AN ABSTRACT OF THE THESIS OF

JOSEPH H. HOTCHKISS for the degree of MASTER OF SCIENCE

in Food Science and Technology presented on September 10, 1976

Title: FORMATION OF BIS(HYDROXYALKYL)N-NITROSAMINES AS PRODUCTS OF THE NITROSATION OF SPERMIDINE

Abstract approved: R. A. Scanlan

The purpose of this study was to characterize the nonvolatile, water soluble, bis(hydroxyalkyl)N-nitrosamines produced from the reaction of spermidine and nitrite.

The biologically occurring polyamine spermidine was reacted with nitrite in the presence of acid. The reaction products were isolated and converted to trimethylsilyl (TMS) derivatives. The derivatized products were separated by gas-liquid chromatography using an all glass, wall coated, capillary column. Mass spectral data were collected on the chromatographic effluent.

Four isomers of bis(hydroxyalkyl)N-nitrosamine were synthesized by combining the appropriate aminopropanol with the appropriate chlorobutanol. Infrared, nuclear magnetic resonance, mass spectral, and thin-layer chromatographic data were collected on the synthetic compounds to assure their structure. Kovat's

indices were collected on the TMS derivative of each authentic compound and compared to the retention data collected on the TMS derivatives of the reaction products of spermidine and nitrite. A comparison of the mass spectra of the TMS derivatives of the authentic compounds to the spectra of the reaction products having the same retention time allowed structural assignments to four compounds. The compounds identified, in order of decreasing amounts were: 4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine, 3-hydroxybutyl(3-hydroxypropyl)N-nitrosamine, 4-hydroxybutyl(2-hydroxypropyl)N-nitrosamine, and 3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine.

Formation of Bis(hydroxyalkyl)N-nitrosamines as Products of the Nitrosation of Spermidine

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

June 1977

APPROVED:

Professor in Food Science and Technology
in charge of major

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Date thesis is presented September 10, 1976

Typed by A and S Bookkeeping/Typing for Joseph H. Hotchkiss

ACKNOWLEDGMENTS

Appreciation is extended to Dr. R. A. Scanlan for his guidance throughout this investigation. Special thanks is also extended to Dr. L. M. Libbey for his help in obtaining and interpreting the mass spectra.

The writer expresses his gratitude to his wife, Kris.

This work was supported by Food and Drug Administration Grant 1 ROl FD-00382.

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I. INTRODUCTION

Two important discoveries have precipitated the intense interest in N-nitrosamines as food adulterants. The first was a report in 1956 that liver tumors could be induced in rats by feeding dimethylnitrosamine. The second report, in 1964, was that dimethylnitrosamine was responsible for liver disorders in domestic animals fed nitrite treated herring meal. Since these reports were first published, concern has mounted that N-nitrosamines can be formed by the interaction of nitrite and compounds containing an amine function. Because amines are ubiquitously distributed in biological materials and nitrite occurs in or is added to some foods, the occurrence of N-nitrosamines in foods has come under investigation. N-nitrosamines have indeed been found to be present in several nitrite preserved foods at low levels. The degree of risk, if any, to the population from these findings has not been fully assessed.

Because of the nature of the N-nitrosation reaction and the polyamine structure of spermidine, their interaction could possibly lead to a wide variety of N-nitrosation products. The polyamine

spermidine is widely distributed in biological materials including those foods preserved with nitrite. For these reasons it appears desirable to determine which N-nitrosamines are formed when spermidine is reacted with nitrite.

To date, only the more volatile products of this reaction have been investigated. The purpose of this study was to characterize the nonvolatile, water soluble, bis(hydroxyalkyl)N-nitrosamines in the reaction products of spermidine and nitrite.

II. REVIEW OF RELATED LITERATURE

The hepatotoxic nature of dimethylnitrosamine was recognized as long ago as 1937 (Freund). Seventeen years later Barnes and Magee (1954) also reported the toxic nature of dimethylnitrosamine and in 1956 (Magee and Barnes) reported that the compound was also a hepatocarcinogen in the rat. Despite these reports nitrosamines received little attention until 1964. In 1964 severe liver disorders in domestic animals fed nitrite preserved herring meal was reported (Ender et al., 1964). The nitrite preserved herring meal was shown to contain dimethylnitrosamine.

A great deal of work has since been undertaken which indicates that a majority of N-nitrosamine compounds tested are carcinogenic and that some are mutagenic and teratogenic as well. Of approximately 100 nitroso compounds tested, 80% have been found to be carcinogenic (Preussmann, 1974). Tumors have been induced in a wide variety of species (Swann, 1975). These carcinogenic effects have also been shown to be transplacental (Mohr et al., 1966). The biological effects of N-nitrosamines have been reviewed elsewhere (Druckrey et al., 1967; Magee and Barnes, 1967; Magee, 1971; Shank, 1975; Swann, 1975).

The possibility that nitrite reacts with amine compounds in our food supply to form carcinogenic N-nitrosamines clearly exists

(Lijinsky and Epstein, 1970). While no cases of cancer in man have been attributed to N-nitrosamines, there is indirect evidence that man, along with several other animal species, would be susceptible (Weisburger and Raineri, 1975). The potential health hazard associated with N-nitrosamines is genuine.

Chemical Properties

N-nitrosamines vary widely in their chemical and physical properties owing to the variety of possible substituents attached to the N-N=0 functional group. They are neutral compounds and may be either liquids or solids at room temperature with the lower molecular weight dialkylnitrosamines being water soluble. The volatility of nitrosamines varies and presents both advantages and disadvantages to the analyst (Foreman and Goodhead, 1975).

Nitrosamines as a group are fairly temperature and pH stable but stability does vary with the substituents on the amine nitrogen. Fan and Tannenbaum (1972) found an average halflife of 21 days for four nitrosamines of greatly different structure at intermediate pH and 110°C. They also reported that the introduction of a carboxyl group to the compound greatly reduced the stability of the compound.

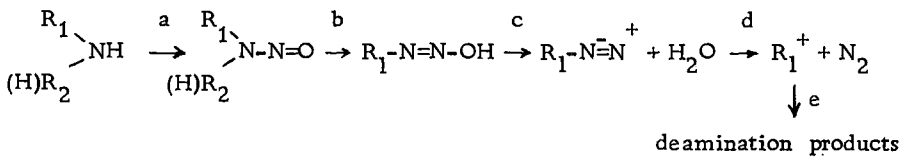
The nitroso group is sensitive to UV radiation. The nitroso group is cleaved producing nitrous acid and a secondary amine (Preussman et al., 1964). The chemical detection of the by products

of the cleavage has been used as a method for the qualitative analysis of nitrosamines.

Nitrosamine Formation

The chemistry of the nitrosation reaction has been studied in detail. Ridd (1961), Ingold (1969), Challis and Butler (1968), and Mirvish (1975) have presented detailed discussions.

The product(s) formed by the action of nitrous acid on amines depends upon the class of amine taking part in the reaction. Primary and secondary amines proceed via the following reaction path:



If the amine is secondary then the reaction stops at the nitrosamine; if the amine is a primary aromatic amine the reaction stops at the diazonium ion; if amine is a primary alkyl amine the reaction proceeds through the carbonium ion to deamination products.

The nitrosation step (step a) is the rate controlling step in all cases except those occurring at very low pHs. This has been demonstrated by the fact that the nitrosation of N-methylaniline follows the same kinetic equation and has a reaction rate similar to the

diazotization of aniline (Kalatzis and Ridd, 1966). An equality in the kinetic equations for diazotization and aliphatic deamination has also been demonstrated in several cases (Challis and Butler, 1968).

Nitrous acid is not the direct nitrosating agent, but must be first converted to a nitrosating species. Depending on the pH and presence of certain anions, aqueous nitrous acid is in equilibrium with one or more of the following nitrosating species (Challis and Butler, 1968).

$ON\text{-}NO_2$	nitrous anhydride
ON-X	nitrosyl halide
$ON\text{-}OH_2^+$	nitrous acidium ion
ON^+	nitrosonium ion

The high acidities required for the generation of the nitrous acidium ion and the nitrosonium ion decrease the probability of their acting as nitrosating agents in foods. In the absence of halide ions, nitrous anhydride is the major nitrosating species. Nitrous anhydride is formed in the following rapid equilibrium; $2HNO_2 \rightleftharpoons N_2O_3 + H_2O$. The overall reaction would then be third order and proceed according to the following rate expression (Hughes et al., 1958).

$$\text{rate} = k_1 [R_2NH] [HNO_2]^2$$

Where k_1 is pH independent but $[R_2NH]$ and $[HNO_2]$ must be

calculated for each pH because a change in pH affects the degree of protonation of the free amine. This protonation also depends in part on the basicity of the amine and explains why amines with weaker basicity are more reactive (Sander et al., 1968). Because nitrous acid is a weak acid (pk_a = 3.37) pH also affects the equilibrium forming nitrous anhydride. The rate equation,

$$\text{rate} = k_2 [\text{amine}] [\text{nitrite}]^2$$

is more useful in application because total concentrations are used but, k_2 changes with pH for the reasons discussed above (Mirvish, 1975).

The overall reaction can be described by the following equations:

$$2\ HNO_2 \overset{\text{fast}}{\rightleftharpoons} N_2O_3 + H_2O$$

$$N_2O_3 + R\text{-}\underset{\underset{H(R)}{|}}{N}\text{-}H \xrightarrow{\text{slow}} R\text{-}\underset{\underset{H(R)}{|}}{N}\text{-}N{=}O + HNO_2$$

$$R\text{-}\underset{\underset{H}{|}}{N}\text{-}N{=}O + H^+ \xrightarrow{\text{fast}} R\text{-}\overset{+}{\underset{-}{N}}{=}N + H_2O$$

$$R\text{-}\overset{+}{\underset{-}{N}}{=}N \xrightarrow{\text{fast}} R^+ + N_2 \xrightarrow{\text{fast}} \text{deamination products}$$

The nitrosyl halide mechanism may also be of importance in food systems. In the presence of halide ions, aqueous nitrous acid reacts in a rapid equilibrium to form nitrosyl halides;

$$HNO_2 + H^+ + X^- \underset{\text{fast}}{\rightleftharpoons} NO\text{-}X + H_2O$$

Nitrosyl halide is an effective nitrosating species. The overall kinetic equation for nitrosation would then become:

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

In food systems the nitrosyl halide and nitrous anhydride mechanisms would probably be operating simultaneously (Fan and Tannenbaum, 1973).

Mirvish (1975) describes catalysis of nitrosation by thiocyanate ion in a similar manner. The nitrosating species nitrosyl thiocyanate is formed in a rapid equilibrium. Unlike halide catalysis, thiocyanate catalysis decreases below pH 2 due to the protonation of NCS^-. Hildrum et al., (1975) has found catalysis by chloride anion to be pH dependent and inhibitory at lower acidities.

Deamination

The existence of the carbonium ion in the deamination of primary amines is based on stereochemical evidence. If the deamination reaction is followed polarimetrically, it can be observed

that the reaction involves racemization and partial inversion (Brewster et al., 1950). This is indicative of an S_n1 type reaction in which a carbonium ion is formed as an intermediate (March, 1968).

The carbonium ion formed has four pathways it may pursue toward stabilization (March, 1968).

1. The carbonium ion may combine with a species possessing an electron pair. This species may be H^-, OH^-, X^- or other negative ions including the nitrite ion which forms the alkyl nitrite. Alkyl nitrite was found to be the major product of the deamination of methylamine (Austin, 1960).
2. The carbonium ion may deprotonate. In this case a double bond is formed if the proton loss is α, or if the elimination is not α a ringed compound is formed.
3. The carbonium ion may rearrange to a more stable position through the migration of a hydrogen, alkyl or aryl group.
4. The carbonium ion may add to a double bond, creating a new positive charge. This mechanism usually involves polymerization.

Pathways 3 and 4 normally go on to react further, usually by pathways 1 or 2.

Precursors of Nitrosamines in Foods

Nitrate and Nitrite

Nitrate is ubiquitous in nature and has been used for fertilizer and as a food additive (Ridder and Oehme, 1974). Nitrates themselves, are not toxic but nitrate can be reduced to nitrite by bacterial action. Nitrite is acutely toxic if administered in sufficient quantity. This toxicity is due to the oxidation of hemoglobin from ferrous to ferric form producing methemoglobin. Methemoglobin does not reversibly bind oxygen and death can occur from anoxia (Swann, 1975).

In addition to that derived from nitrate reduction, nitrite is a permitted additive to foods at a residual level of 200 ppm (Code of Federal Regulations). This additive has three functions in the curing of meats: 1) color development, 2) production of cured flavor and 3) antibacterial activity (Wolf and Wasserman, 1972). Nitrite inhibits the outgrowth and hence toxin production of Clostridium botulinum (Duncan and Foster, 1968; Hustad et al., 1973). The level of added and/or residual nitrite necessary for inhibition of Clostridium botulinum has not been established due to the number of variables such as the spore level, salt concentration, and pH present in the food products to be preserved (Ingram, 1973). The

Federal Government has, however, proposed new standards of 156 ppm added sodium nitrite in canned cured products and an added level of 125 ppm in bacon (Federal Register, 1975).

Nitrite is also found in human saliva and may be related to in vivo formation of N-nitrosamines (Tannenbaum et al., 1974).

The nitrite anion is in equilibrium with nitrous acid ($pK_a = 3.34$) in acidic aqueous solutions

$$NO_2^- + H^+ \rightleftharpoons HNO_2$$

Because nitrous acid is the precursor to the various nitrosating agents, this equilibrium is necessary and hence nitrosation is considered to be an acid dependent reaction. This coupled with the necessity for unprotonated amine explains the pH and amine basicity dependence of the reaction.

Amines

With the possible exception of fish, the amine content of foods has received relatively little attention until recently. It has however become apparent that amines which are possible precursors to N-nitroso compounds are widely distributed in foods. Miller et al. (1973) found up to 150 ppm dimethylamine in fish protein concentrate and Patterson and Mottram (1974) followed the concentration of 5 volatile amines present in pork at various stages of

curing and storage. Dimethylamine continued to increase in concentration throughout the experiment and at 43 days averaged 348 mg/kg. Another worker has reported the trimethylamine, dimethylamine, and trimethylamine oxide content of 11 foods (Ruiter, 1974). Canned mackerel contained the greatest amount of dimethylamine (145 mg/kg) while banana contained the lowest (0.2 mg/kg). Certain amino acids can also serve as nitrosamine precursors (Eisenbrand et al., 1975).

In addition to the classical nitrosamine formation from secondary amines, primary, tertiary and quaternary amines can also form nitrosamines. Warthesen et al., (1975) have demonstrated the formation of N-nitrosamines from primary diamines but the yields were very low and make primary amines an unlikely source of nitrosamines in foods. Lijinsky (1974) has reported that 12 drugs containing a tertiary amine function reacted to give carcinogenic nitrosamines.

Polyamines

Polyamine compounds are widely distributed throughout biological tissues (Bachrach, 1973). At least seven of these type compounds occur naturally. All contain two or more amine groups; some being only primary while others are mixed primary and secondary. Spermine, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ and

spermidine $H_2N(CH_2)_3NH(CH_2)_4NH_2$ are found in the highest concentrations with spermidine being greater in many cases (Bachrach, 1973).

The polycationic nature of these polyamines impart several unique properties to the compounds in biological systems. The polyamines bind ionically to the polybasic nucleic acids (DNA) in the cell increasing the stability of the DNA (Lehninger, 1970). Spermine and spermidine have been shown to be growth factors for both bacterial and mammalian cells. In higher concentrations the compounds are toxic to many species including man (Bachrach, 1973).

Russell (1971) has found the urine of cancer patients to have a polyamine concentration up to 50 times that of a control group. After surgery the concentration returns to near normal. This increased polyamine excretion may be the basis of a new diagnostic tool for cancer.

Because spermidine contains a secondary amine function, it may form a nitroso compound under the proper conditions. The primary amine function could deaminate and produce a variety of substituents on the alkyl group. Hildrum (1975) has in fact identified four new volatile nitrosamines from the nitrosation of spermidine. The compounds are in order of decreasing yield: 3-butenyl(2-propenyl)N-nitrosamine, 3-butenyl(3-hydroxypropyl)N-nitrosamine,

4-hydroxybutyl(2-propenyl)N-nitrosamine, and 4-chlorobutyl(2-propenyl)N-nitrosamine. Nitrosopyrrolidine had previously been identified as a reaction product of the nitrosation of spermidine (Bills et al., 1973).

Ferguson et al., (1974) found spermidine to be rapidly nitrosated at pH 3.4 and 24°C. Higher nitrite to amine ratios gave higher yields. Specific products were not identified.

Hildrum (1975) found maximum accumulation of nitrosamines between pH 3.0 and 4.5, when reacting spermidine for 1 hour at 50°C. The activation energy for the formation of 3-butenyl(2-propenyl)N-nitrosamine was estimated to be 19 kcal/mol.

The spermidine content of various foods has been reported (Table 1). Concentrations of over 1% spermidine were observed for some putrefied pork samples. It must be pointed out that the same workers have reported spermidine concentrations which were lower by a factor of 10^2. The authors attribute this variability to a lack of knowledge concerning the history of the samples (Lakritz et al., 1975). These same authors conclude that cooking decreases the amine content of pork, while putrefaction causes a significant increase.

Analysis of Nitrosamines

Three major obstacles confront the analyst in determining nitrosamines in foods. The first is the low levels at which N-nitroso

Table 1. Contents of spermidine in various foods.

Food	Spermidine	References
Barley (germ)	29.1[a]	Moruzzi and Caldarera (1964)
Rice (germ)	15.3	"
Oats (germ)	30.7	"
Corn (germ)	12.4	"
Wheat (germ)	25.4	"
Sorghum (germ)	8.3	"
Soybean (flour)	1.64	Wang (1972)
Apple (fruit)	0.99	Smith (1970)
Spinach (leaves)	3.45	"
Pork (fresh)	0.57-1.45	Spinelli et al. (1974)
Pork (pickled)	0.36-1.07	"
Pork (bacon)	0.20-1.49	"
Pork (fresh)	13.4-125.0	Lakritz et al. (1975)
Pork (cooked)	9.7-70.0	"
Pork (putrified)	20.1-1013.0	"
Ham (smoked and cured)	15.1-127.3	"

[a] mg amine/100 g of wet tissue.

compounds have been found. The second problem is the complex matrix from which the nitrosamine must be separated. The last obstacle involves the need for absolute confirmation. Several authors have reviewed the various approaches taken by workers in their attempt to surmount these problems (Eisenbrand, 1973; Bogovski and Walker, 1974; Fiddler, 1975; Foreman and Goodhead, 1975).

The majority of the analytical procedures have been directed towards the more volatile nitrosamines. The preliminary cleanup in most analyses has been an atmospheric (Crosby et al., 1972) or vacuum distillation (Telling et al., 1971). Eisenbrand et al. (1970) found both methods comparable in recovery.

While some workers have used extraction as a preliminary cleanup method (Fazio et al., 1972) others have used extraction from basic and/or acidic distillates to remove non-neutral components (Fiddler et al., 1971; White et al., 1974). Dichloromethane is the most widely used solvent.

Further purification has generally been accomplished by chromatography. Thin-layer chromatography has been used (Eisenbrand et al., 1970a) but column chromatography is more prevalent (Eisenbrand, 1974).

Separations of nitrosamines has involved thin-layer chromatography (Sen and Dalpe', 1972) but more often gas-liquid

chromatography has been used (Foreman and Goodhead, 1975). In addition to the normal attributes of gas-liquid chromatography, it has the added advantage of being capable of interfacing with a mass spectrometer (Gough and Sawyer, 1974).

The detection of nitrosamines may be either by chemical methods or physical methods. Spray reagents have been used in thin-layer chromatography by some workers (Sen and Dalpe', 1972) while others have used derivitization as a means of detection (Eisenbrand, 1974). Several nitrogen sensitive or specific detectors have been coupled to the gas chromatograph. These detectors include the Coulson Electrolytic Conductivity Detector, Alkali Flame Ionization Detector, and the Election Capture Detector (Foreman and Goodhead, 1975). It must be emphasized however, that none of these methods of detection provide absolute proof for the presence of a nitrosamine. The only generally acceptable method of confirmation is mass spectrometry. Because of the public health implications, confirmation must be as nearly absolute as possible.

The analysis of nonvolatile nitrosamines has been especially difficult. High pressure liquid chromatographic methods have been described (Cox, 1973) but the lack of a sensitive, specific detector has been a major obstacle. Eisenbrand et al. (1975) have derivatized nonvolatile N-nitrosamino acids with trimethylsilyl derivatives rendering the compounds volatile.

A new method of detection which appears to be specific for the -N-N=O group has been described (Fine et al., 1974; 1975). Fine et al. (1975a) recently reported the analysis of a standard mixture of 14 nitrosamines at the sub ppb level using this same thermo-luminenscence technique. The instrument may be coupled to a chromatographic system and used as a highly specific detector.

Occurrence of Nitrosamines in Foods

Nitrosamines have occurred in foods sporadically and at the lower ppb range. The potential hazards of this class of compound have been established but the public health significance of the low levels which usually occur in foods is not known. There is however cause for concern. The subject of nitrosamines in foods has been reviewed in detail (Scanlan, 1975).

The methods of analyses used to confirm several reports of nitrosamines in foods prior to 1970 used nonspecific methods and are open to suspicion (Fiddler, 1975). After 1970 the majority of reports concerning nitrosamines in food were in fish and cured meats.

Marine fish would seem particularly susceptible to nitrosamine formation due to their high content of free amines (Keay and Hardy, 1972). Crosby et al. (1972) and Fazio et al. (1971) found levels of 1-26 ppb dimethylnitrosamine in raw fish and various fish products.

Crosby et al. (1972) found more dimethylnitrosamine positive samples and higher concentrations in heat processed fish than in fresh fish.

In two large survey studies Panalaks et al. (1973, 1974) showed that 96 of 277 cured meat samples were positive for one or more nitrosamines. Generally it can be concluded that nitrosamines occur sporadically in cured meats (Scanlan, 1975). The occurrence of nitrosopyrrolidine in fried bacon is however, an exception. Nitrite treated cooked bacon consistently contains an analyzable amount of this carcinogen (Fazio et al., 1973). Bills et al. (1973) has shown that the amino acid proline as well as four other compounds produce nitrosopyrrolidine under conditions designed to simulate bacon frying. A close review of the literature reveals that a total of nine compounds have been shown to produce nitrosopyrrolidine in the presence of nitrite and heat (Scanlan, 1975). These compounds include the polyamine spermidine (Bills et al., 1973).

The food additive ascorbate has been reported to partially block the formation of nitrosamines in food systems (Fiddler et al., 1973). Mirvish et al. (1972) attributes this to the fact that the ascorbate anion is more rapidly nitrosated and effectively competes for the nitrosating species present. The addition of these or other compounds to selected foods may reduce N-nitrosamine formation.

III. EXPERIMENTAL PROCEDURES

Reaction, Extraction and Concentration Procedure

A modification of the spermidine reaction procedure detailed by Hildrum et al., (1975a) was used. Ten mmol of the free base spermidine (Sigma Chemical Company) was dissolved in 20 ml distilled water and the pH of the solution adjusted to pH 3.5 (± 0.2) with 2 N H_2SO_4 and 0.1 N KOH, respectively. The solution was placed in a 100 ml three-neck round bottom flask fitted with a dipping thermometer, condenser, and magnetic stirring bar. The solution was cooled to 0°C over an ice and salt mixture. Ninety mmol sodium nitrite (Mallinckrodt Chemical Works) was dissolved in 20 ml distilled water and the pH of the solution adjusted to 3.5 (± 0.2) with 2 N H_2SO_4. The nitrite solution was added dropwise to the spermidine solution, keeping the temperature below 5°C.

The mixture was allowed to react one hour with the temperature between 0° - 5°C, then the temperature was slowly raised to 80°C and the reaction allowed to proceed for one more hour. The solution was saturated with anhydrous sodium sulfate (Mallinckrodt Chemical Works) and extracted with redistilled ethyl acetate (4 x 20 ml) (Mallinckrodt Chemical Works). The combined extracts were dried over anhydrous sodium sulfate for eight hours and then dried over

anhydrous calcium sulfate (Mallinckrodt Chemical Works) for 24 hours. The solvent was removed on a rotary evaporator at reduced pressure and 35°C.

Derivatization

Trimethylsilyl (TMS) derivatives were made by placing 5 to 10 mg of the compound or reaction mixture to be derivatized in a Silli-vial (Pierce Chemical Company) fitted with a teflon and silicon septum. One half ml acetonitrile (silylation grade, Pierce Chemical Company) and 0.5 ml Regisil(bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane, Regis Chemical Company) was added. The sealed vial was sonicated five minutes and heated to 100°C for 15 minutes.

The derivatized products from the nitrosation of spermidine were concentrated before analysis. When 0.5 ml of the silylation mixture was cooled to -5°C two layers separated. One tenth ml of the upper darker layer was concentrated to ca. 0.01 ml in a Concentratube (Laboratory Research Company). One half µl of of this material was injected onto the gas-liquid chromatography column.

Analytical Procedures

Thin-layer Chromatography

Thin-layer chromatography of the nitrosamines was accomplished on precoated 10 x 50 cm x 0.25 mm thick Silica gel G plates (Macherey-Negel and Company). The plates were developed in n-butanol:acetic acid:water (4:1:1, V/V/V) as the mobile phase. Some plates were developed in duplicate with one set of plates being sprayed with ninhydrin (0.3% ninhydrin in ethanol containing 2% pyridine) while the other was sprayed with Griess reagent as modified by Fan and Tannenbaum (1971). Both plates were exposed to UV radiation for color development. By comparison of those spots which are both ninhydrin and Griess positive, a more definitive confirmation of the presence of a nitrosamine can be achieved (Sen et al., 1968).

Gas-liquid Chromatography

All gas-liquid chromatographic analyses were performed on a Varian Aerograph Series 1400 Gas Chromatograph equipped with a flame ionization detector. An 80 m x 0.75 mm i.d. glass open tubular column coated with SE-30 (6.35 mg/ml coating solution) was used to facilitate most separations. The column was prepared in

accordance with procedures outlined elsewhere (Jennings et al., 1974). The entire system was made glass by the use of glass injection liners and glass lined stainless steel tubing for connecting the column to the detector. The chromatographic conditions for the analysis of TMS derivatives were as follows: flow N_2, 12 ml/min; temperature injector, 210°C; detector, 220°C; column, 180°C isothermal.

Kovat's indices were assigned to known and unknown peaks using the retention times of an externally chromatographed series of n-hydrocarbons (Alltech Associates).

Amines were analyzed for purity on a stainless steel column (0.13 in. o.d. x 1 ft.) packed with 28% Pennwalt 223, 4% KOH on Gaschrom R. Conditions were as follows: N_2 flow 30 ml/min; temperatures, injector 210°C, detector 270°C, and the column 160°C isothermal.

Spectrophotometric Analyses

Mass spectral data were collected on a Finnigan Quadrupole Mass Spectrometer Model 1015C coupled to a Varian Model 1400 Gas Chromatograph by an all glass jet separator. Gas-liquid chromatographic conditions were identical to those described above with the exception that helium was the carrier gas. Data were collected by a System Industries System/150 dedicated computer. Mass

spectrometer conditions were as follows; filament current, 1000 μA; electron voltage, 70 eV; analyzer pressure, 10^{-6} torr; multiplier voltage, 2.90 KV. Spectra were scanned from m/e 25 to m/e 400.

Infrared spectra were collected neat between NaCl discs by a Beckman Model IR-18A infrared spectrophotometer.

Nuclear magnetic resonance data were collected on a Varian Model HA-100 spectrometer at 100 MC. Tetramethylsilane was the external marker and D_2O was the solvent.

Synthesis of Authentic Compounds

Chlorobutanols

4-Chloro-2-butanol was synthesized by a modification of Sondheimer and Woodward's method (1953). One hundred eleven g anhydrous aluminum chloride (Baker Chemical Company) were dissolved in 300 ml chloroform and cooled to 5°C over ice and salt. Seventy-eight g of acetylchloride (Mallinckrodt Chemical Works) were added dropwise over a 20 minute period. Ethylene (Matheson Company) was bubbled through the mixture until no more was absorbed; ca. three hours. The temperature was kept below 10°C. The entire mixture was poured over one kg ice and 100 ml concentrated HCl. The organic layer was separated and washed with 50

ml 10% (V/V) HCl followed by 50 ml 5% sodium bicarbonate followed by 50 ml distilled water. The organic layer was dried over sodium sulfate overnight. A 40 cm Vigreux column was used to distill off the chloroform. The 4-chloro-2-butanone was distilled over the same Vigreux column under reduced pressure. Reduction to the alcohol was accomplished by dissolving 40 g of the ketone in 40 ml anhydrous ether. This mixture was added slowly to 5 g lithium aluminum hydride in 150 ml anhydrous ether. An ice and salt bath was used to keep the temperature below 20°C. After reacting 0.5 hour distilled water was added dropwise to destroy excess reagent. One hundred fifty ml 10% (V/V) sulfuric acid was added. The organic layer was removed and the aqueous phase washed with anhydrous ether (2 x 100 ml). The organic layers were combined, washed with 50 ml 5% sodium bicarbonate followed by 50 ml distilled water, and dried over anhydrous sodium sulfate. The ether was removed by distillation and the product distilled under reduced pressure. The chlorobutanol was redistilled over a 20 cm Vigreux column at 18 mm. The bp was 62°C. Yield was 32 g or 79% of theoretical.

4-Chloro-1-butanol was synthesized as outlined elsewhere (Blatt, 1943). A 500 ml three necked flask was fitted with a reflux condenser, dipping thermometer, and gas diffusion tube. One hundred fourteen g of tetrahydrofuran (Baker Chemical Company) was heated to reflux. Technical grade hydrogen chloride (Merck Chemical Company) was bubbled through the diffuser into the liquid. Diffusion was continued for five hours, at which time the

reflux temperature was 104°C. The liquid was cooled and fractionally distilled at reduced pressure. The fraction distilling at 80-90°C at 18 mm was collected and redistilled over a 40 cm Vigreux column at reduced pressure. Yield was 94 g (55% theoretical) and the bp at 18 mm was 86°C.

Aminopropanols

Practical grade 3-amino-1-propanol (Eastman Organic Chemicals) was purified by distillation over a 20 cm Vigreux column. Ninety-five percent 1-amino-2-propanol (Aldrich Chemical Company) was purified in a similar manner.

Bis(hydroxyalkyl)amines

The method of Falbe et al., (1965) was modified for the synthesis of the secondary amines. To a 100 ml round bottom three-neck flask fitted with a dipping thermometer, condenser and funnel was added 48.6 g (0.64 mole) of the appropriate aminopropanol. The flask was cooled to 0°C over ice and salt and 10 g (0.093 mole) of the appropriate chlorobutanol added dropwise. The temperature was raised to 80°C over a one hour period while the mixture was magnetically stirred. The reaction was allowed to proceed for three more hours at 80°C. The reaction mixture was next cooled and excess reagent distilled off at reduced pressure. The remaining

residue was dissolved in 100 ml 10% (V/V) hydrochloric acid. Solid sodium hydroxide was added until the amine oiled out of solution. The amine was separated and distilled under reduced pressure using a short path distillation head. The fraction distilling at 160-165°C at 15 mm was collected. Yield was 5.5 g or 82% of theoretical.

Bis(hydroxyalkyl)N-nitrosamines

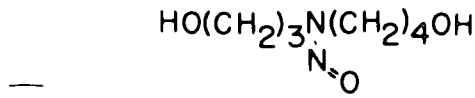
The method presented by Dutton and Heath (1956) was modified for the nitrosation of the amines. Ten mmol (1.5 g) of the free amine was dissolved in 10 ml distilled water. The pH of the solution was adjusted to 3.5 (± 0.2) with 2 N H_2SO_4 and 0.1 N KOH and cooled to 0°C over an ice salt mixture. Forty mmol (2.8 g) sodium nitrite (Mallinckrodt Chemical Works) was dissolved in 10 ml distilled water and the pH adjusted to 3.5 (± 0.2) with 2 N H_2SO_4 acid and 0.1 N KOH. The sodium nitrite solution was added dropwise to the cooled amine solution in a three-neck round bottom flask. The reaction mixture was reacted at 0°C for one hour and heated to 60°C for two additional hours. The reaction mixture was cooled, saturated with sodium sulfate and extracted with ethyl acetate (4 x 20 ml). The extracts were combined and dried first over anhydrous sodium sulfate, then over anhydrous calcium sulfate. The solvent was removed by rotary evaporation at reduced pressure. The compound was further purified by distillation over a short path

distillation head at reduced pressure. The compound formed was confirmed by tlc, ms, gc-ms, nmr and ir in most cases. Kovat's indices were determined on the TMS derivatives using pure n-hydrocarbons (Alltech Associates) as standards.

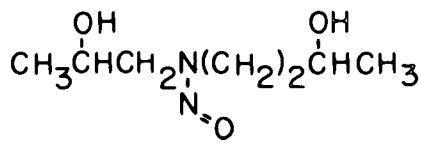
The structures of the four N-nitrosamines synthesized in this manner are given in Figure 1.

Safety

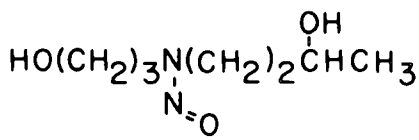
Because of the potent carcinogenicity demonstrated by several N-nitroso compounds in laboratory animals, extreme caution was used in handling the compounds. All work was done under fume hoods, laboratory coats and gloves were worn, and absorbent covers were used in all work areas. All glassware and solutions were decontaminated as outlined by Eisenbrand and Preussman (1970b). Gas-liquid chromatographic effluents were vented to a fume hood. Respirators were kept available for emergency use in case of a mishap.



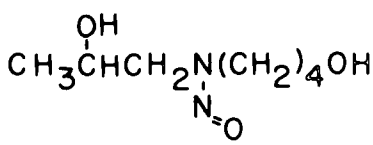
4-HYDROXYBUTYL(3-HYDROXYPROPYL)N-NITROSAMINE



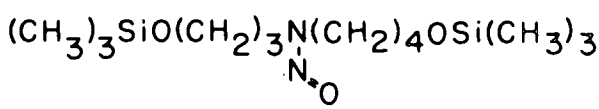
3-HYDROXYBUTYL(2-HYDROXYPROPYL)N-NITROSAMINE



3-HYDROXYBUTYL(3-HYDROXYPROPYL)N-NITROSAMINE



4-HYDROXYBUTYL(2-HYDROXYPROPYL)N-NITROSAMINE



BIS-TMS-4-HYDROXYBUTYL(3-HYDROXYPROPYL)N-NITROSAMINE

Figure 1. Structures of bis(hydroxyalkyl)N-nitrosamines.

IV. RESULTS AND DISCUSSION

Reaction Products

The derivatized products of the nitrosation of spermidine gave several peaks when gas-liquid chromatographed. A plot of the total ion current with overlays of limited mass searches for the molecular ion m/e 320 and ions of mass 305 and 303 was made by the data system (Figure 2). This revealed that five peaks could possibly correspond to the bis-TMS-bis(hydroxyalkyl)N-nitrosamines. Based solely on the spectra obtained from the reaction products it was not possible to definitively determine if the hydroxyl functions were primary, secondary or mixed.

Peaks 1, 2, 3, 4, and 5 had Kovat's indices of 1678, 1725, 1750, 1766 and 1843 respectively.

Thin-layer chromatography revealed that the underivatized nitrosation products gave a strong positive spot with a Rf value equal to authentic 4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine.

Authentic Compounds

Mass spectral analyses conducted on the synthesized 4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine and 3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine which were introduced into the mass

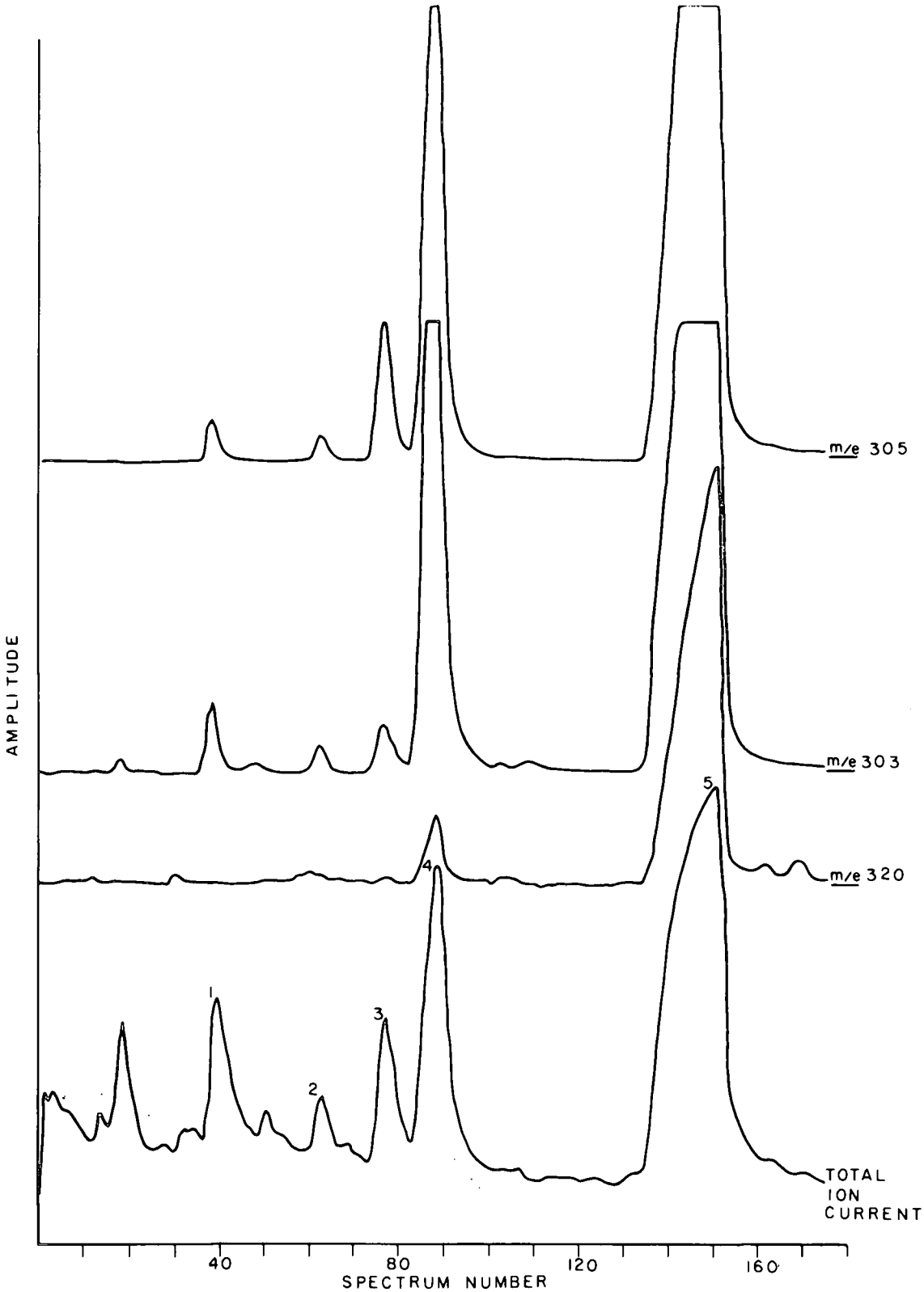


Figure 2. Total ion current plot and limited mass searches for m/e 320, 305, 303.

spectrometer by solid probe revealed the presence of only a weak molecular ion, m/e 176. A weak molecular ion is a common occurrence with alcohols and especially secondary alcohols (Silverstein et al., 1974). The M+1 ion is, however, clearly visable in the disecondary hydroxy compound. This is presumably due to the high inlet pressure of the instrument and is a common occurrence in hetero atomic compounds (Silverstein et al., 1974). The fragmentation pattern of both the diprimary bis(hydroxyalkyl) and the disecondary bis(hydroxyalkyl)N-nitroso compounds were consistent with the scheme outline by Saxby (1972) for the fragmentation of alkyl nitrosamines. Both compounds showed the loss of a hydroxyl radical to produce the m/e 159 ion. Both compounds also showed the loss of the NO radical to produce an m/e 146 ion. Cleavage at the α-carbon of the longest chain produced the m/e 117 ion which subsequently lost HNO to produce the m/e 86 ion. The most significant difference in the two spectra was at m/e 161. This would correspond to M-15. Even though the disecondary bis(hydroxyalkyl) compound gave a weaker total spectrum, the m/e 161 ion was clearly present. The m/e 161 ion did not appear in the spectrum of the diprimary bis(hydroxyalkyl) compound. Because M-15 corresponds to the loss of CH_3, evidence was provided that one compound contains only primary hydroxyl groups while the other compound must contain secondary hydroxyl groups. The suggested fragmentation scheme of

the diprimary can be seen in Figure 3. The abbreviated (Hertz et al., 1971) spectra[1] are given in Table 2.

Infrared spectra (Figure 4) collected on both the 4-hydroxybutyl (3-hydroxypropyl)N-nitrosamine and the 3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine showed broad absorption bands centered about 3380 cm^{-1}, which is typical of alcohols in a pure liquid state. The molecule containing only primary hydroxyl groups gave a strong band at 1065 cm^{-1}, which is in the area of primary alcohols. The compound containing secondary alcohols demonstrated absorption at 1130 cm^{-1} which was not seen in the primary alcohol compound. The N-N stretch of the nitroso group absorbs in the region of 1065-1015 cm^{-1} (Colthup et al., 1964) and thus may be confused with the primary alcohols but does not interfere with the secondary stretch. The strong absorptions in the region of 3000-2800 cm^{-1} were assigned to the aliphatic C-H stretching vibrations.

Thin-layer chromatography of the authentic diprimary and disecondary alcoholic nitrosamines indicated the presence of only one nitroso compound. The diprimary alcoholic nitrosamine had an Rf value of 0.60 while the disecondary alcoholic nitrosamine had a slightly lower Rf value of 0.57.

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[1] Spectra were abbreviated by listing the two largest ions every 14 AMU beginning at m/e 20. Ions with less than 0.11% of total intensity were not listed, with the exception of the molecular ion which is italicized.

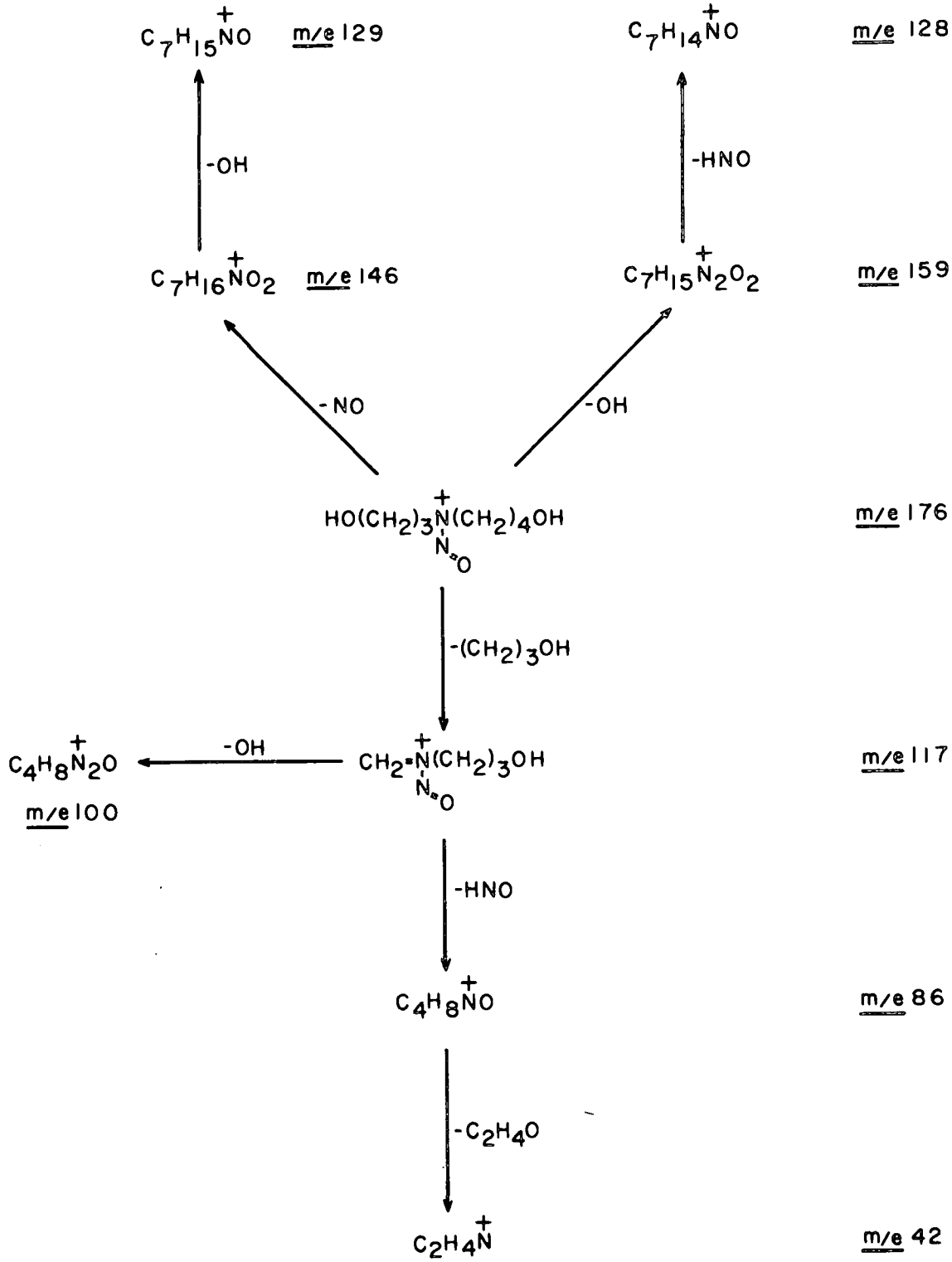

Figure 3. Suggested mass spectrometric fragmentation scheme of 4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine.

Table 2. Abbreviated mass spectra.

m/e	intensity[a]	m/e	intensity	m/e	intensity
4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine (Authentic)					
29	44.23	100	29.60	160	1.95
30	68.34	102	26.70	176	0.22
42	100.00	104	5.94		
44	65.97	117	3.26		
55	66.42	128	33.87		
56	40.34	129	4.61		
70	19.63	132	1.81		
71	49.22	145	0.89		
84	23.42	146	7.10		
86	68.49	159	24.80		
3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine (Authentic)					
29	73.92	86	36.42	141	0.42
30	86.77	88	24.89	145	0.40
42	99.77	100	53.66	146	1.85
45	99.88	102	16.01	159	2.56
55	100.00	114	1.53	160	0.31
56	75.44	117	1.33	161	0.72
70	14.01	119	11.04	176	<0.10
71	10.10	128	1.52	177	0.21

Table 2. Continued

m/e	intensity	m/e	intensity	m/e	intensity
Bis-TMS-3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine (Authentic)					
29	10.35	101	9.28	160	8.87
30	9.86	103	10.47	172	1.43
42	37.54	115	37.39	174	1.20
45	45.95	117	99.06	175	0.35
55	16.18	130	67.81	189	0.22
59	30.95	131	32.99	200	0.21
73	97.49	144	36.59	303	0.40
75	100.00	145	7.35	305	0.38
76	7.08	147	8.62	320	<0.10
84	6.28	158	21.99		
Bis-TMS-4-hydroxybutyl(2-hydroxypropyl)N-nitrosamine (Authentic)					
29	2.70	76	2.06	144	23.45
30	0.69	84	13.67	145	8.33
45	16.53	99	1.08	147	6.76
47	5.23	103	4.09	158	4.28
59	13.14	115	10.73	174	1.58
61	3.73	117	100.00	184	0.25
73	73.85	118	16.08	200	4.13
75	31.44	130	41.38	201	0.53

Table 2. Continued

m/e	intensity	m/e	intensity	m/e	intensity
246	0.31	291	0.16	305	1.86
290	0.62	303	0.32	320	<0.10

Bis-TMS-3-hydroxybutyl(3-hydroxypropyl)N-nitrosamine (Authentic)

29	6.50	115	16.14	189	1.23
30	10.12	117	24.74	200	0.63
43	38.08	129	7.68	213	0.40
44	85.51	131	14.49	215	0.24
55	23.98	143	10.65	233	0.11
56	35.77	144	19.04	273	0.61
73	100.00	147	2.90	275	0.24
75	48.27	158	11.75	303	1.61
83	8.54	160	3.86	305	1.79
84	17.84	171	1.33	320	<0.10
101	13.23	174	0.99		
103	18.83	175	0.57		

Table 2. Continued

m/e	intensity	m/e	intensity	m/e	intensity
Bis-TMS-4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine (Authentic)					
29	12.82	117	13.32	202	0.86
30	12.94	129	9.30	213	2.23
42	43.43	131	25.25	219	0.24
45	40.09	144	36.21	231	0.34
55	38.84	145	35.60	232	0.12
59	42.09	147	13.53	274	0.12
73	99.11	158	16.42	290	1.28
75	76.03	160	1.66	291	0.42
84	100.00	172	3.55	303	2.53
85	25.51	174	7.52	305	4.52
101	33.11	185	2.16	320	<0.10
103	74.72	200	10.67		
110	15.54	201	2.00		
Bis-TMS-3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine (spermidine)					
29	2.54	54	6.75	82	4.77
30	5.63	59	6.87	84	11.64
42	10.07	73	100.00	101	2.08
45	8.63	75	29.50	103	3.78

Table 2. Continued

m/e	intensity	m/e	intensity	m/e	intensity
115	8.53	158	9.34	208	0.13
117	57.72	160	2.25	212	0.15
123	11.61	172	1.22	226	0.87
130	14.55	183	0.76	228	0.64
143	3.12	185	0.35	303	0.83
144	5.67	195	0.53	305	0.81
155	2.50	197	0.48	320	<0.10
Unidentified peak (spermidine)					
27	1.26	101	6.10	160	0.92
29	1.60	103	3.60	165	1.57
43	14.07	115	2.06	174	1.13
44	9.74	116	2.44	180	0.36
59	9.64	129	3.68	261	0.37
61	2.69	131	66.84	290	0.30
71	31.49	132	7.96	303	0.46
73	100.00	144	22.14	305	0.96
76	1.28	148	2.19		
84	1.73	158	9.04		

Table 2. Continued

m/e	intensity	m/e	intensity	m/e	intensity
Bis-TMS-4-hydroxybutyl(2-hydroxypropyl)N-nitrosamine (spermidine)					
29	3.68	103	6.68	174	1.74
30	2.76	115	6.83	175	0.25
42	10.11	117	91.74	200	3.24
45	15.41	118	9.94	201	0.62
55	9.30	130	23.36	202	0.17
59	12.12	144	14.25	246	0.20
73	100.00	145	6.04	290	0.39
75	27.34	147	3.61	303	0.22
84	22.84	158	3.11	305	1.26
85	3.43	172	0.93	306	0.31
101	4.19	173	0.26	320	<0.10
Bis-TMS-3-hydroxybutyl(3-hydroxypropyl)N-nitrosamine (spermidine)					
29	9.55	73	64.49	115	24.19
30	12.19	75	86.74	117	35.06
42	59.05	83	14.53	129	15.09
44	100.00	84	12.82	131	30.53
56	44.44	101	22.41	143	25.55
59	31.36	103	35.34	144	31.84

Table 2. Continued

m/e	intensity	m/e	intensity	m/e	intensity
147	6.03	189	3.05	233	0.24
158	28.27	190	0.42	274	0.17
160	8.42	213	0.60	303	3.29
173	2.79	215	0.49	305	3.38
174	2.33	218	0.12	320	<0.10
175	1.16	231	0.25		
Bis-TMS-4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine (spermidine)					
29	15.93	117	14.59	202	0.88
30	14.87	129	10.32	213	2.39
42	53.91	131	28.18	219	0.23
45	49.81	144	41.10	231	0.34
55	47.58	145	40.41	242	0.98
59	52.59	147	15.04	274	0.11
73	88.77	158	18.38	290	1.22
75	92.36	170	1.92	291	0.36
84	100.00	172	3.99	303	2.33
85	29.57	174	7.74	305	4.05
101	41.65	185	2.50	320	<0.10
103	87.02	200	11.28		
110	17.60	201	2.04		

[a] Intensity is given as percent of base peak.

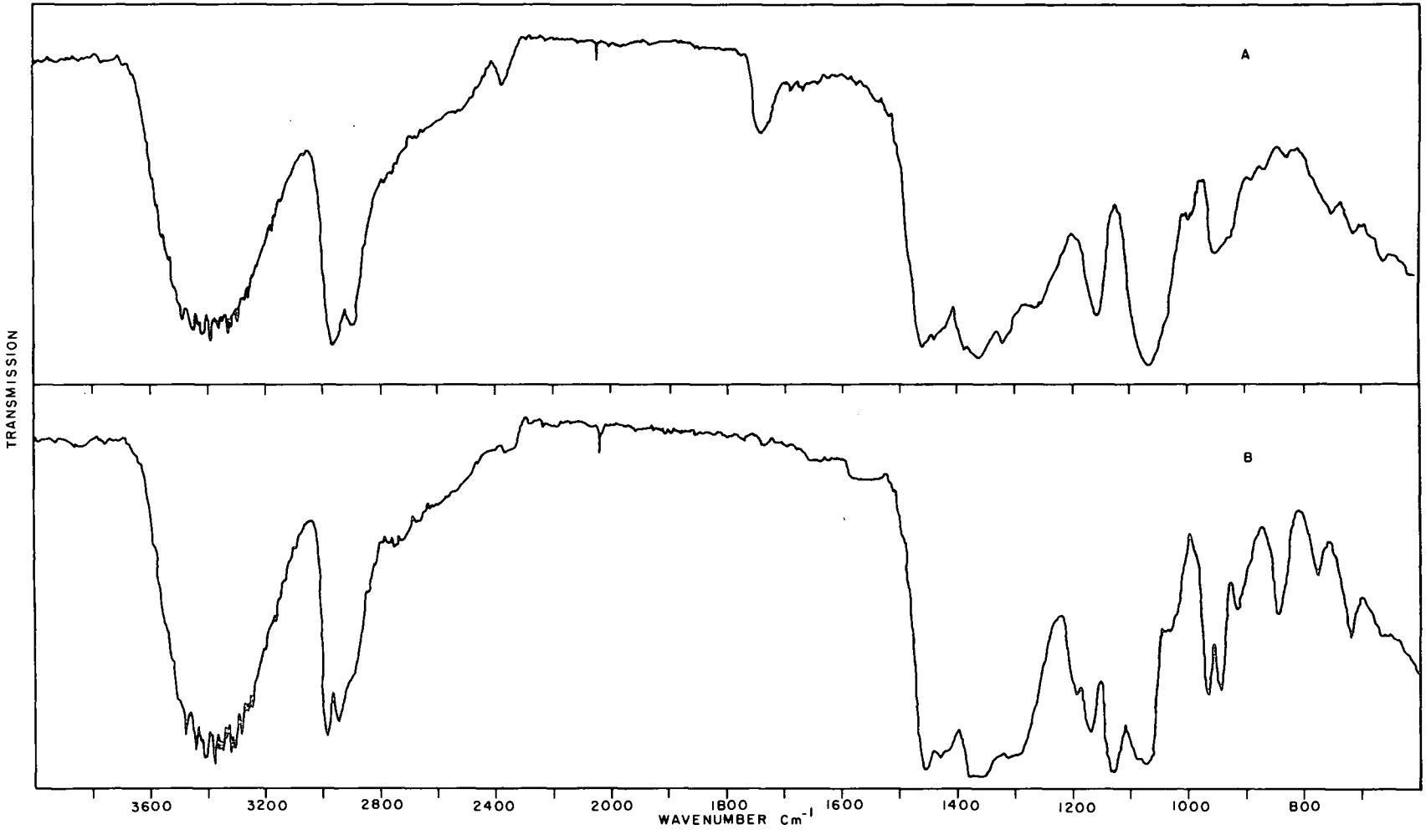

Figure 4. Infrared spectra of A. 4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine and, B. 3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine.

The nuclear magnetic resonance spectra of the diprimary bis(hydroxyalkyl)N-nitrosamine and disecondary bis(hydroxyalkyl)N-nitrosamine further confirmed their structure (Figures 5 and 6). Their spectra were complicated by the fact that the partial double bond character of the N-N bond restricts the rotation of the nitroso group. This forms two conformers because one nitrogen substituent is *syn* to the oxygen atom while the other is *anti* to the oxygen atom (Looney *et al.*, 1957). The *syn* and *anti* conformations of each proton are not magnetically equivalent.

The spectrum of 3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine can be interpreted as follows; the singlet at 4.86 τ is due to the D_2O solvent, the methylene group which is not bound to a hetero atom gives two sets of triplets (corresponding to the *syn* and *anti* forms) at 7.88 τ and 7.60 τ. An area equivalent to six protons appears at the expected shift for methyl protons (Silverstein *et al.*, 1974). The methyl protons resonated between 8.46-8.30 τ and exhibited the expected four sets of doublets. The remaining protons resonated as two similar complex patterns (*syn* and *anti* forms) centered about 5.79 and 5.37 τ.

The spectrum of 4-hydroxybutyl(3-hydroxpropyl)N-nitrosamine may be interpreted in a similar manner. There is however one striking difference; the absence of absorption in the region occupied by methyl proton resonance. This leads to the conclusion that

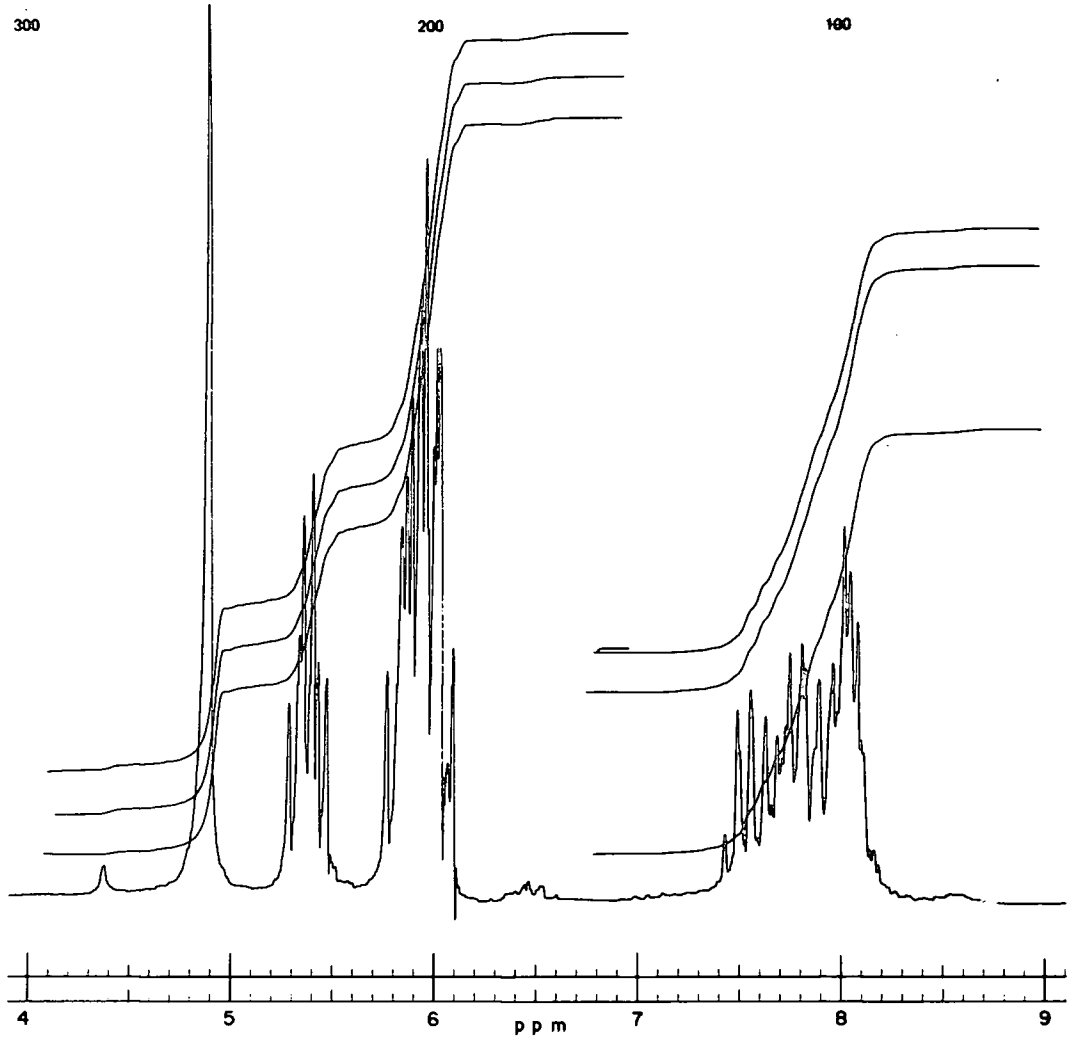

Figure 5. Nuclear magnetic resonance spectrum of 4 hydroxybutyl (3-hydroxypropyl) N-nitrosamine.

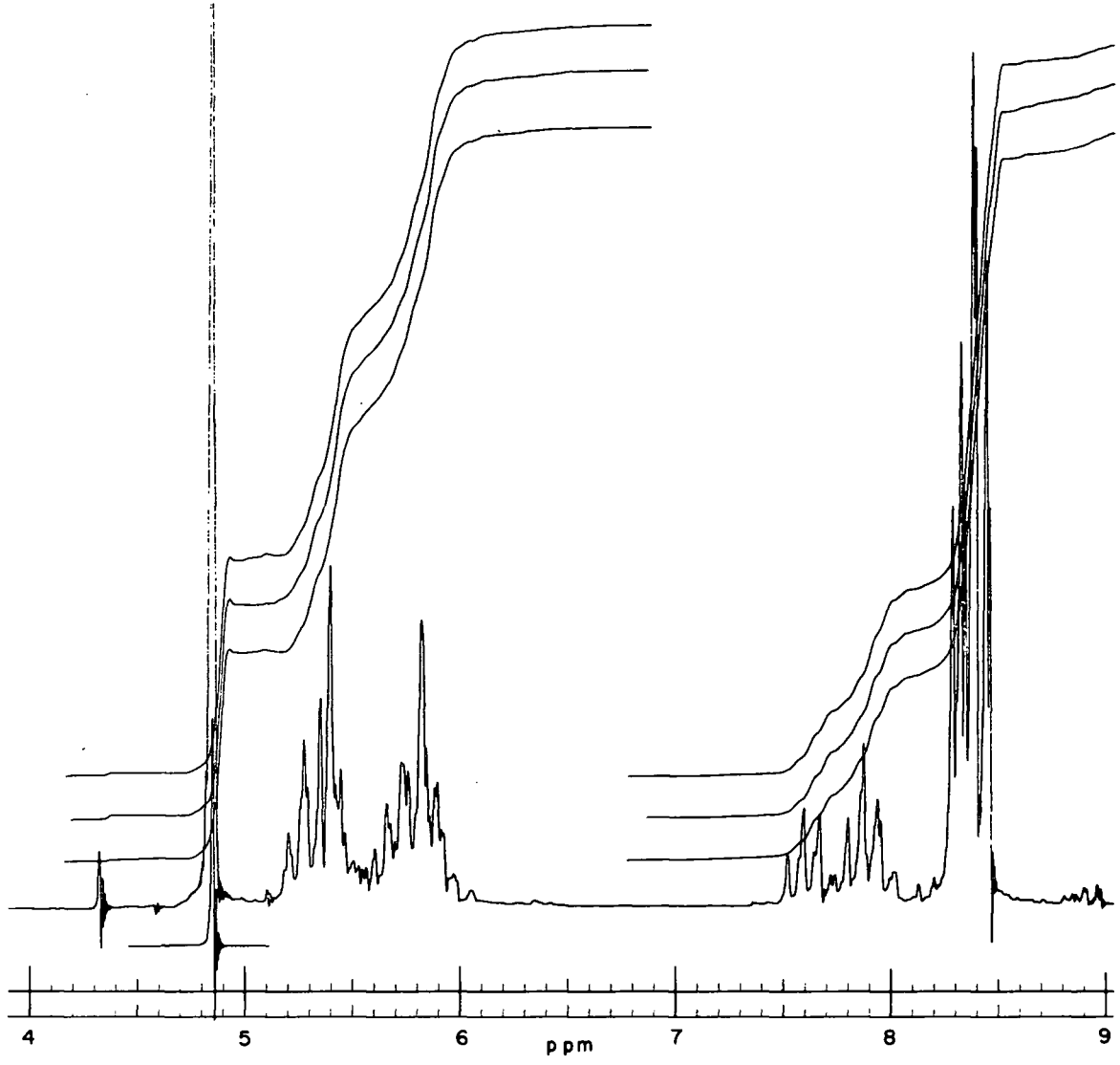

Figure 6. Nuclear magnetic resonance spectrum of 3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine.

methyl groups, even as contaminants, are absent.

Mass spectral data were collected on the bis-trimethylsilyl derivatives of each of the four bis(hydroxyalkyl)N-nitrosamines synthesized. The compounds were introduced to the mass spectrometer via gas-liquid chromatography. All four isomers gave very similar spectra. Only weak molecular ions were observed but the M-15 ions, resulting from the loss of CH_3, were prominent. This is a common occurrence with trimethylsilyl ether compounds (Pierce, 1968). In addition to the M-15 ion, M-17 ion corresponding to the loss of OH is visible in all spectra. The loss of OH is a common occurrence with dialkyl nitrosamines (Saxby, 1972). The abbreviated spectra for all four isomers are presented in Table 2.

The retention times of the four isomers differed substantially. Kovat's indices were assigned to the bis-trimethylsilyl derivatives of the four compounds as follows: bis-TMS-3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine I_x = 1684; bis-TMS-4-hydroxybutyl(2-hydroxypropyl)N-nitrosamine I_x = 1751; bis-TMS-3-hydroxybutyl(3-hydroxypropyl)N-nitrosamine I_x = 1772; bis-TMS-4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine I_x = 1853.

Identification of Reaction Products

A comparison of the Kovat's indices of the four authentic compounds and the Kovat's indices of the five peaks in the derivatized

nitrosated spermidine which contained the proper ions was made. Each known compound compared closely with a peak from the derivatized, nitrosated spermidine. Peaks were identified as follows (Figure 2); peak 1, bis-TMS-3-hydroxybutyl(2-hydroxypropyl) N-nitrosamine; peak 3, bis-TMS-4-hydroxybutyl(2-hydroxypropyl)N-nitrosamine; peak 4, bis-TMS-3-hydroxybutyl(3-hydroxypropyl)N-nitrosamine; peak 5, bis-TMS-4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine.

Peak 2 (Figure 2) had a Kovat's index which did not correspond to the index of any authentic compound, yet the peak gave a spectrum characteristic of a bis(hydroxyalkyl)N-nitrosamine. Both the M-15 and M-17 ions as well as a large m/e 73 were present. The structure of this compound was not determined but it may be a β carbonium ion rearrangement product.

Mass spectra of the peaks eluting from the chromatography of the reaction further confirmed that they were in fact the same four compounds synthesized (Table 2). As an example the suggested fragmentation scheme of bis-TMS-4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine is shown in Figure 7. The molecular ion is weak as would be expected for a bis-TMS derivative (Pierce, 1968). The M-15 ion is apparent at m/e 305. Cleavage of this species α to the nitroso group on the longest chain produced the m/e 174 ion. The molecular ion can also lose m/e 17 to produce m/e 303 which

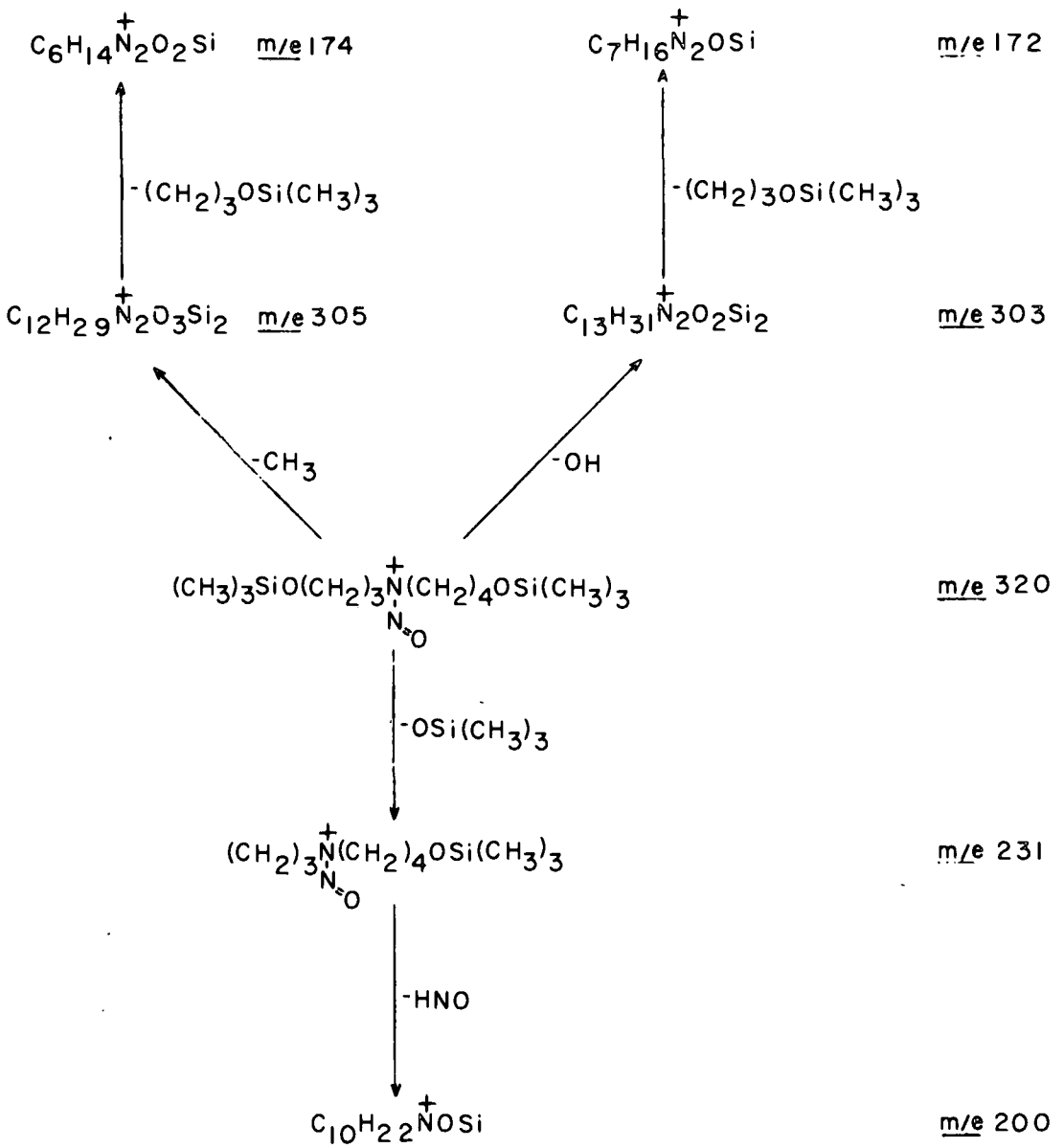

Figure 7. Suggested mass spectrometric fragmentation scheme of bis-TMS-4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine.

subsequently loses m/e 131 to produce the m/e 172 ion. The M+ ion can cleave between the oxygen and carbon and produce the m/e 231 ion which subsequently loses HNO to produce a strong m/e 200. The spectrum of each peak was compared to the spectrum of the authentic compound having the same retention time and with few exceptions appeared nearly identical.

Pathway of Nitrosamine Formation

The proposed pathway for the formation of bis(hydroxyalkyl)N-nitrosamines as products of the nitrosation of spermidine is presented in Figure 8. Because spermidine contains both primary and secondary amine groups, both nitrosation and deamination can occur within any one single molecule.

The reaction proceeds as outlined by Ridd (1961). The N-nitrosation of the secondary amine stops at the formation of the N-nitrosamine. The primary amines, however, proceed to the diazonium ion which subsequently deaminates to a carbonium ion. The carbonium ion may either rearrange to a secondary carbonium ion or it may react through solvolysis with water to produce a primary hydroxylated product. The secondary carbonium ion may also react through solvolysis to produce a secondary hydroxylated product. Because spermidine is unsymmetrical and each primary amine can react independently, four hydroxylated products are

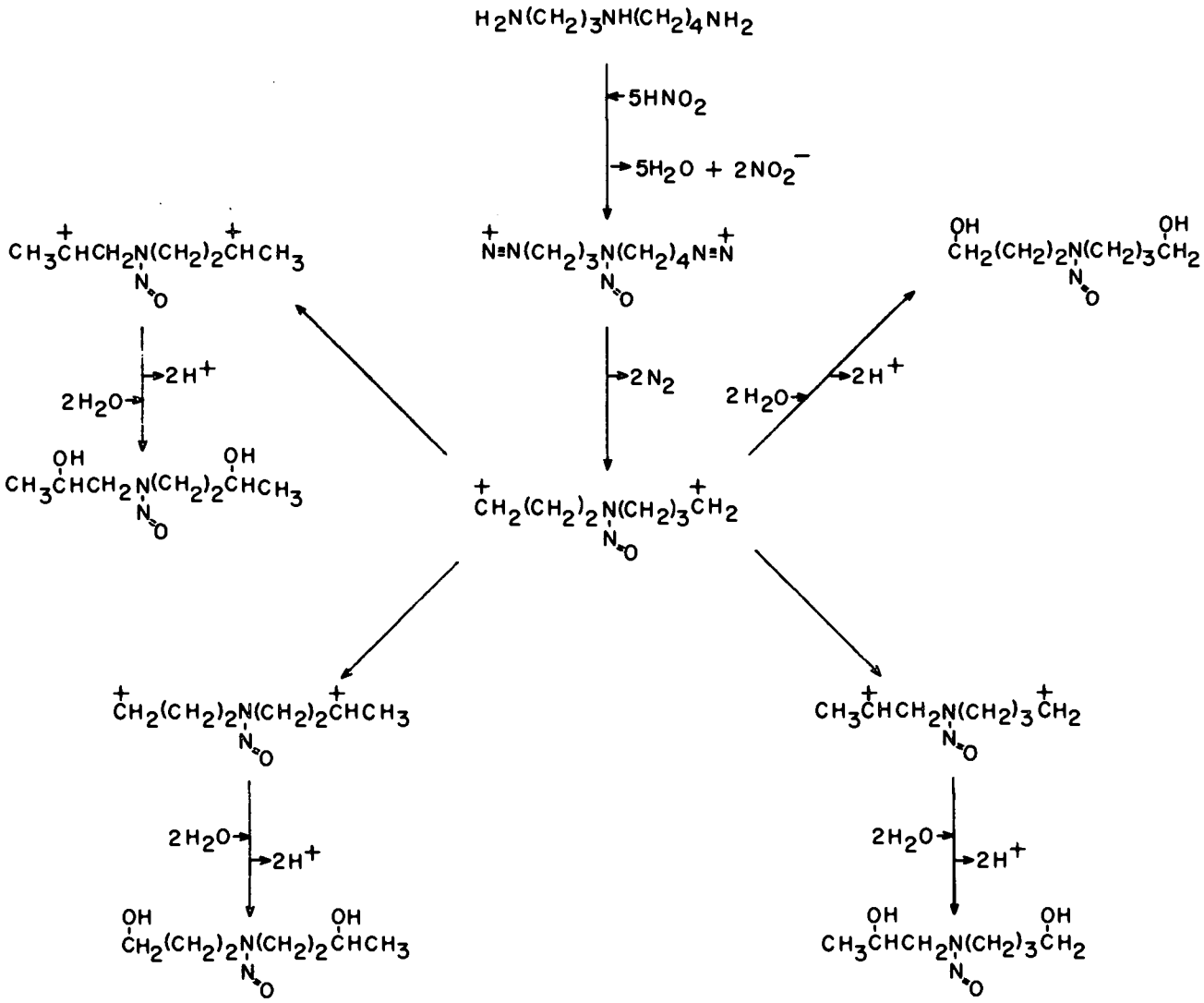

Figure 8. Suggested pathway of formation of bis(hydroxyalkyl) nitrosamines from the nitrosation of spermidine.

possible considering only α-carbon carbonium ion rearrangements.

Amine Purity

Gas-liquid chromatography revealed that the spermidine contained approximately 2% volatile impurities. These volatile impurities would however, elute much earlier in the chromatogram than the compounds under study.

Artifacts

The possibility that the bis(hydroxyalkyl)amines were artifacts or contaminants in the spermidine at low levels was considered. Ten mg spermidine and 10 mg 4-hydroxybutyl(3-hydroxypropyl)amine were separately derivatized and chromatographed. Based on retention times and mass searches of the spermidine chromatogram, it could be concluded that the spermidine contained essentially no 4-hydroxybutyl(3-hydroxypropyl)amine. The amine spectrum contained m/e 363 (M^+) and m/e 348 (M-15) ions. The lack of the m/e 363 and m/e 348 ions in the spermidine chromatogram was taken as evidence that the other bis(hydroxyalkyl)amines were also absent.

Bis(hydroxyalkyl)N-nitrosamines in Foods

The high concentrations of spermidine found in nitrite treated pork (Lakritz et al., 1975) coupled with the fact that

bis(hydroxyalkyl)N-nitrosamines can be formed from spermidine would indicate that these compounds may exist in some heat treated foods. Current analytical procedures which are based on a distillation step would not detect the dihydroxy compounds due to their low volatility. The dihydroxy nature of these N-nitrosamines would probably also exclude their removal from food in most analytical procedures involving extraction from aqueous phase with dichloromethane. Even if these problems were not present, the compounds cannot be gas-liquid chromatographed without derivatization. It can be concluded that present methods of analysis of N-nitrosamines in foods would probably not detect the bis(hydroxyalkyl)N-nitrosamines.

Two general approaches might be taken in the analysis of a food matrix for bis(hydroxyalkyl)N-nitrosamines. The first is high pressure liquid chromatography. As the detectors and interfacing apparatuses become more refined this method will probably become the method of choice. The other approach would be a combination of extraction with a water immiscible yet polar solvent such as ethyl acetate, and possibly cleanup by column chromatography. After concentration and derivatization, gas-liquid chromatography-mass spectrometry could be used for separation and identification.

V. SUMMARY AND CONCLUSIONS

The biologically occurring, polyamine compound spermidine was reacted with nitrite in the presence of acid. The reaction products were isolated and derivatized to the trimethylsilyl ether derivatives. Mass spectra of the effluent of a gas-liquid chromatographic separation of the reaction products were obtained. Limited mass searches for those ions which may indicate bis(hydroxyalkyl)N-nitrosamines revealed that five peaks could possibly be isomeric forms of the bis(hydroxyalkyl)N-nitrosamines. Four possible isomers which differed only in the location of the hydroxyl groups were synthesized. Infrared, nuclear magnetic resonance, mass spectral, and thin-layer chromatographic data were collected on the synthetic 4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine and 3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine. Comparison of the Kovat's indices of the known compounds and the reaction products allowed the assignment of structure to four of the five peaks in the chromatogram. That the synthetic compounds were also products of spermidine nitrosation was further confirmed by tandem gas-liquid chromatography-mass spectrometric examination.

The diprimary bis(hydroxyalkyl)N-nitrosamine was formed in the greatest amount and the disecondary bis(hydroxyalkyl)N-nitrosamine

was formed in the least amount. The ratios of the four products are as follows: 3-hydroxybutyl(2-hydroxypropyl)N-nitrosamine: 4-hydroxybutyl(2-hydroxypropyl)N-nitrosamine: 3-hydroxybutyl(3-hydroxypropyl)N-nitrosamine: 4-hydroxybutyl(3-hydroxypropyl)N-nitrosamine, 2:5:21:78.

This work has shown that under certain conditions a widely dispersed, naturally occurring compound can react with nitrite to produce at least four water soluble, nonvolatile N-nitrosamines. Because these compounds have not been previously studied, no information regarding their toxicity is available. Until such data are collected, the compounds should be considered as potentially dangerous based on the number of other nitroso compounds which have demonstrated carcinogenic activity.

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