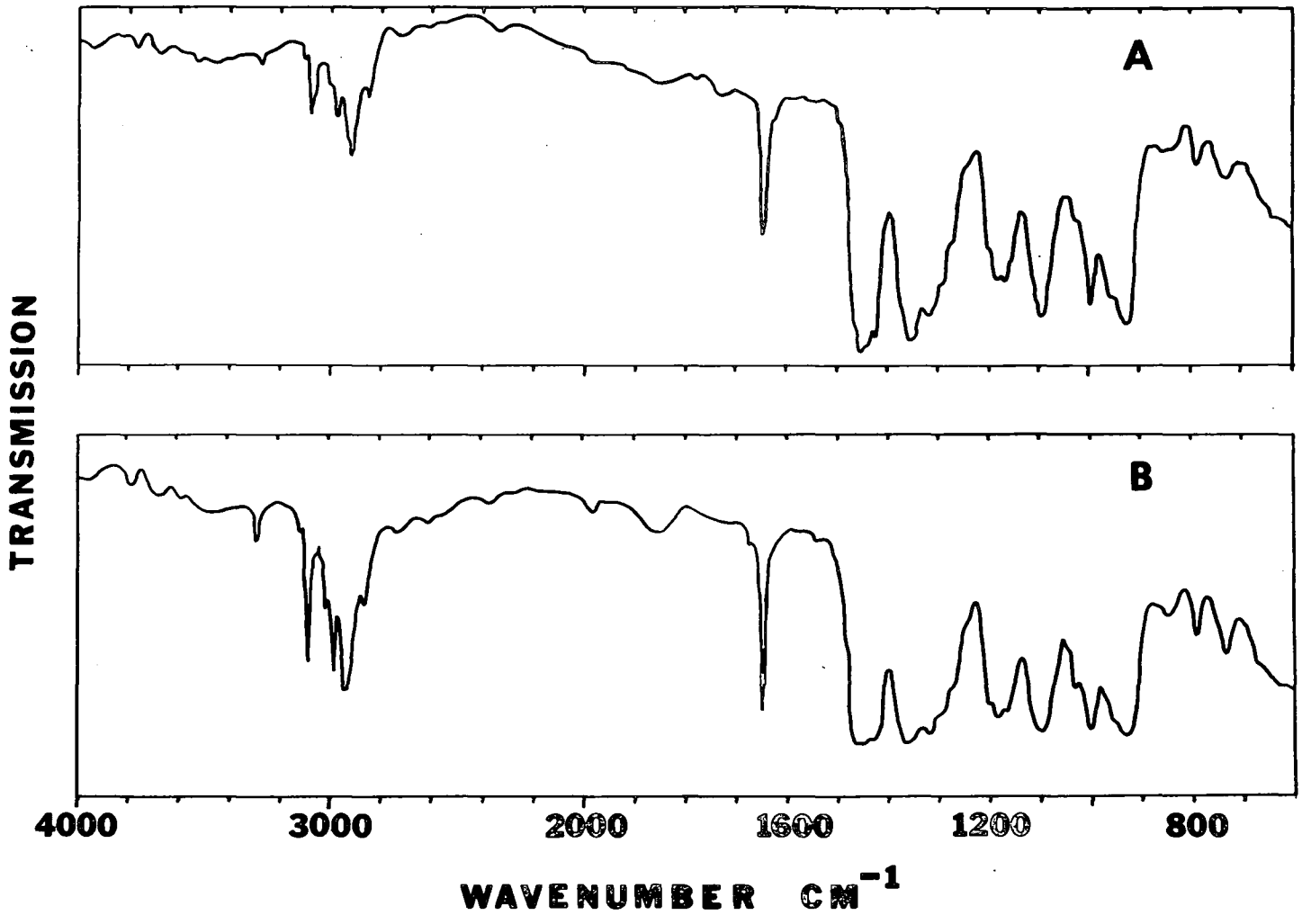


Figure 6. Infrared spectra of γ-butenyl(β-propenyl)nitrosamine from A. spermidine·3HCl B. γ-butenyl(β-propenyl)amine.

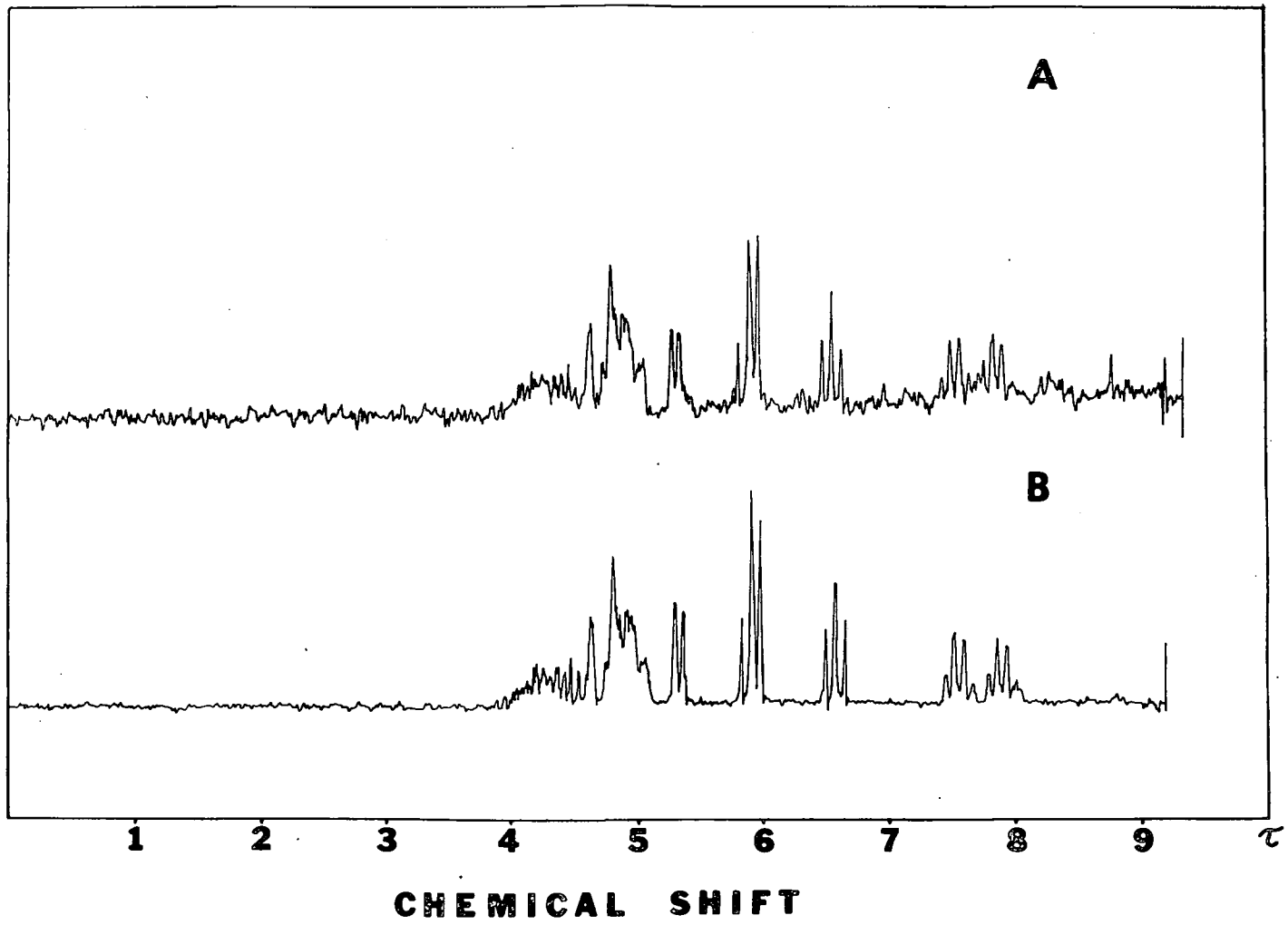


Figure 7. Nuclear magnetic resonance spectra of γ-butenyl(β-propenyl)nitrosamine from A. spermidine·3HCl, B. γ-butenyl(β-propenyl)amine.

butenyl side chain of BPN. Two triplets centered at 6.62 τ and 5.95 τ were assigned to the syn and anti conformations of the α-methylene group in the butenyl side chain. The latter triplet overlapped with the resonance of the syn-α-methylene of the propylene side chain. The resonance of the corresponding anti-α-methylene occurred at lower fields, giving a doublet centering at 5.40 τ. The vinylic hydrogens in both groups resonated between 3.9 τ and 5.2 τ.

The yields of BPN from the hydrochlorides of spermidine and spermine in this experiment were estimated to be 1.7% and 1.4%, respectively. The yield of BPN from free spermidine was 2.4% (Table 3).

Hydroxylated Nitrosamines

The mass spectrum of peak 18 in the gas chromatogram of the nitrosation products from spermidine suggested the presence of hydroxylated nitrosamines (Figure 8). Peak 18 did not appear in the gas chromatogram from spermine. On trapping peak 18 from the column, it was found to give a red color with the Griess reagent. 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 BPN, except that instead of a double elimination, substitution with a hydroxy group would have taken place at one of the carbonium ions. The hydroxy group could conceivably be located on either side chain.

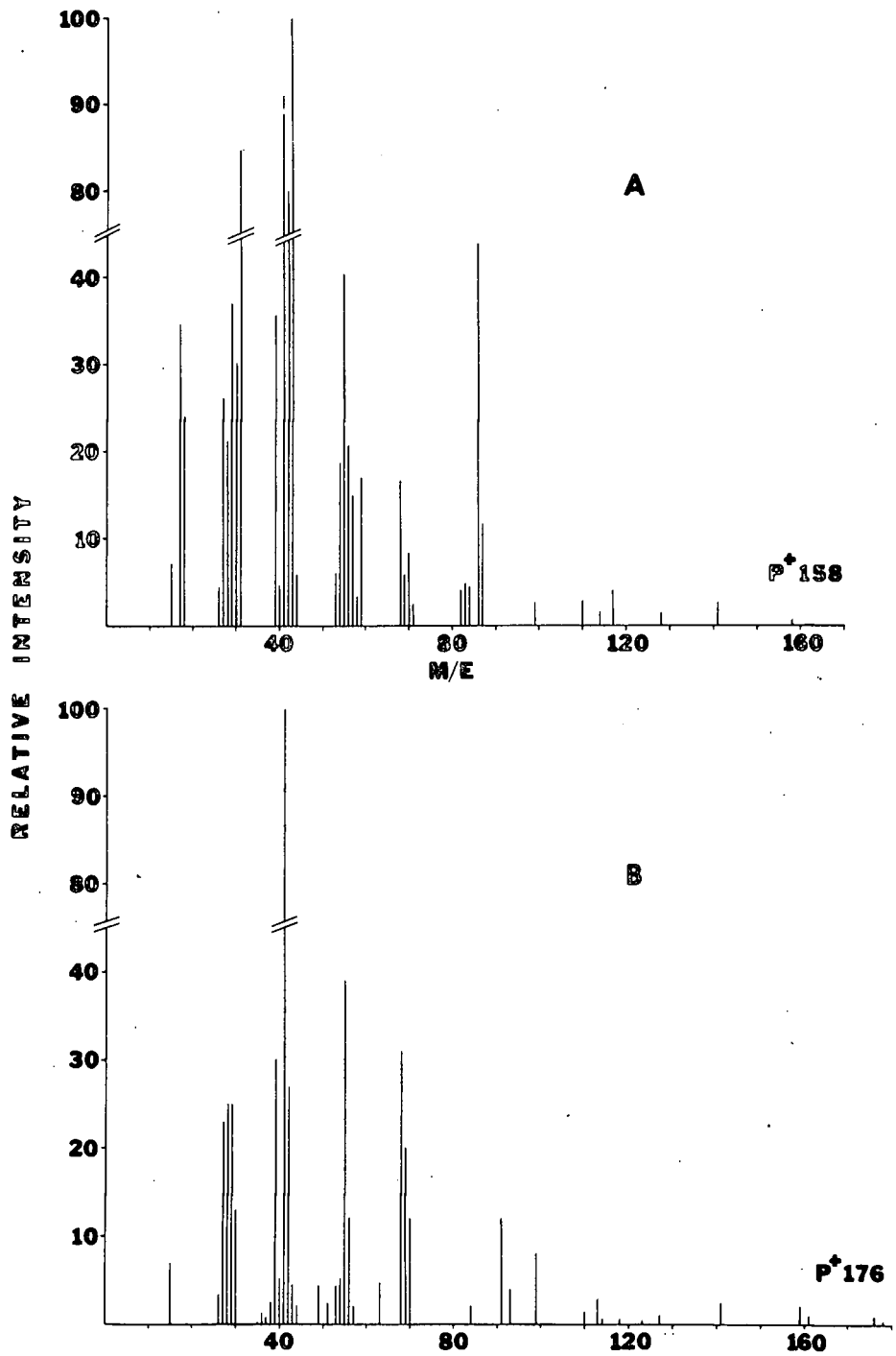


Figure 8. Mass spectra of A. a mixture of the isomers γ-butenyl-(γ-propanol)nitrosamine and δ-butanol(β-propenyl)-nitrosamine (peak 18, Figure 3), B. δ-butylchloride (β-propenyl)-nitrosamine[a] (peak 16, Figure 3).
[a]possibly a mixture of isomers.

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 beta 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 (Figure 8), it was concluded that the compound probably contained 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 is 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 of the presence of primary monohydroxylated nitrosamines (Figure 2), 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 20 M column and obtaining its ir spectrum (Figure 9). Alcohols in the liquid state usually exist as hydrogen bonded polymers, the existence of which showed up in the neat spectrum as a broad, strong absorption band near 3300 cm^{-1} due to stretching of the O-H$\cdots$O bonds (Colthup et al., 1964). The band at 1040-1070 cm^{-1} which involves asymmetric C-C-O stretch, supported the conclusion that the structure contained a primary alcohol. A double bond stretch occurred at 1633 cm^{-1}. As previously stated, the C-H bend bands at 985 cm^{-1} and 910 cm^{-1} are typical in compounds containing vinyl unsaturation.

To obtain additional information on the isomeric monohydroxylated nitrosamines, trifluoroacetate (TFA) derivatives were made from trapped material 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 10), which correspond to the molecular weight of the TFA derivative of the monohydroxylated nitrosamine (Figure 2). The base

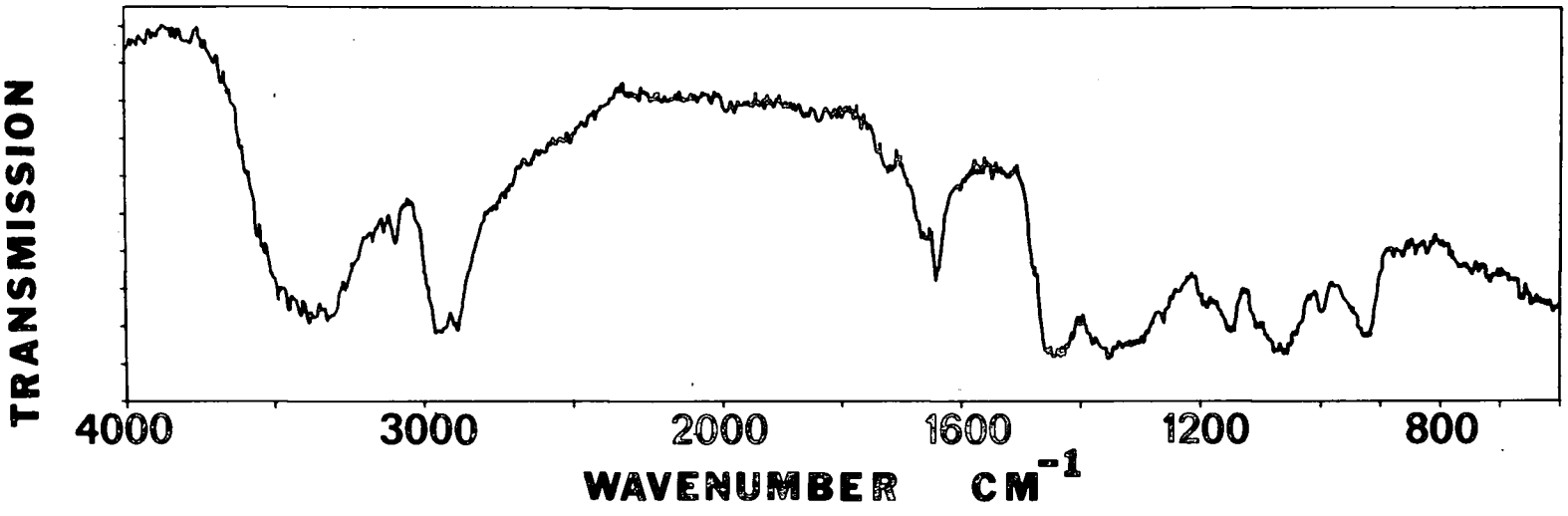


Figure 9. Infrared spectrum of a mixture of the isomers γ-butenyl(γ-propanol)nitrosamine and δ-butanol(β-propenyl)nitrosamine (peak 18, Figure 3).

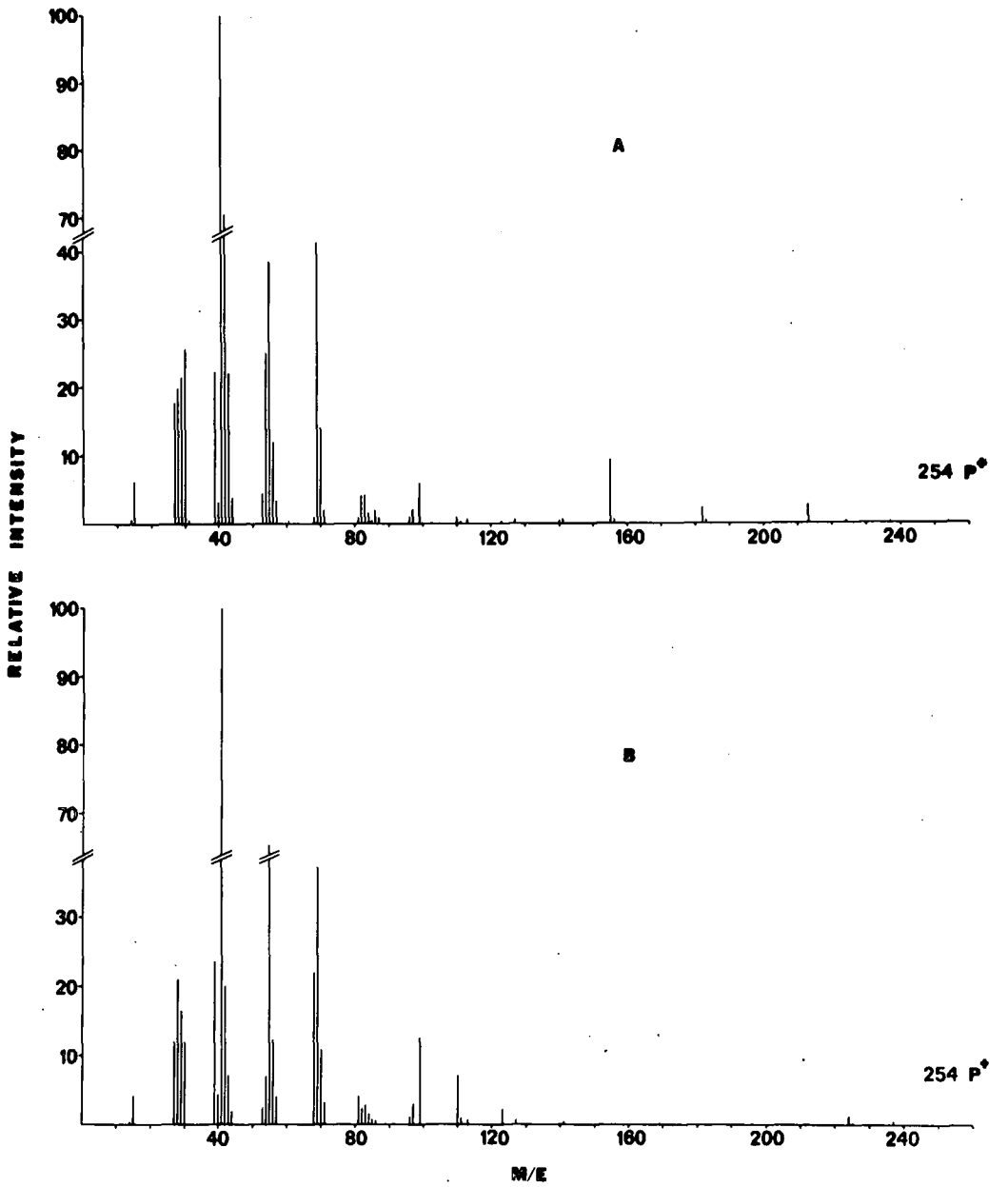


Figure 10. Mass spectra of the trifluoroacetate derivatives of A. γ-butenyl(γ-propanol)nitrosamine, B. δ-butanol (β-propenyl)nitrosamine.

peak in the mass spectra of both compounds was m/e 41, 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 (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. Similar fragment ions arose from the cleavages at other positions of the carbon chains.

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 10A) 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 γ-butenyl (γ-propanol) nitrosamine, fragmentation at the α-carbon of the longest 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 δ-butanol (β-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, which both appeared as prominent ions in the mass

spectrum of this isomer (Figure 10B). 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 γ-butenyl (γ-propanol) nitrosamine, while the isomer with the longer retention time was the TFA derivative of δ-butanol (β-propenyl) nitrosamine.

From the estimated peak areas, the relative amounts of γ-butenyl (γ-propanol) nitrosamine, and δ-butanol (β-propenyl) nitrosamines were 61% and 39%, respectively, on the basis of the total amount of primary, monohydroxylated nitrosamines from spermidine and nitrite. The total yield of primary, monohydroxylated nitrosamines from spermidine was 0.47%, calculated on the basis of the amount of amine precursor added (Table 3).

Secondary monohydroxylated nitrosamines were expected to appear from rearrangements of carbonium ion intermediates from spermidine. A small peak with retention time of 89 min (not shown in Figure 3) had mass spectral fragmentation characteristics suggesting a secondary alcohol. This mass spectrum was very similar to the mass spectrum of peak 18 except that the apparent molecular ion of m/e 158 was absent or extremely weak, which is typical of secondary alcohols (Silverstein and Bassler, 1964). Cleavage of the bond beta to the oxygen atom gave the m/e 45 ion, which was an intense ion in the present spectrum. The m/e 143 and m/e 15 ions

were prominent indicating the loss of CH_3 from the parent. The trapped compound gave a red color with the Griess reagent. Insufficient amount of compound was obtained for an ir spectrum. More information is needed for a confident identification of this compound.

Dihydroxylated nitrosamines, where substitution with hydroxy groups had occurred at both carbonium ions, were probably also products from the nitrosation of spermidine. These compounds would be relatively nonvolatile, and could most likely not be analyzed directly by gas chromatography. Separation could probably be achieved after suitable derivatization, or directly by high pressure liquid chromatography.

Chlorinated Nitrosamines

When spermidine·3HCl was used as the amine precursor, chlorinated nitrosamines were formed as products in the nitrosation reaction. Since sodium chloride is an important food additive in meat products cured with sodium nitrite, it was relevant to include these compounds in the study. The gas chromatograms of the nitrosation products from spermidine and spermidine·3HCl showed essentially the same yields for most of the volatile compounds (Figure 11). Peak 16 was one of the few peaks that increased strongly in the presence of chloride ions. The trapped compound gave a red color with the Griess reagent.

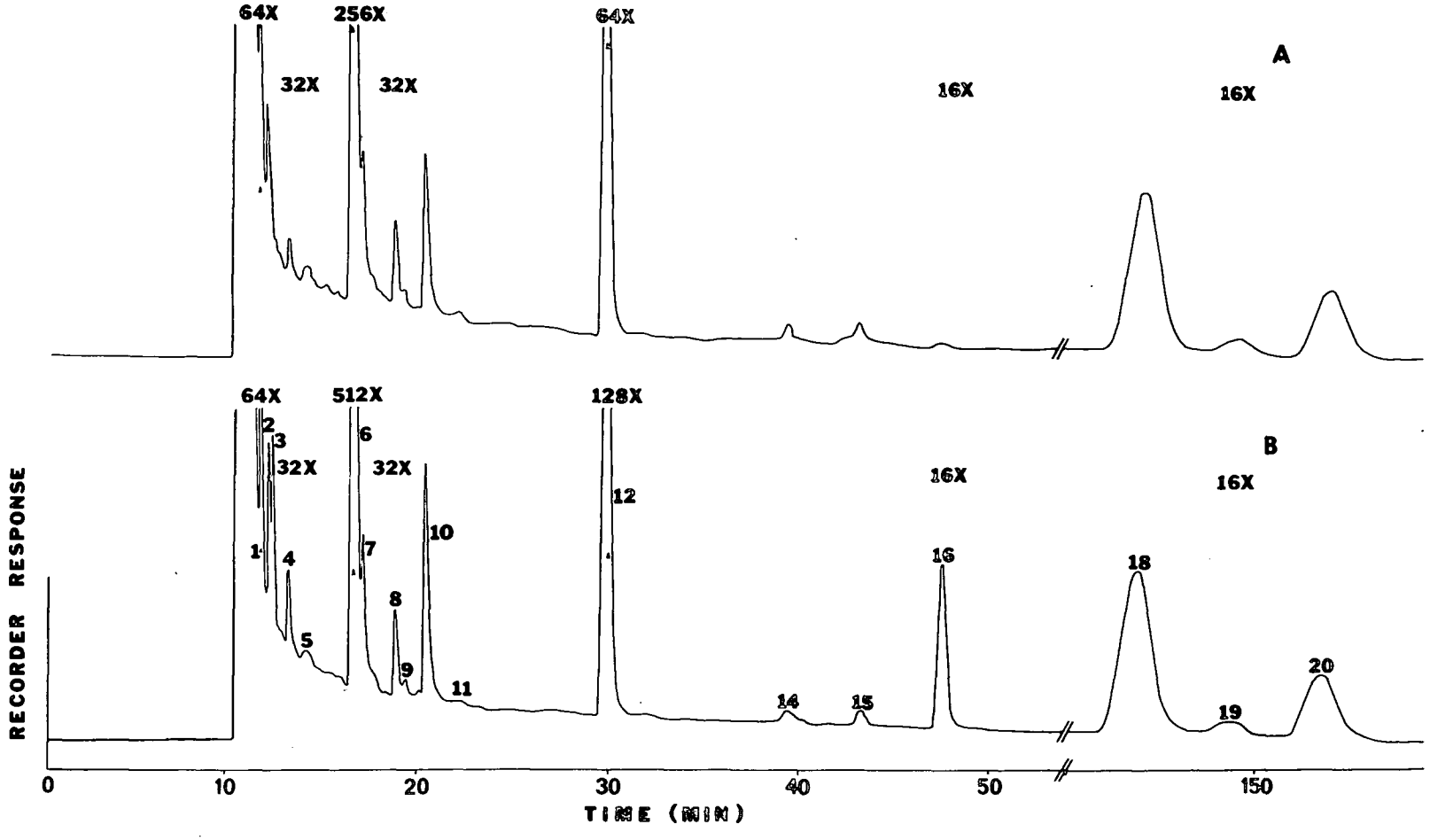


Figure 11. Gas chromatograms on 8% Carbowax 20 M and 1% Versamid 900 capillary column of the volatile products from the nitrosation of A. spermidine·3HCl, B. spermidine.

The mass spectrum of peak 16 suggested the presence of monochlorinated nitrosamines corresponding to the monohydroxylated nitrosamines (Figure 8). The parent ion appeared to be m/e 176, which is the molecular weight of a nitrosamine derived from spermidine, but where elimination has taken place at one carbonium ion, and substitution with chloride at the other carbonium ion (Figure 2). The mass spectrum of the smaller peak produced from spermidine with the same retention time as peak 16 (Figure 11A) revealed that this was not the same compound as was formed from spermidine· 3HCl.

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 Cl^{37} isotope (Silverstein and Bassler, 1964). Such isotope peaks were clearly present in the mass spectrum of peak 16 (Figure 8). The P+2/P mass ratio was estimated to be 0.39, matching well with theoretical values. The presence of the m/e 36 ion 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. Loss of hydroxy radical 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 δ-butylchloride (β-propenyl) nitrosamine was an important isomer (Figure 2). 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 butylchloride fragment. However, the existence of more than one of the monochlorinated isomers in peak 16 (Figure 11B) is possible.

The ir spectrum of trapped peak 16 was inconclusive. This was probably due to an apparent heat instability of the substance, causing a partial breakdown on trapping the compound from the gc column. Shortening the packed gc column to 2 ft and reducing the temperatures of the injection port and column to 160°C and 150°C, respectively, did not solve the problem. The yield of monochlorinated nitrosamines (peak 16) from spermidine·3HCl was 0.12%, calculated on the basis of amount of amine precursor added.

Nitrosamines Not Identified

Nitrosoazetidine, a four-membered cyclic nitrosamine, was expected to form in the nitrosation of spermidine and spermine by

cyclization of a three-carbon fragment including an amine group (Figure 1). However, no nitrosoazetidine was found in the extracts from either spermidine or spermine. Standard nitrosoazetidine was synthesized from azetidine and nitrite. Nitrosoazetidine is reported to be unstable in the presence of mineral acid (Howard and Marckwald, 1899), which might be the reason for the negative finding.

Peak 11 in the gas chromatograms from spermine and spermidine (Figure 3) showed nitrosamine characteristics with high m/e 30 and m/e 41 ions in its mass spectrum. The molecular ion was at m/e 116. Both the mass spectrum and the retention time on gc for peak 11 were very similar to those for nitrosomorpholine, which was synthetized from morpholine and nitrite. However, repetitive runs of both compounds on the capillary gc column revealed a small but reproducible difference in the retention times.

A compound with a parent ion of m/e 116 also appeared as a product in the nitrosation of the diamines putrescine, cadaverine, and diaminopropane (Warthesen, 1974). The substance had a similar mass spectrum and retention time on gc as the product from spermidine. The yield of the unknown compound was highest from diaminopropane. The substance from diaminopropane was trapped from the gc column, and its nmr spectrum obtained. This nmr spectrum was far more complex than the nmr spectrom for nitrosomorpholine. More information is needed to identify the substance.

Thin Layer Chromatography

Thin layer chromatography (tlc) of the concentrated dichloromethane extract of the reaction mixture of the nitrosation of spermidine revealed eight distinct spots after spraying with Griess reagent and developing the plate under ultraviolet light (Table 3). As considerable streaking occurred on the plate, additional minor spots may not have been detected. The major spots had R_f values of 0.55 and 0.10, corresponding to peak 6 (BPN) and peak 18 (monohydroxylated nitrosamines)(Figure 3). Tlc of the residue after distillation of the reaction mixture from spermidine revealed that four of the eight Griess-positive compounds remained in the non-distillable residue. Nonvolatility correlated well with low R_f values on tlc.

Pathway of Formation of Nitrosamines from Spermidine

The pathway proposed for the formation of the volatile nitrosamines from spermidine and nitrite is shown in Figure 12 and assumes that the reaction at the secondary amine function will stop at the nitrosamine stage. 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 BPN. Solvolysis with water at one carbonium ion and elimination of a proton

Table 3. Thin layer chromatography of nitrosamines formed in the nitrosation of spermidine on Silica gel with hexane:diethylether:dichloromethane (4:3:2 v/v/v) as the mobile phase. Sprayed with Griess reagent, developed under ultraviolet light.

		R_f
Dichloromethane extract of reaction mixture		0.55, 0.46, 0.42, 0.32, 0.18, 0.12, 0.10, 0.07
Dichloromethane extract of residue after steam distillation		0.18, 0.12, 0.10, 0.07
Trapped peaks (Figure 3)	peak 6	0.55 (BPN)
	peak 16	0.45 (BPN·Cl)
	peak 18	0.10 (BPN·OH)
Standards:		
Dipropylnitrosamine		0.52
Nitrosopyrrolidine		0.29

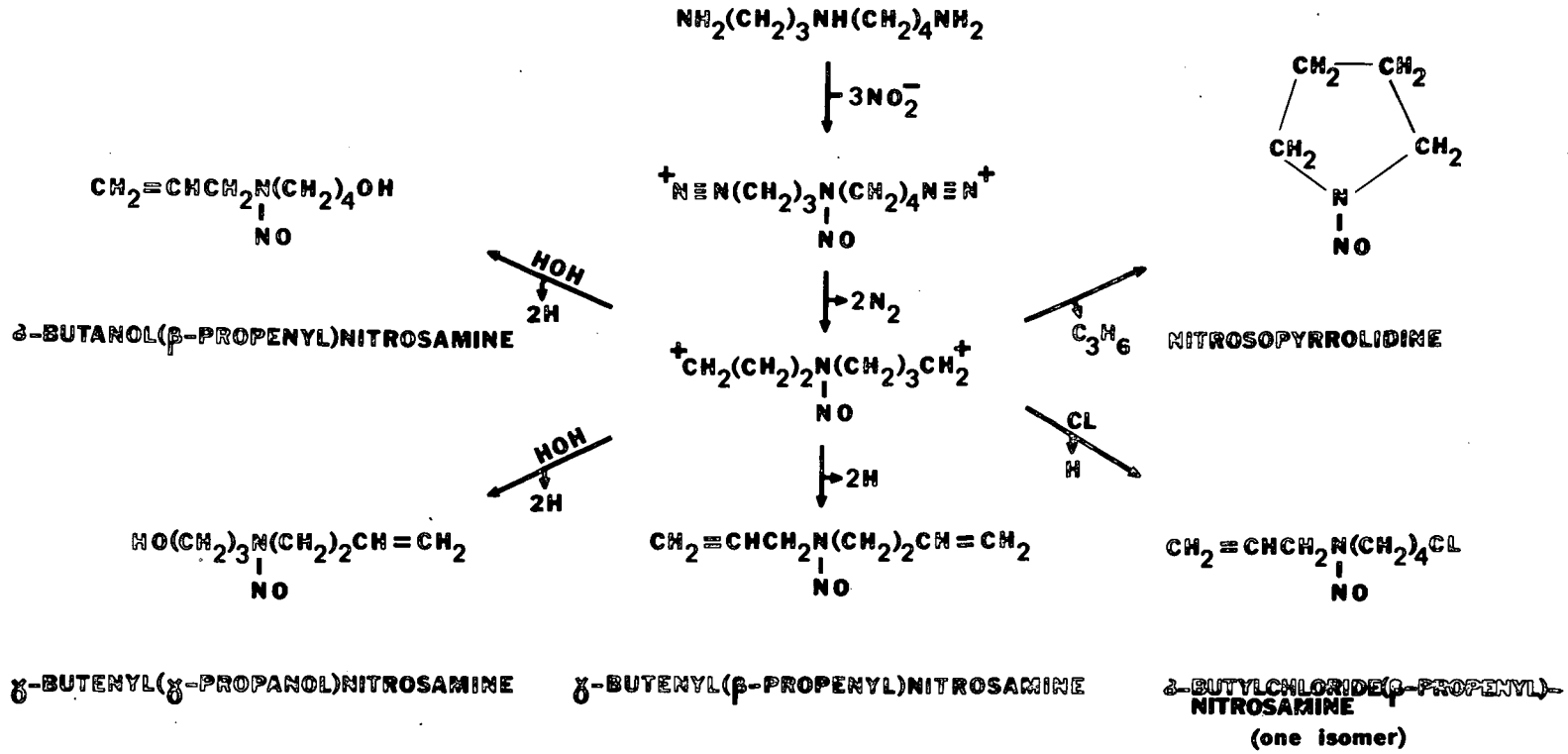


Figure 12. Pathway of formation suggested for several nitrosamines in the nitrosation of spermidine.

at the other carbonium ion yields monohydroxylated nitrosamines (BPN· OH). Correspondingly, in the presence of chloride ions, nucleophilic addition of the chloride ion to the carbonium ion would produce monochlorinated nitrosamines (BPN· Cl). Secondary hydroxy derivatives and secondary chlorides would be formed through rearrangements of carbonium ions, followed by solvolysis with water or addition of chloride. Nitrosopyrrolidine may be formed by electrophilic attack on the secondary amine nitrogen by 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, 1970). An alternate route is the formation of pyrrolidine from spermidine which is subsequently nitrosated.

In the reaction between sodium nitrite and a secondary amine with no additional functional groups in the molecule, one nitrosamine is usually formed. To the knowledge of the author, this study is the first instance 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 only one nitrosamine, is a difficult task. The fact that a single secondary amine can form a range of different nitrosamines makes the toxicological and analytical aspects of the nitrosamine in the environment problem more complex.

Influence of Selected Parameters on the Nitrosation of Spermidine

General Remarks

The primary monohydroxylated nitrosamines were quantified together (Figure 3, peak 18), and are abbreviated BPN· OH. The monochlorinated nitrosamines are denoted BPN· Cl (Figure 3, peak 16), and were quantified as a group. The partition coefficients between dichloromethane and distilled water saturated with sodium sulfate were estimated to be 18, 26, and 14 for BPN, BPN· Cl, and BPN· OH, respectively. Extracting twice with an equal volume of dichloromethane was therefore adequate to remove the nitrosamines from the reaction mixtures.

Relatively high recoveries during the extraction and concentration steps were found for both BPN and BPN· Cl, while the average recovery for BPN· OH was 32% (Table 4). All of the recoveries were reproducible. The low recovery of BPN· OH might have been caused by polymerization of the compound during concentration, which was suggested by sample darkening during this step. Denitrosation of BPN· OH or interaction with other substances in the system might also have taken place. The recovery of the internal standard, methyl myristate, was 98%. All yields of nitrosamines were corrected for their respective recoveries.

Table 4. Recoveries during the extraction and concentration steps of several nitrosamines formed in the nitrosation of spermidine.

Nitrosamine	Recovery in percent		
	Replicate 1	Replicate 2	Mean
BPN[a]	87	90	89
BPN· Cl	94	92	93
BPN· OH	33	31	32

[a]BPN : γ-butenyl (β-propenyl) nitrosamine

BPN· OH : γ-butenyl (γ-propanol) nitrosamine + δ-butanol (β-propenyl) nitrosamine

BPN· Cl : δ-butylchloride (β-propenyl) nitrosamine + other isomers of primary and secondary chlorides

Estimates of the variability between replications were calculated for each nitrosamine from the standard deviations of four replications. In percent of the mean values, the estimated standard deviations for BPN, BPN· Cl, and BPN· OH were 7.9%, 5.3%, and 14.2%, respectively. The response factor f between BPN and methylmyristate was 1.215. The standard deviation for f, determined from five replications, was 0.045, which is 3.7% of the mean value of f.

Effect of pH

Table 5 shows the effect of pH of several nitrosamines in the nitrosation of spermidine. In all samples, the pH changed during the reaction period, particularly at pH 3.0, where a large decrease in the

pH value was observed. To stop the reaction after 1 hr, ammonium sulfamate was added to the reaction flask, which caused a vigorous liberation of nitrogen gas from the remaining nitrite. In samples with low pH levels (pH 3.0, 3.5), little or no nitrogen gas was liberated, which indicated depletion of nitrite during the reaction period. In the sample of an initial pH of 3.0, a relatively large amount of H_2SO_4 had been previously added to adjust the nitrite to pH 3.0. By the depletion of the nitrite during the reaction, the remaining H_2SO_4 caused a shift towards higher acidity in the sample. In all other samples than that of pH 3.0, the pH increased moderately during the reaction.

Table 5. Effect of pH on the yields of several nitrosamines in the nitrosation of spermidine. Reaction conditions: 1 mmol spermidine, 9 mmol sodium nitrite, 0.2 M acetate buffer, 0.33 M sodium chloride, 1 hr, 50°C.

Initial pH	Final pH	BPN	BPN·Cl	BPN·OH
3.0	2.0	0.387[a]	0.0297	0.0155
3.5	3.85	0.803	0.0410	0.0189
4.0	4.26	0.987	0.0392	0.0270
4.5	4.78	0.643	0.0227	0.0056
5.0	5.32	0.206	0.0100	0
6.0	6.02	0.035	0.0034	0

[a] yield in % based on spermidine added

As the pH changed during the reaction in this experiment, no true pH optima for the formation of nitrosamines could be reported. However, the results indicated that the highest yields of the

nitrosamines, under these experimental conditions, were formed between pH's 3.0 and 4.5.

Effect of Reaction Time

Two different pH levels (pH 3.5, 5.0) were included when studying the effects of lengthening the reaction time (Figure 13). At pH 3.5 the rate of formation of BPN sharply declined after 1 hr reaction time due to depletion of nitrite. The highest yield of BPN was observed after 5 hrs at this pH, while after 25 hrs at pH 3.5 the yield of BPN had decreased. At pH 5.0 the rate of formation of BPN was constant up to 5 hrs. The yield of the nitrosamine increased only slightly by increasing the reaction time from 5 hrs to 25 hrs. The yield of BPN after 1 hr at pH 3.5 was thus four times the yield at pH 5.0. However, after 25 hrs 45% more BPN had been formed at pH 5.0, as compared to pH 3.5. The effects of reaction time on the yields of BPN·Cl and BPN·OH followed similar patterns as for BPN, although the yields at pH 5.0 for these nitrosamines never exceeded the corresponding yields at pH 3.5.

Secondary reactions with losses of nitrosamines were observed for all nitrosamines at longer reaction times at pH 3.5. The losses may be due to interactions of nitrite with the hydroxy, chloride, or vinyl groups in the nitrosamines (Norman, 1968; Ridd, 1961). Polymerization or denitrosation of the nitrosamines formed might also

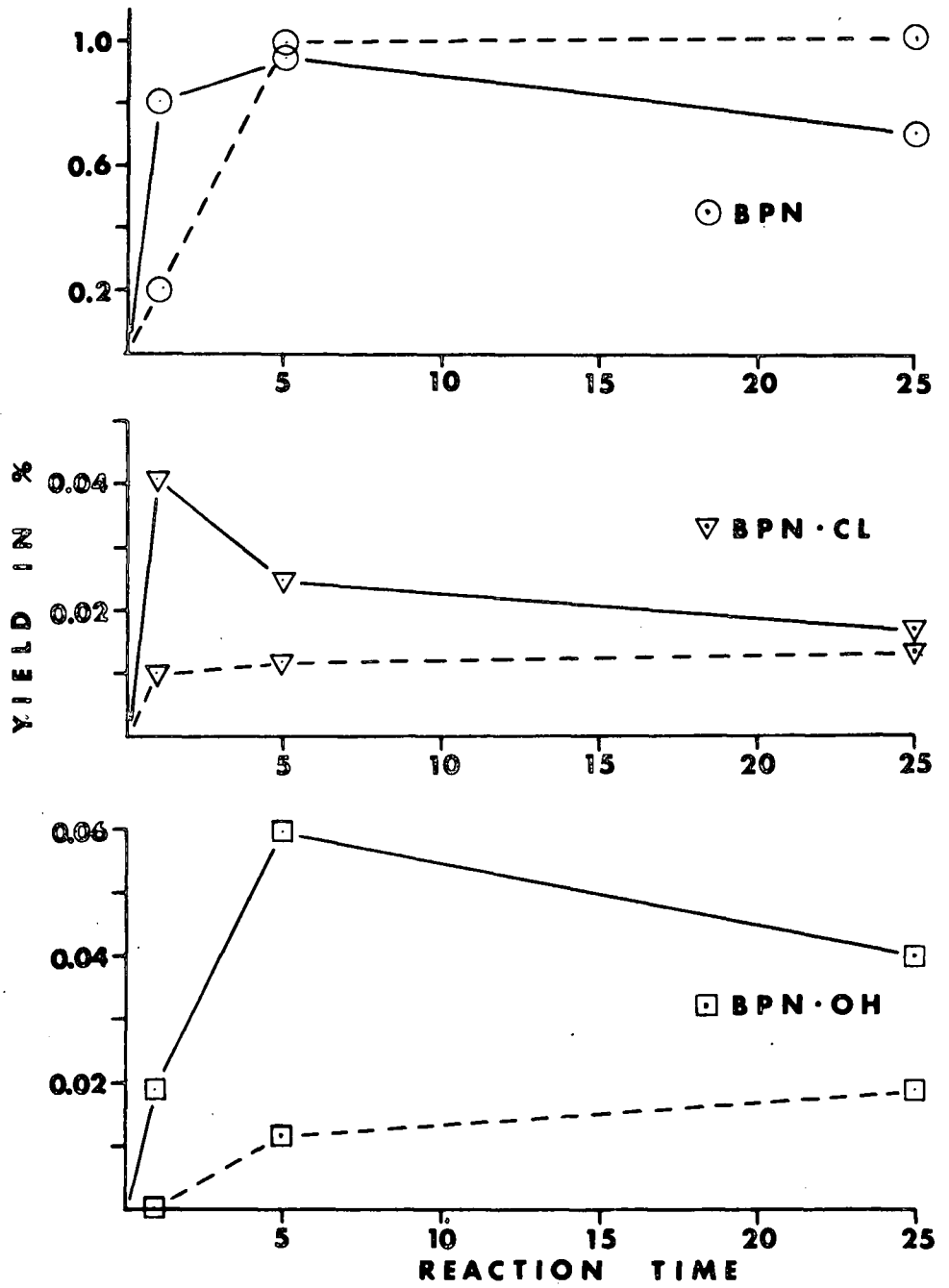


Figure 13. Effect of reaction time in hours at pH 3.5 and pH 5.0 on the yields of several nitrosamines in the nitrosation of spermidine. _____ pH 3.5 ------- pH 5.0 Reaction conditions: 1 mmol spermidine, 9 mmol sodium nitrite, 0.2 M acetate buffer, 0.33 M sodium chloride, 50°C.

cause losses of nitrosamines at longer reaction times and higher temperatures.

The pH optimum for the formation of nitrosamines has been determined from initial rates of formation of the compounds (Fan and Tannenbaum, 1973; Mirvish et al., 1973). The optimal yields of nitrosamines were reported to range between pH's 2.5 and 3.5. When using longer reaction times, maximum accumulation of nitrosamines might take place at higher pH levels, partly due to the instability of nitrite solutions under acidic conditions (Ewing and Bauer, 1958). This observation might have implications when predicting nitrosamine formation in mildly acidic or neutral food materials during storage.

Effect of Temperature

The effect of temperature in the range 24°C to 48.5°C on the yields of nitrosamines from spermidine after 1 hr at pH 5.0 is shown in Table 6. Since the rate of formation of BPN was constant up to 5 hrs at pH 5.0 (Figure 13), measuring reaction rate at 1 hr at pH 5.0 was assumed to give initial rates of formation of BPN. The results on the effect of temperature on the rate of formation of BPN was used to construct an Arrhenius plot (Figure 14). The activation energy was calculated from the slope of the curve, and was found to be 19. kcal/mol for this reaction. In comparison, the activation energy for the nitrosation of morpholine is reported to be approximately 10 kcal/mol

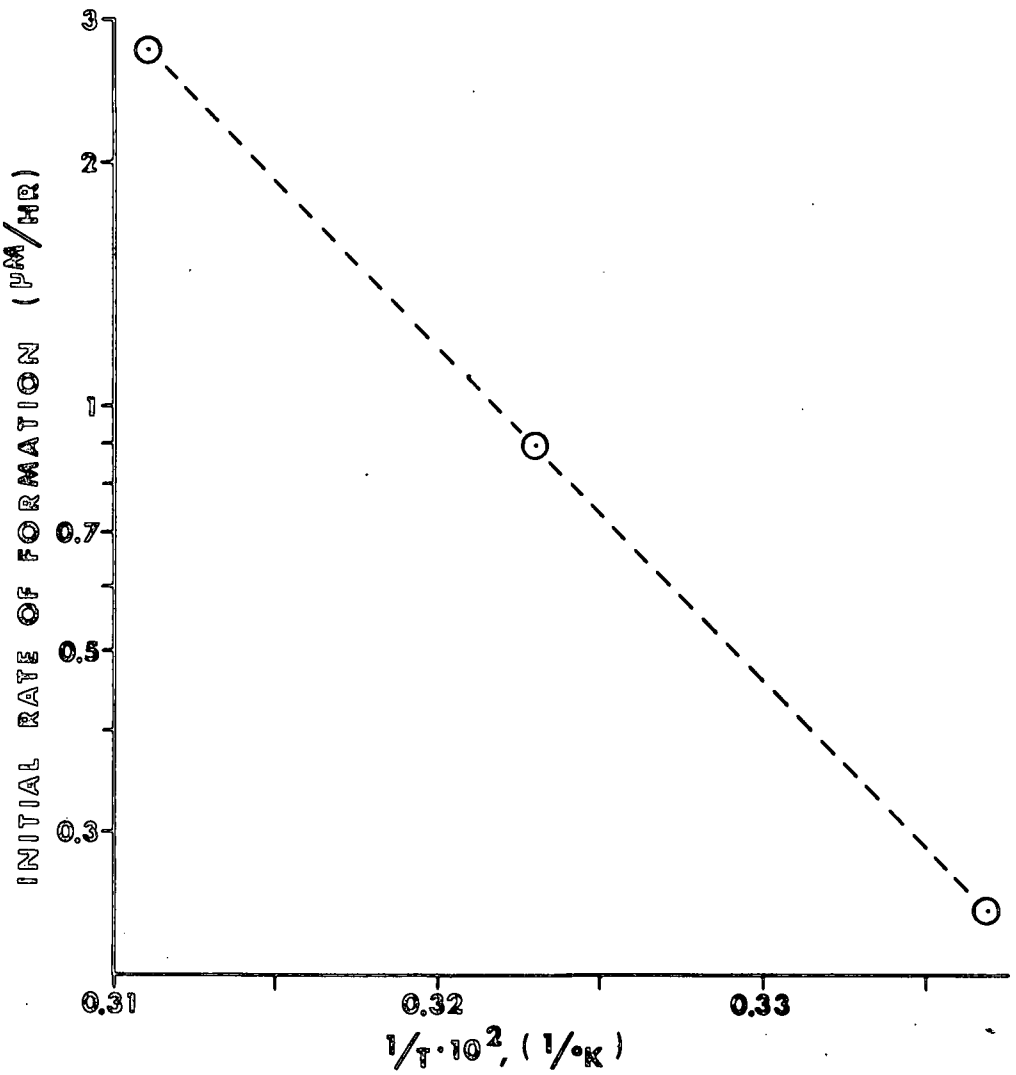


Figure 14. Effect of temperature on the formation of BPN in the nitrosation of spermidine (Arrhenius plot). Reaction conditions: 1 mmol spermidine, 9 mmol sodium nitrite, 0.2 M acetate buffer, 0.33 M sodium chloride, pH 5.0, 1 hr.

(Fan and Tannenbaum, 1973).

Table 6. Effect of temperature on the yields of several nitrosamines in the nitrosation of spermidine. Reaction conditions: 1 mmol spermidine, 9 mmol sodium nitrite, 0.2 M acetate buffer, 0.33 M sodium chloride, pH 5.0, 1 hr.

Nitrosamine	24°C	37°C	48.5°C
BPN	0.0241[a]	0.0891	0.2730
BPN·Cl	0.0004	0.0023	0.0082
BPN·OH	0	0	0.0090

[a]yield in % based on spermidine added

Effect of Sodium Chloride Concentration

No significant effects on the yields of BPN and BPN·OH from spermidine were observed at pH 4.0 in the presence of 0.1-1 M sodium chloride (Figure 15). The acetate buffer concentration was reduced to 0.1 M. However, 1.5 M or 2.0 M sodium chloride in the system inhibited the nitrosamine formation strongly. The yield of BPN·Cl increased steadily with increasing sodium chloride concentration in the range 0-1 M salt. At 1.5 M or 2.0 M sodium chloride only trace amounts of BPN·Cl were found.

Since it was suspected that the presence of acetate buffer might mask the inhibiting effects of sodium chloride at the lower salt concentrations, the reaction was also performed in a buffer-free system. A small but significant inhibition on the formation of BPN was found in

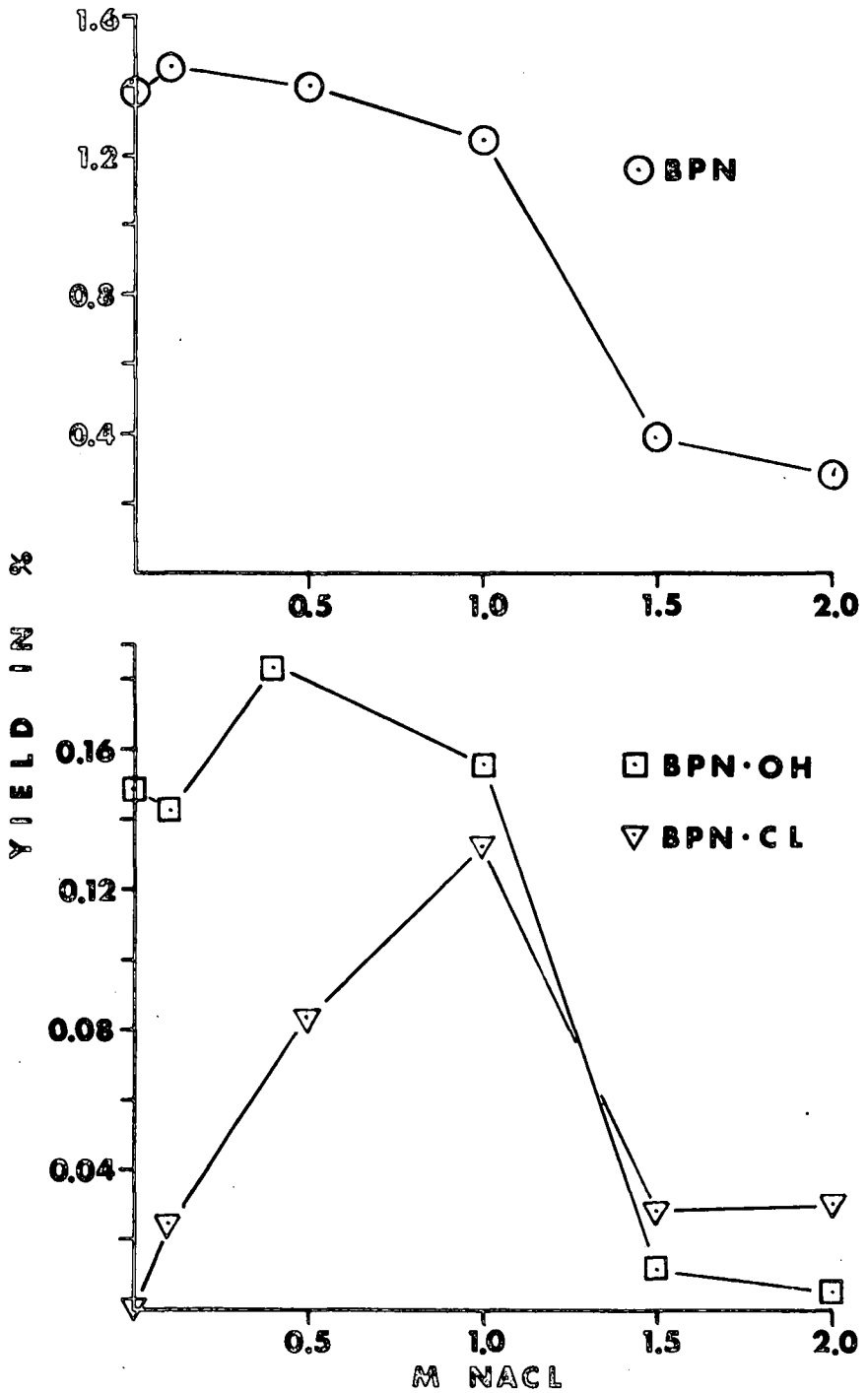


Figure 15. Effect of sodium chloride concentration on the yields of several nitrosamines in the nitrosation of spermidine. Reaction conditions: 1 mmol spermidine, 9 mmol sodium nitrite, 0.1 M acetate buffer, pH 4.0, p hr, 50°C.

the presence of 0.5 M sodium chloride (Table 7). The inhibiting effects both by acetate buffer and sodium chloride were probably due to primary and secondary salt effects.

Table 7. Effect of sodium chloride on the yields of several nitrosamines in the nitrosation of spermidine in a buffer-free system. Reaction conditions: 1 mmol spermidine, 9 mmol sodium nitrite, initial pH 4.0, pH after reaction 6.5, 1 hr, 50°C.

	0 M NaCl		0.5 M NaCl	
Nitrosamines	Rep. 1	Rep. 2	Rep. 1	Rep. 2
BPN	0.665[a]	0.695	0.578	0.564
BPN·Cl	0	0	0.0163	0.0186
BPN·OH	0.0184	0.0120	0.0213	0.0157

[a]yield in % based on spermidine added.

Purity of Amine Precursors

No contaminating amines were found in spermidine·3HCl and spermine·4HCl (Nutritional Biochemicals Co.) as tested by thin layer chromatography. The detection limit of amine impurities by this method was 0.5%.

The purity of the free bases spermidine (Sigma Chemical Co.) and spermine (Nutritional Biochemical Co.) as examined by gas chromatography, revealed that both commercial samples contained several impurities. The spermine sample contained appreciable amounts of spermidine, and was not used in these experiments. Free spermidine contained a total of 2.2% volatile impurities.

One of the impurities in spermidine co-chromatographed with γ-butenyl (β-propenyl)amine (BPA). In order to substantiate the formation of BPN from spermidine and not from a BPA impurity in the spermidine, the spermidine sample was purified by vacuum distillation (bp. 94°C at 2 mm Hg) over a 20 cm Vigreaux column. The concentration of all volatile impurities was strongly reduced. The concentration of the impurity which co-chromatographed with BPA decreased from 370 ppm to 40 ppm. The yield of BPN from the nitrosation of the purified spermidine, however, remained the same as before the purification, proving that BPN was a genuine product from spermidine. The yields of NPy and BPN·OH from the purified precursor also remained unchanged.

Pyrrolidine was not found as an impurity in the commercial sample of spermidine. The possibility of δ-butylamine (γ-propanol) amine or δ-butanol(γ-propylamine) amine as impurities was considered, as nitrosation of these amines would lead to the formation of BPN·OH. However, since these amines are much less volatile than spermidine, fractional distillation was considered appropriate to separate them from the spermidine. BPN·Cl was only formed from spermidine in the presence of chloride ions. The formation of BPN·Cl from an impurity in the spermidine was therefore not considered very probable.

Artifact Formation in Extraction, Concentration and gc Analysis

Formation of artifacts in the isolation and separation of substances is frequently a problem in chemical analysis. In the present work, this possibility deserves attention, since several of the nitrosation products from spermidine conceivably could be interconverted.

One possibility is that BPN· OH might partly dehydrate during the clean-up steps to yield BPN. The yields of BPN· OH in the quantitative experiments were low, and even if all the BPN· OH lost during the clean-up steps was converted to BPN, the effect on the yield of BPN would be very small. BPN· Cl might also be converted to BPN by the elimination of HCl. The high recovery of BPN· Cl, however, indicated that this can only happen to a very small extent during the extraction and concentration steps.

Gas chromatography can cause problems by heat-induced changes which can take place in the injector or in the column. Apparently degradation did not occur to a significant extent, however, as the peaks observed in the gc analysis at a column temperature of 170°C were symmetrical and showed little tailing (Figure 3), which indicates that no serious degradation took place during gc analysis.

Effect of Sodium Chloride Concentration on the Nitrosation of Proline at Different pH Levels

The assay used for measuring the rate of formation of nitrosoproline, the direct spectrophotometric method, was compared at pH 2.5 to the procedure by Mirvish et al. (1973), which will be referred to as the ammonium sulfamate method (Table 8). The ammonium sulfamate method gave slightly lower values for the rates of formation of nitrosoproline, and the intrinsic variability in the ammonium sulfamate assay was larger than for the direct spectrophotometric method. As a consequence, a small but significant correlation between sodium chloride concentration and rate was detected at pH 2.5 using the direct spectrophotometric method, whereas no significant correlation was observed when the ammonium sulfamate assay was used.

Table 8. Rate of nitrosation of proline measured by two methods at pH 2.5.

NaCl M	Nitrosoproline, initial rate (μM/min)	
	Direct spectrometric method	Ammonium sulfamate method
0	14.95	11.33
0.1	14.26	11.38
0.25	13.83	9.55
0.50	13.71	10.81
0.75	13.86	11.10
1.00	13.86	9.36

The effect of sodium chloride on the rate of nitrosation of proline at different pH levels is shown in Figure 16. Strong activation was observed at pH 0.5, very slight inhibition at pH 2.5, and moderate inhibition at pH's 4.0 and 5.5. Table 9 shows that the best fitted regression model at different pH levels was of the form log(rate) = a + b[NaCl]+ c[NaCl]^2. These equations could be used to estimate rates of nitrosation in systems with known sodium chloride concentrations and pH levels. The effect of sodium chloride was statistically significant at the 95% level at all pH levels, and the quadratic effect was significant except at pH 5.5 (Table 10). The small residual mean square terms show that the variability between replicates in the experiment was very low. The highest nitrosation rates of proline were at pH 2.5, which confirms earlier results by Mirvish et al. (1973).

In the absence of an anionic promotor, the rate of nitrosation of amines under acidic conditions is third order, while decreasing to second order with decreasing acidity (Ingold, 1969). In the presence of promoting anions the rate expression is described by the following equation (Hughes and Ridd, 1958).

$$\text{Rate} = k[HNO_2][H^+][X^-][R_2NH]$$

In the presence of bromide or thiocyanate, the anion promoting mechanism is prevalent at pH values below 2 (Fan and Tannenbaum,

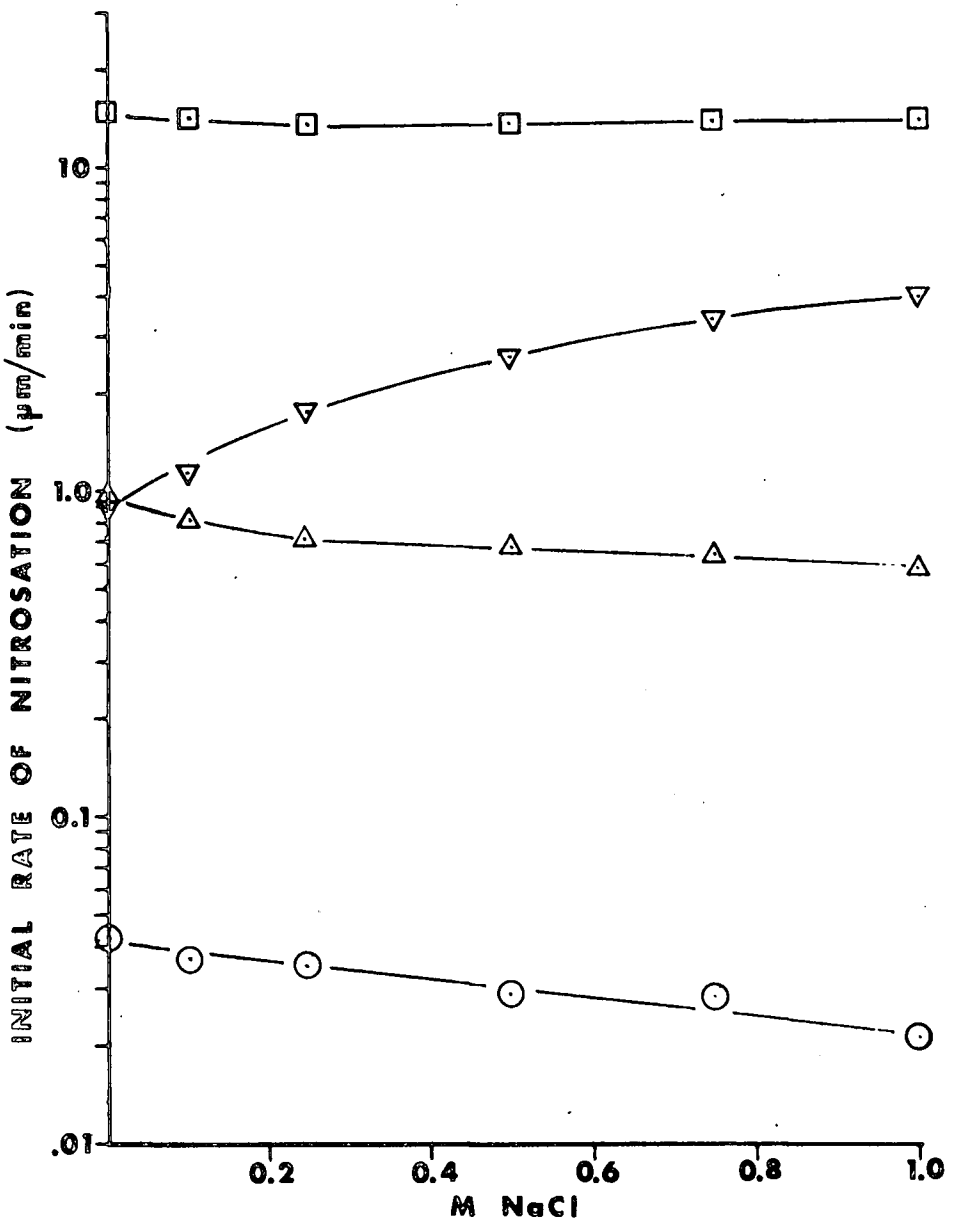


Figure 16. Effect of sodium chloride concentration on the nitrosation of proline at different pH levels: ▽ pH 0.5; □ pH 2.5; △ pH 4.0; ○ pH 5.5.

Table 9. Regression models describing the relationship between sodium chloride concentration and rate of nitrosation of proline at different pH levels.

pH	Model
0.5	$\text{Log(Rate)}^{a} = -0.097 + 2.773[\text{NaCl}] - 1.323[\text{NaCl}]^2$
2.5	$\text{Log(Rate)} = -2.690 - 0.248[\text{NaCl}] + 0.195[\text{NaCl}]^2$
4.0	$\text{Log(Rate)} = -0.078 - 0.865[\text{NaCl}] + 0.425[\text{NaCl}]^2$
5.5	$\text{Log(Rate)} = -3.194 - 0.594[\text{NaCl}]^{b}$

[a]Log base 10

[b]Quadratic term not significant

Table 10. Statistical description of regression models.

	pH 0.5	pH 2.5	pH 4.0	pH 5.5
Mean squares total	0.3117	0.00146	0.0313	0.0574
Mean squares regression	2.6042	0.00674	0.2420	0.4551
Mean squares residual	0.00607	0.000751	0.00318	0.00434
t-values of the quadratic term $[\text{NaCl}]^2$	-6.930	2.903	3.075	-0.240

1973). In addition, these investigations demonstrated that at pH's greater than 2, the nitrous anhydride mechanism also becomes operative. In the presence of chloride, it is likely that similar effects would be observed. However, as chloride is a weaker promotor than bromide or thiocyanate (Fan and Tannenbaum, 1973), the nitrous anhydride mechanism may start exerting a dominant influence at a pH level lower than 2.

In this experiment, at pH 0.5, the rate of formation of nitrosoproline was strongly enhanced by increased sodium chloride concentrations (Figure 16), which was probably due to the formation of activating nitrosyl chloride. As the concentration of nitrosyl halide is proportional to the square of the hydrochloric acid concentration, the rate of nitrosation with nitrosyl chloride increases rapidly with acidity (Challis and Butler, 1968). The rate of nitrosation increased linearly with sodium chloride concentration, which agrees with equation 2 where the anion is a first-order participant.

At pH 2.5 the situation is more complex as both nitrosation mechanisms were probably operative. A slightly inhibiting effect of sodium chloride on the rate of nitrosation was observed at this pH. The activating effect of nitrosyl chloride would be less at pH 2.5 than at pH 0.5, and was apparently counterbalanced by the inhibiting effect of the high ionic strength caused by sodium and chloride ions in the medium.

By increasing the sodium chloride concentration at pH 4.0 and 5.5, moderately inhibiting effects were observed. The nitrous anhydride mechanism was predominant at these pH levels, and the promoting effect by nitrosyl chloride was negligible. Thus the inhibiting effects by high ionic strength at pH's 4.0 and 5.5 became more evident than observed at pH 2.5.

The effects on the reacting rate of the addition of a salt to a reacting system can be at least of two different types. The increase in the ionic strength of the medium caused by salt changes the activity coefficients of neutral molecules, which can lead to decreased reaction rates (Frost and Pearson, 1961). This effect is called the primary salt effect. For reactions involving catalysis by acids or bases there is a secondary salt effect which has to do with the effect of ionic strength on the dissociation of weak acids or bases (Frost and Pearson, 1961).

The influence of salt on the activity coefficients of both the reactants and the activated complex must be considered when evaluating the primary salt effect.

As the nitrosation of amines is an acid-catalyzed reaction, secondary salt effects are likely in the presence of sodium chloride. When nitrous anhydride is the nitrosating species, the concentration of this species in the system affects the reaction rate. At higher pH levels, the rate limiting step in the nitrosation reaction is the

formation of nitrous anhydride (Hughes et al., 1958).

$$ONO^- + H_3O^+ \rightleftharpoons HONO + H_2O \overset{HONO}{\rightleftharpoons} H_2O^+NO + ONO^- \overset{slow}{\rightleftharpoons}$$

$$ONONO + H_2O$$

Increasing the ionic strength will favor the stability of charged species, which will shift the rate limiting step, the formation of nitrous anhydride, to the left. The inhibition observed by sodium chloride on the nitrosation of proline at higher pH levels probably is mainly due to secondary salt effects.

This experiment describes the relationship between sodium chloride concentration and rate of nitrosation of proline at different pH levels. Strong activation of the nitrosation of proline was seen at high acidities (pH 0.5), and inhibiting effects were observed at lower acidities. In recent publications (Boyland, 1972; Sander, 1973) the point has been made that halide ions are catalytic agents in the nitrosation reactions. As shown by this work, one must consider the pH of the system to determine whether a catalytic or inhibiting effect from chloride ions would be predicted. Whereas chloride might activate nitrosations in strongly acidic, human gastric juice, the same ion would be expected to produce a moderately inhibiting effect on nitrosation in mildly acidic or neutral food systems.

SUMMARY AND CONCLUSIONS

The reactions between sodium nitrite and the polyamines spermidine and spermine were investigated. When reacted at 80°C for 1 hr at pH 3.5 with a 1:3 molar ratio of amine groups to sodium nitrite, five volatile nitrosamines were identified as products in the nitrosation of spermidine·3HCl, and two volatile nitrosamines were identified from spermine.4HCl.

The principal volatile nitrosation product both from spermidine and spermine was γ-butenyl(β-propenyl)nitrosamine (BPN). The identification of this compound was based on the spectral characteristics of the compound, using mass, infrared, and nuclear magnetic resonance spectrometry. To confirm the identity, BPN was synthesized from γ-butenyl(β-propenyl)amine and sodium nitrite, and the spectra obtained for this substance and the unknown compound were compared. The amine precursor was synthesized from allylamine and 1-Br-4-butene.

Two hydroxylated, dialkyl nitrosamines were identified as products from spermidine·3HCl: γ-butenyl(γ-propanol)nitrosamine and δ-butanol(β-propenyl)nitrosamine. The compounds were characterized by mass and infrared spectrometry, and by the Griess test for nitrosamines. The structures were confirmed by making the trifluoroacetate derivatives of the compounds, and obtaining their mass spectra.

In the presence of chloride ions, chlorinated dialkyl nitrosamines were tentatively identified as nitrosation products from spermidine. The identification was based on mass spectrometry, particularly the isotope effects by chlorine, and by the Griess test. It appeared that δ-butylchloride(β-propenyl)nitrosamine probably was a major isomer, but other structural isomers may also be formed.

The nitrosamines described above have previously not been synthesized or characterized. Their individual carcinogenic potency therefore is not known. Nitrosopyrrolidine, which was a nitrosation product both from spermidine and spermine, is a potent carcinogen. The yields of the individual nitrosamines from spermidine·3HCl and spermine·4HCl were, respectively: BPN, 1.7%, 1.4%; γ-butenyl-(γ-propanol)nitrosamine, 0.29%, 0%; δ-butanol(β-propenyl)nitrosamine, 0.18%, 0%; δ-butylchloride(β-propenyl)nitrosamine (and its isomers), 0.12%, 0%; nitrosopyrrolidine, 0.60% and trace amounts. The yields were estimated on the basis of the amount of polyamine precursor.

Maximum accumulation of all nitrosamines from spermidine was observed between pH 3.0 and 4.5, when reacting for 1 hr at 50°C. Increasing the reaction time to 25 hrs at 50°C, the yield of BPN at pH 5.0 exceeded the yield at pH 3.5. From the temperature effect on the nitrosation rate, activation energy for the formation of BPN from spermidine and nitrite was estimated to be 19 kcal/mol. In the

presence of 0.1-1 M sodium chloride at pH 4.0, no significant effects on the yields of BPN, γ-butenyl(γ-propanol)nitrosamine and δ-butanol-(β-propenyl)nitrosamines were observed, while the yield of δ-butyl-chloride(β-propenyl)nitrosamine (and its isomers) was strongly enhanced with increased sodium chloride concentration. The yields of all nitrosamines were drastically reduced in the presence of 1.5 M or 2.0 M sodium chloride in the system.

In the nitrosation of proline, sodium chloride in concentrations up to 1 M strongly activated the reaction at pH 0.5. Small inhibiting effects were observed at pH 2.5, however, and moderate inhibition by sodium chloride was seen at pH's 4.0 and 5.5. Multiple regression analysis showed the best fitted model was of the form, log (initial rate of nitrosation) = $a + b\,[NaCl] + c\,[NaCl]^2$ at all pH levels tested.

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