destabilization of the protonated form of the amine with respect to the unprotonated form. This type of electrostatic effect explains, in part, the rate enhancements for nitrosation in the presence of cationic micelles (Okun and Archer, 1977). Moreover, cationic regions of the cell surface would attract nitrite ions, thereby producing high concentrations of the nitrosating agent in the vicinity of the amine.

The effect of amine structure on nitrosation in the presence of cells follows a similar pattern to nitrosamine formation in saliva, which we have previously investigated (Tannenbaum et al., 1977). Thus, the formation of nitrosamines in saliva—at close to neutral pH in this study—may be subject to the same kind of microbial rate acceleration. Similar microbial effects on nitrosamine formation may be expected in other situations of environmental importance, such as in the food itself, in the stomach, or in the intestinal tract. At pH 3.5, we did not observe rate enhancements for formation of dimethylnitrosamine or diethylnitrosamine in the presence of cells. Since microorganism-dependent formation of these products has been reported at higher pHs (Sander, 1968; Hawksworth and Hill, 1971, 1974; Klubes and Jondorf, 1971; Klubes et al., 1972; Thacker and Brooks, 1974; Mills and Alexander, 1976), mechanisms other than the one described here are clearly operative.

Nonenzymatic cellular rate enhancement of chemical reactions facilitated by hydrophobic forces has not previously been described. In addition to its potential importance in nitrosamine formation, it may also be a more general phenomenon for other reactions in which hydrophobic interactions between the cell, reactants, transition states, and products are possible.

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LITERATURE CITED

Ayanaba, A., Alexander, M., *Appl. Microbiol.* **25**, 862 (1973).
Ayanaba, A., Verstraete, N., Alexander, M., *J. Natl. Cancer Inst.* **50**, 811 (1973).
Collins-Thompson, D. L., Sen, N. P., Aris, B., Schwinghamer, L., *Can. J. Microbiol.* **18**, 1968 (1972).
Druckrey, H., Preussmann, R., Ivankovic, S., Schmähl, D., *Z. Krebsforsch.* **69**, 103 (1967).
Fine, D. H., Roubehler, D. P., *J. Chromatogr.* **109**, 271 (1975).
Hawksworth, G., Hill, M., *Brit. J. Cancer* **25**, 520 (1971).
Hawksworth, G., Hill, M. J., *Brit. J. Cancer* **29**, 353 (1974).
Klubes, P., Cerna, I., Rabinowitz, A. D., Jondorf, W. R., *Food Cosmet. Toxicol.* **10**, 757 (1972).
Klubes, P., Jondorf, W. R., *Res. Commun. Chem. Pathol. Pharmacol.* **2**, 24 (1971).
Mills, A. L., Alexander, M., *Appl. Environ. Microbiol.* **31**, 892 (1976).
Okun, J. D., Archer, M. C., *J. Natl. Cancer Inst.*, **58**, 409 (1977).
Sander, J., *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 429 (1968).
Sherbet, G. V., Lakshmi, M. S., *Biochim. Biophys. Acta* **298**, 50 (1973).
Tannenbaum, S. R., Archer, M. C., Wishnok, J. S., Correa, P., Cuello, C., Haenszel, W., "Origins of Human Cancer", Vol. 4, Cell Proliferation Series, Cold Spring Harbor Press, New York, in press (1977).
Thacker, L., Brooks, J. B., *Infect. Immun.* **9**, 648 (1974).
White, S. H., *Nature (London)* **262**, 421 (1976).

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

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The biologically occurring polyamine spermidine was reacted with nitrite in the presence of acid. Reaction products were isolated and converted to trimethylsilyl (Me_3Si) 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 determined for the Me_3Si derivative of each authentic compound and compared to the retention data collected on the Me_3Si derivatives of the reaction products of spermidine and nitrite. A comparison of the mass spectra of the Me_3Si 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.

The polyamine spermidine, $H_2N(CH_2)_3NH(CH_2)_4NH_2$, is ubiquitous in biological materials and is found in many food materials including several cereal germs (Moruzzi and Caldarera, 1964) and soybean flour (Wang, 1972). The levels found in pork and pork products are of special interest. Lakritz et al. (1975) reported concentrations of spermidine as high as 1013 mg/100 g of wet tissue in putrefied pork ham-butt portions. Fresh pork ham-butt portions ranged in concentration from 13.4 to 125 mg/100 g of tissue. Spinelli et al. (1974) reported spermidine

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concentrations in bacon of 0.17 to 1.49 mg/100 g of wet tissue. Fresh pork bellies ranged from 0.57 to 1.45 mg/100 g of wet tissue.

Spermidine contains one secondary and two primary amine groups which may react with nitrite. Nitrosation of the secondary amine and deamination of the primary amines could produce a variety of *N*-nitrosamines, some or all of which may be carcinogenic. Bills et al. (1973) demonstrated that *N*-nitrosopyrrolidine is a volatile product of spermidine nitrosation, and Hildrum et al. (1975) identified 3-butenyl-(2-propenyl)-*N*-nitrosamine as the principle volatile product. Hildrum (1976) also identified 4-hydroxybutyl-(2-propenyl)-*N*-nitrosamine, 3-butenyl-(3-hydroxypropyl)-*N*-nitrosamine, and 4-chlorobutyl-(2-propenyl)-*N*-nitrosamine as volatile spermidine nitrosation products.

Nonvolatile products could conceivably result from the nitrosation of spermidine. The present work describes the identification and characterization of the nonvolatile bis(hydroxyalkyl)-*N*-nitrosamine products resulting from the nitrosation of spermidine.

EXPERIMENTAL PROCEDURE

Reagents. Practical grade 3-amino-1-propanol (Eastman Chemical Co.) and 95% 1-amino-2-propanol (Aldrich Chemical Co.) were purified by distillation over a 20-cm Vigreux column. The method outlined by Blatt (1943) was used for the synthesis of 4-chloro-1-butanol. The method of Sondheimer and Woodward (1953) was used to synthesize 4-chloro-2-butanol.

Synthesis of Bis(hydroxyalkyl)-*N*-nitrosamines. A modification of the method of Falbe et al. (1965) was used to synthesize the bis(hydroxyalkyl)amines. The appropriate aminopropanol (48.6 g) was placed in a round-bottom flask and cooled to 0 °C. Ten grams of the appropriate chlorobutanol was added dropwise. The temperature was raised to 80 °C over a 1-h period and held for 1 additional h. The reaction mixture was cooled and excess amine distilled off at reduced pressure. The residue was dissolved in 100 mL of 10% HCl and solid NaOH added until the product oiled out. The amine was separated and fractionally distilled over a short-path distillation head at 165 °C and 15 mm. Yield was 5.5 g or 82% of theoretical.

Nitrosation of the amines was accomplished by dissolving 1.5 g of the amine in distilled water, adjusting the pH to 3.5 (±0.2) with 2 N H_2SO_4, and cooling to 0 °C. Sodium nitrite (2.8 g) dissolved in 10 mL of distilled water and adjusted to pH 3.5 (±0.2) was added dropwise with continued cooling. The mixture was reacted at 0 °C for 1 h, raised to 60 °C, and reacted 2 additional h. The reaction mixture was cooled, saturated with sodium sulfate, and extracted with ethyl acetate (4 × 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 and the residue distilled over a short-path distillation head at reduced pressure. Each compound formed was confirmed by TLC, MS, GC–MS, NMR, and IR in most cases. Kovat's indices were determined using *n*-hydrocarbons (Alltech Associated) as external standards. The structures of the four *N*-nitrosamines synthesized and of a $(Me_3Si)_2$ derivative are given in Figure 1.

Reaction, Extraction, Concentration. Ten millimoles of the free base spermidine was dissolved in 20 mL of distilled water, and the pH of the solution adjusted to 3.5 (±0.2) with 2 N H_2SO_4. The solution was cooled to 0 °C and 90 mmol of sodium nitrite, dissolved in 20 mL of distilled water and adjusted to pH 3.5 (±0.2), was added slowly with continued cooling. The reaction was allowed to proceed for 1 h at 0 to 5 °C, then the temperature was raised to 80 °C for 1 additional h. The reaction products were extracted, dried, and the solvent removed in the same manner as the authentic compounds above.

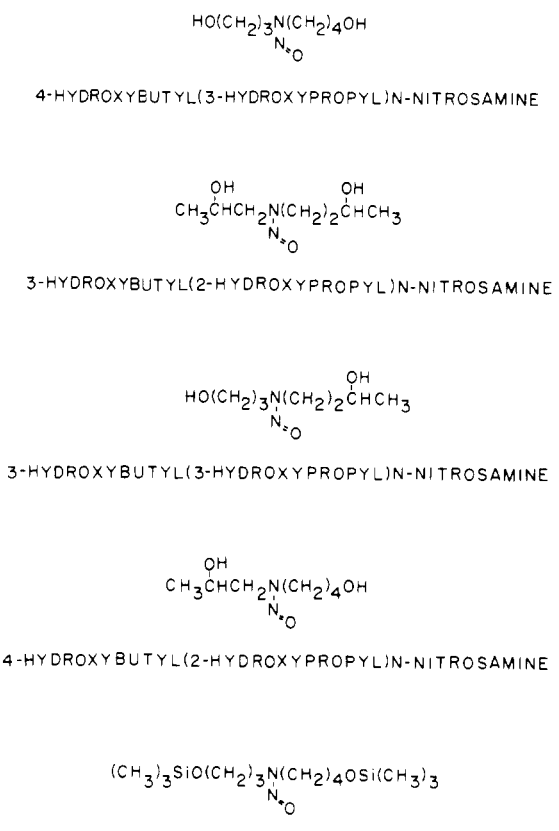

Figure 1. Structures of bis(hydroxyalkyl)-*N*-nitrosamines.

Derivatization. Trimethylsilyl (Me_3Si) derivatives were made by placing 5–10 mg of the compound(s) to be derivatized in a Silli-vial (Pierce Chemical Co.) fitted with a Teflon and silicone septum. Acetonitrile (0.5 mL) (silylation grade, Pierce Chemical Co.) and 0.5 mL of Regisil (bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane, Regis Chemical Co.) were added. The sealed vial was sonicated 5 min and heated to 100 °C for 15 min.

The derivatized products were concentrated approximately tenfold under a stream of N_2 at room temperature in a Concentratube (Laboratory Research Co.).

Thin-Layer Chromatography. Reaction products and authentic compounds were chromatographed on 0.25-mm thick Silica Gel G plates (Macherey-Nagel and Co.) using 1-butanol–acetic acid–water (4:1:1, v/v/v) as the mobile phase. Griess reagent as modified by Fan and Tannenbaum (1971) and UV irradiation were used for color development.

Gas–Liquid Chromatography. A Varian Aerograph series 1400 gas chromatograph (GC) equipped with a flame ionization detector was used for most separations. An 80 m × 0.75 mm i.d. open tubular wall-coated glass column, coated with SE-30 as outlined by Jennings et al. (1974) was used. A glass injection port and glass-lined stainless steel tubing for connection to the detector completed the system. Temperatures of injector, column, and detector were 210, 180, and 220 °C, respectively, and the flow rate of carrier gas (N_2) was 12 mL/min.

Kovat's indices were assigned using the corrected retention times of an externally chromatographed series of

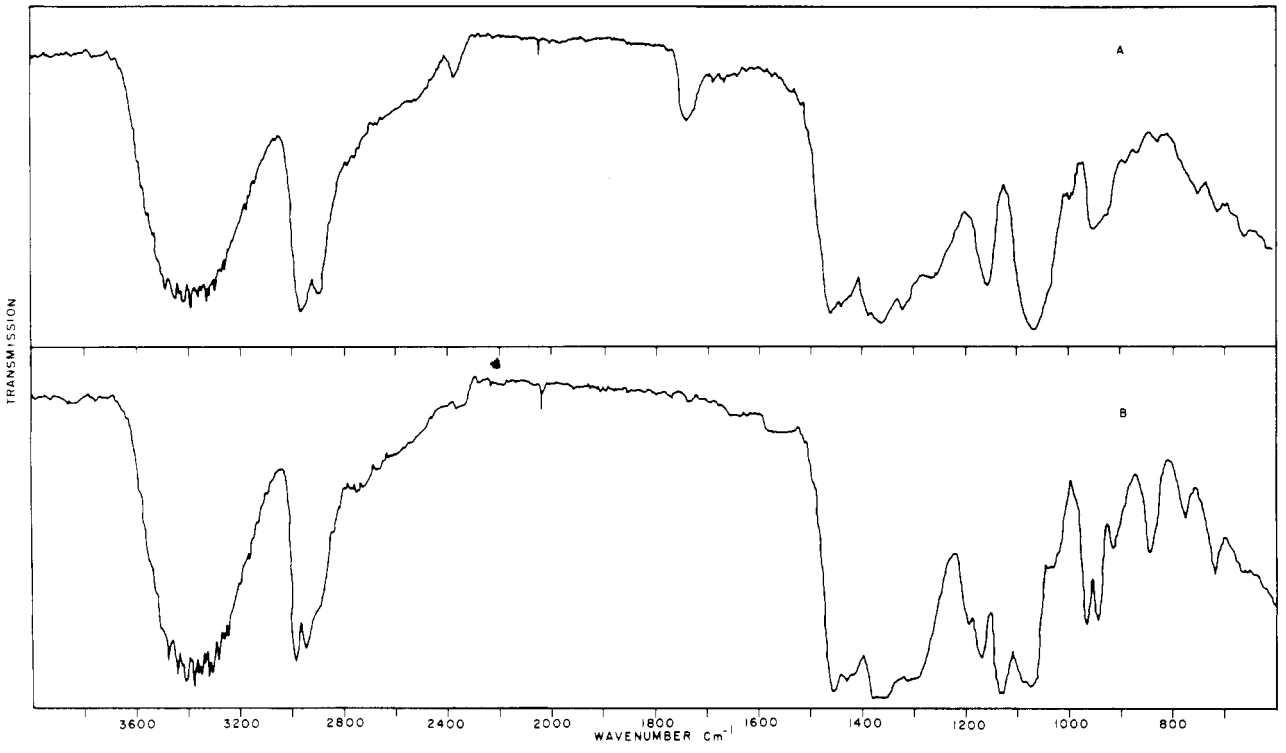

Figure 2. Infrared spectra of (A) 4-hydroxybutyl-(3-hydroxypropyl-*N*-nitrosamine and (B) 3-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrsoamine.

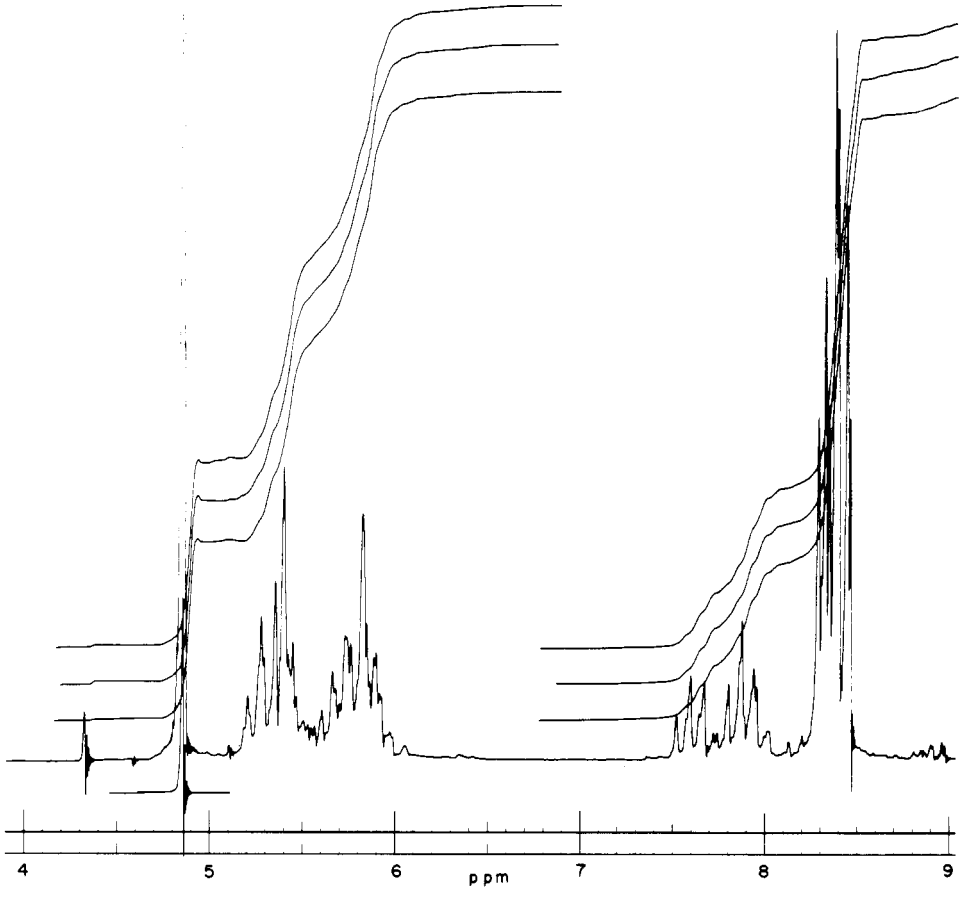

Figure 3. Nuclear magnetic resonance spectrum of 3-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine.

n-hydrocarbons (Alltech Associates).

Spectrometric Analyses. A Finnigan Model 1015C quadrupole mass spectrometer coupled to a Varian Model 1400 GC by an all glass jet separator was used to collect mass spectral (MS) data. The GC conditions were identical with those described above except helium was the carrier gas. Data was collected on a Systems Industries System/150 computerized data system. Mass spec-

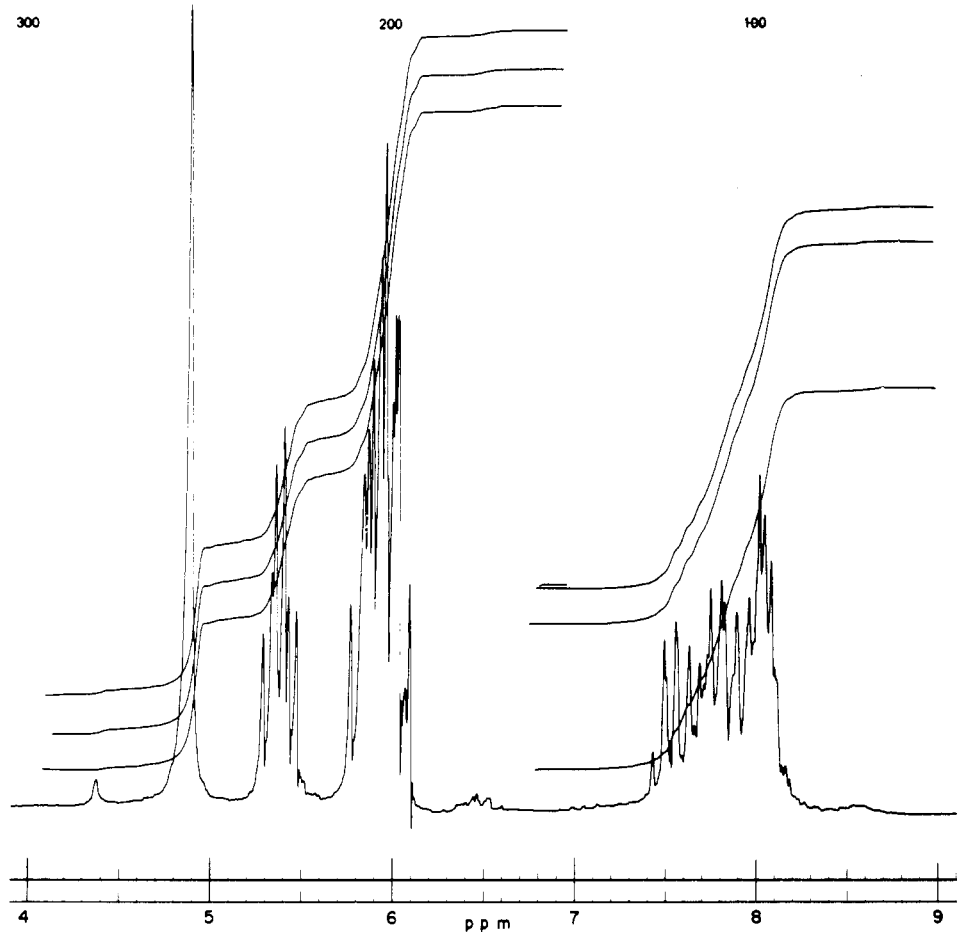

Figure 4. Nuclear magnetic resonance spectrum of 4-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine.

trometer conditions were: filiment current, 1000 μA; electron voltage, 70 eV; analyzer pressure, 10^{-6} Torr; and multiplier voltage, 2.90 kV. Spectra were scanned from *m/e* 25 to 400.

Samples were analyzed for infrared (IR) absorption neat between NaCl discs by a Beckman Model IR-18A spectrophotometer.

Nuclear magnetic resonance (NMR) data were collected by a Varian Model HA-100 spectrometer at 100 mc. Tetramethylsilane was an external marker.

Safety. The compounds synthesized are of unknown toxicity but should be handled as carcinogens.

RESULTS AND DISCUSSION

Mass spectra (Table I) of the synthesized 4-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine [4-OHB(3-OHP)N] and 3-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine [3-OHB(2-OHP)N], which were obtained by introducing the samples via solid probe, were consistent with those presented by Saxby (1972) for alkyl nitrosamines. A weak molecular ion at *m/e* 176 and large ions at *m/e* 159 and 146 were produced. The most significant difference in the two spectra occurred at *m/e* 161. This ion was absent from the isomer containing two primary hydroxyl groups but was present in the spectrum of the isomer containing secondary hydroxyls. The *m/e* 161 ion corresponds to the loss of CH_3 and is suggestive of the presence of methyl group(s) in one compound and the lack of methyl groups in the other.

Infrared spectra were collected on the neat 4-OHB(3-OHP)N and 3-OHB(2-OHP)N (Figure 2). The broad absorption at 3380 cm^{-1} was assigned to the hydrogen bonding in neat alcohols, and the sharp absorptions at 3000–2800 cm^{-1} was assigned to aliphatic C–H stretching.

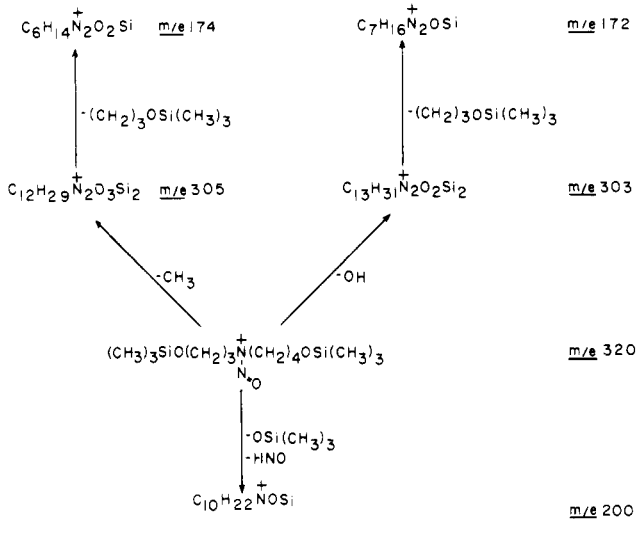

Figure 5. Suggested mass spectrometric fragmentation scheme of bis(trimethylsilyl)-4-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine.

The 1065 cm^{-1} absorption in both compounds was due to the N–N stretch of the nitroso group. This obscured the C–O stretch of the primary alcohol, but the secondary C–O stretch can be seen in the spectrum of 3-OHB(2-OHP)N at 1130 cm^{-1}.

The NMR spectra of 3-OHB(2-OHP)N (Figure 3) and 4-OHB(3-OHP)N (Figure 4) were complicated by the fact that the restricted rotation of the N–N bond creates syn and anti isomers (Karabatsos and Taller, 1964) which are not magnetically equivalent. In the spectrum of the compound containing secondary hydroxyl groups (Figure

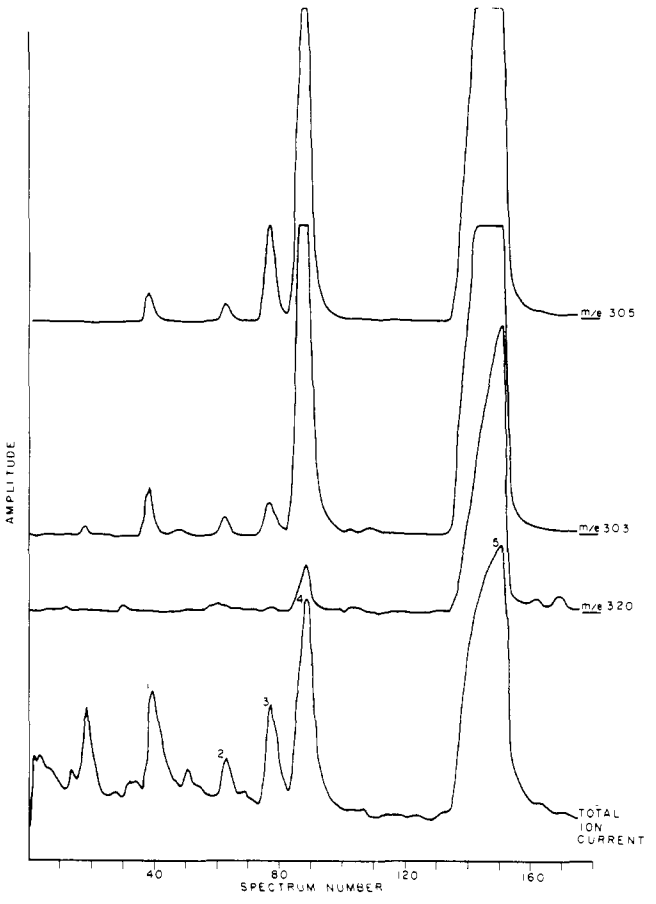

Figure 6. Total ion current plot and limited mass searches for m/e 320, 305, 303.

3), the expected four sets of doublets resulting from the two methyl group protons were seen at 8.46 to 8.30 ppm. These absorptions were lacking in the spectrum of 4-OHB(3-OHP)N (Figure 4), demonstrating the absence of methyl groups.

Mass spectral data was collected on the $(Me_3Si)_2$ derivatives of each of the four synthetic bis(hydroxylalkyl)-*N*-nitrosamines as well as the peaks eluting from the gas chromatography of the derivatized reaction products (Table I). Only weak molecular ions at m/e 320 were seen, but larger ions at m/e 303 and 305 were evident. The m/e 303 ion corresponds to the loss of OH, which is common to alkyl nitrosamines (Saxby, 1972), and the m/e 305 ion corresponds to the loss of CH_3, which is common to Me_3Si derivatives (Pierce, 1968).

The mass spectra of four of the peaks eluting from the gas chromatography of the reaction products confirmed that they were identical with the four authentic compounds (Table I). As an example, a suggested fragmentation scheme for $(Me_3Si)_2$-4-OHB(3-OHP)N is shown in Figure 5. The molecular ion is weak as would be expected for a $(Me_3Si)_2$ derivative (Pierce, 1968). The M–15 and M–17 ions are both visible. The m/e 303 and 305 cleave α to the nitroso group on the longest chain producing the m/e 172 and 174 ions, respectively. The molecular ion can also cleave between the oxygen and carbon atoms and produce the m/e 231 ion which subsequently loses HNO, producing the m/e 200 ion.

The chromatogram of the derivatized reaction products contained several peaks. Computer generated plots of the total ion current with overlays of limited mass searches for the molecular ion (m/e 320) and ions at m/e 305 and 303 indicated that five peaks could possibly correspond to the bis(trimethylsilyl)bis(hydroxyalkyl)-*N*-nitrosamines (Figure 6).

A comparison of Kovat's indices of the five peaks from the chromatogram of the reaction products and the Kovat's indices of the four authentic isomers allowed structural assignments as follows (Figure 6): peak 1, $(Me_3Si)_2$-3-OHB(2-OHP)N, I_x = 1684; peak 3, $(Me_3Si)_2$-4-OHB(2-OHP)N, I_x = 1751; peak 4, $(Me_3Si)_2$-3-OHB(3-OHP)N, I_x

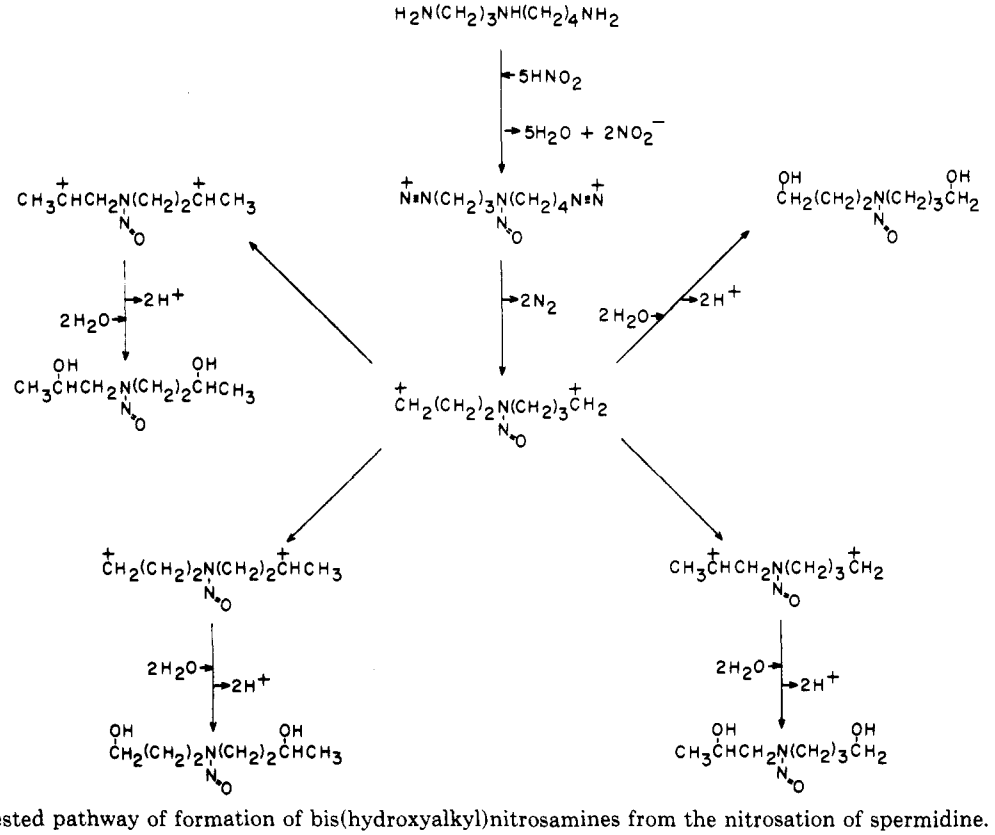

Figure 7. Suggested pathway of formation of bis(hydroxyalkyl)nitrosamines from the nitrosation of spermidine.

Table I. Mass Spectra of Bis(hydroxyalkyl)-*N*-nitrosamines and $(Me_3Si)_2$ Derivatives[a,b]

4-Hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine (authentic)

29 (44), 30 (68) 42 (100), 44 (66), 55 (66), 56 (40), 70 (20), 71 (49), 84 (23), 86 (68), 100 (30), 102 (27), 104 (6), 117 (3), 128 (34), 129 (5), 132 (2), 145 (1), 146 (7), 159 (25), 160 (2), *176* (0.2)

3-Hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine (authentic)

29 (74), 30 (87), 42 (99), 45 (99), 55 (100), 56 (75), 70 (14), 71 (10), 86 (36), 88 (25), 100 (54), 102 (16), 114 (2), 117 (1), 119 (11), 128 (2), 146 (2), 159 (3), 161 (1), *176* (<0.1)

Bis(trimethylsilyl)-3-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine (authentic)

29 (10), 30 (10), 42 (38), 45 (46), 55 (16), 59 (31), 73 (97), 75 (100), 76 (7), 84 (6), 101 (9), 103 (10), 115 (37), 117 (99), 130 (68), 131 (33), 144 (37), 145 (7), 147 (9), 158 (22), 160 (9), 172 (1), 174 (1), 303 (0.4), 305 (0.4), *320* (<0.1)

Bis(trimethylsilyl)-4-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine (authentic)

29 (3), 30 (1), 45 (17), 47 (5), 59 (13), 61 (4), 73 (74), 75 (31), 76 (2), 84 (14), 99 (1), 103 (4), 115 (11), 117 (100), 118 (16), 130 (41), 144 (23), 145 (8), 147 (7), 158 (4), 174 (2), 200 (4), 201 (1), 290 (1), 303 (0.3), 305 (2), *320* (<0.1)

Bis(trimethylsilyl)-3-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine (authentic)

29 (7), 30 (10), 43 (38), 44 (86), 55 (24), 56 (36), 73 (100), 75 (48), 83 (9), 84 (18), 101 (13), 103 (19), 115 (16), 117 (25), 129 (8), 131 (14), 143 (11), 144 (19), 147 (3), 158 (12), 160 (4), 171 (1), 174 (1), 175 (1), 189 (1), 200 (1), 273 (1), 303 (2), 305 (2), *320* (<0.1)

Bis(trimethylsilyl)-4-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine (authentic)

29 (13), 30 (13), 42 (43), 45 (40), 55 (39), 59 (42), 73 (99), 75 (76), 84 (100), 85 (26), 101 (33), 103 (75), 110 (16), 117 (13), 129 (9), 131 (25), 144 (36), 145 (36), 147 (14), 158 (16), 160 (2), 172 (4), 174 (8), 185 (2), 200 (11), 201 (2), 202 (1), 213 (2), 290 (1), 303 (3), 305 (5), *320* (<0.1)

Bis(trimethylsilyl)-3-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine (spermidine)

29 (3), 30 (6), 42 (10), 45 (9), 54 (7), 59 (7), 73 (100), 75 (30), 82 (5), 84 (12), 101 (2), 103 (4), 115 (9), 117 (58), 123 (12), 130 (15), 143 (3), 144 (6), 155 (3), 158 (9), 160 (2), 172 (1), 183 (1), 195 (1), 226 (1), 228 (1), 303 (1), 305 (1), *320* (<0.1)

Unidentified peak (spermidine)

27 (1), 29 (2), 43 (14), 44 (10), 59 (10), 61 (3), 71 (31), 73 (100), 76 (1), 84 (2), 101 (6), 103 (4), 115 (2), 116 (2), 129 (4), 131 (67), 132 (8), 144 (22), 148 (2), 158 (9), 160 (1), 165 (2), 174 (1), 303 (0.5), 305 (1),

Bis(trimethylsilyl)-4-hydroxybutyl-(2-hydroxypropyl)-*N*-nitrosamine (spermidine)

29 (4), 30 (3), 42 (10), 45 (15), 55 (9), 59 (12), 73 (100), 75 (27), 84 (23), 85 (3), 101 (4), 103 (7), 115 (7), 117 (92), 118 (10), 130 (23), 144 (14), 145 (6), 147 (4), 158 (3), 172 (1), 174 (2), 200 (3), 201 (1), 303 (0.2), 305 (1), *320* (<0.1)

Bis(trimethylsilyl)-3-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine (spermidine)

29 (10), 30 (12), 42 (59), 44 (100), 56 (44), 59 (31), 73 (64), 75 (87), 83 (15), 84 (13), 101 (22), 103 (35), 115 (24), 117 (35), 129 (15), 131 (31), 143 (26), 144 (32), 147 (6), 158 (28), 160 (8), 173 (3), 174 (2), 175 (1), 189 (3), 213 (1), 303 (3), 305 (3), *320* (<0.1)

Bis(trimethylsilyl)-4-hydroxybutyl-(3-hydroxypropyl)-*N*-nitrosamine (spermidine)

29 (16), 30 (15), 42 (54), 45 (50), 55 (48), 59 (53), 73 (89), 75 (92), 84 (100), 85 (30), 101 (42), 103 (87), 110 (18), 117 (15), 129 (10), 131 (28), 144 (41), 145 (40), 147 (15), 158 (18), 170 (2), 172 (4), 174 (8), 185 (3), 200 (11), 201 (2), 213 (2), 242 (1), 290 (1), 303 (2), 305 (4), *320* (<0.1)

[a] Mass spectra are condensed by the method of Hertz et al. (1971). Molecular ions are italicized. [b] *m/e* (rel %).

= 1772; peak 5, $(Me_3Si)_2$-4-OHB(3-OHP)N, I_x = 1853. Peak 2 had a Kovat's index which did not correspond to the index of any authentic compound yet the spectrum was characteristic of a bis(hydroxyalkyl)-*N*-nitrosamine (Table I). The structure of peak 2 was not determined, but it may be a product of the β shift of either carbonium ions.

In order to eliminate the possibility that bis(hydroxyalkyl)amines were contaminants in the spermidine, 10 mg of 4-hydroxybutyl-(3-hydroxypropyl)amine were converted to a $(Me_3Si)_3$ derivative, chromatographed, and its mass spectrum obtained. Ions of *m/e* 363 (M^+) and 348 (M − 15) were apparent. A similar procedure of derivatization, chromatography, and mass spectral analysis was made on the spermidine. Limited mass searches for *m/e* 363 and 348 revealed the absence of these ions in the spectra obtained from the chromatography of the derivatized spermidine. This gave evidence that the bis(hydroxylalkyl)amines were not contaminants in the spermidine.

The mechanism (Figure 7) proposed for the formation of bis(hydroxylalkyl)-*N*-nitrosamines from the nitrosation of spermidine assumes the secondary amine reacts to give a stable *N*-nitrosamine. The primary amines, however, proceed to the diazonium ion (Ridd, 1961) which subsequently deaminates to a carbonium ion. The carbonium ion may either rearrange to the secondary carbonium ion or it may react through solvolysis with water to produce a primary hydroxyl product. The secondary carbonium ion may also react with water through solvolysis producing a secondary hydroxyl product. Because spermidine is unsymmetrical and each primary amine can act independently, four hydroxylated products are possible.

A total of nine *N*-nitroso compounds have been identified as products of the nitrosation of spermidine. Whether or not any or all of these products occur in foods has yet to be determined.

LITERATURE CITED

Bills, D. D., Hildrum, K. I., Scanlan, R. A., Libbey, L. M., *J. Agric. Food Chem.* **21**, 876 (1973).

Blatt, A. H., "Organic Syntheses", Wiley, New York, N.Y., 1943, p 571.

Falbe, J., Schultz-Steinen, H. J., Korte, F., *Chem. Ber.* **98**, 1923 (1965).

Fan, T. V., Tannenbaum, S. R., *J. Agric. Food Chem.* **19**, 1267 (1971).

Hildrum, K. I., Scanlan, R. A., Libbey, L. M., *J. Agric. Food Chem.* **25**, 252 (1977).

Hildrum, K. I., Williams, J. L., Scanlan, R. A., *J. Agric. Food Chem.* **23**, 439 (1975).

Jennings, W. G., Yabumoto, K., Wohleb, R. H., *J. Chromatogr. Sci.* **12**, 344 (1974).

Karabatsos, G. J., Taller, R. A., *J. Am. Chem. Soc.* **86**, 4373 (1964).

Lakritz, L., Spinelli, A. M., Wasserman, A. E., *J. Agric. Food Chem.* **23**, 344 (1975).

Moruzzi, G., Caldarera, C. M., *Arch. Biochim. Biophys.* **105**, 209 (1964).

Pierce, A. E., "Silylation of Organic Compounds", Pierce Chemical Co., Rockford, Ill., 1968, p 34.
Ridd, J. H, *Q. Rev. Chem. Soc.* **15**, 418 (1961).
Saxby, M. J., *J. Assoc. Off. Anal. Chem.* **55**, 9 (1972).
Sondheimer, F., Woodward, R. B., *J. Am. Chem. Soc.* **75**, 5438 (1953).
Spinelli, A. M., Lakritz, L., Wasserman, A. E., *J. Agric. Food Chem.* **22**, 1026 (1974).
Wang, L. C., *Plant Physiol.* **50**, 152 (1972).

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Bovine Liver Metabolism and Tissue Distribution of Aflatoxin B_1

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Studies were undertaken to determine which of the aflatoxin (AF) metabolites may occur as significant residues in edible meat products from cows fed AFB_1. In the first experiment, ^{14}C-labeled AFB_1 was incubated with bovine liver preparations to determine which metabolites could be produced. About 15–22% of the AFB_1 was metabolized to AFQ_1, AFM_1, and two unidentified metabolites; 61–64% of the original AFB_1 was converted to material which was soluble in the aqueous phase. Of the two chloroform-soluble unidentified metabolites, one had chromatographic properties intermediate to those of AFQ_1 and AFM_1; the other was very polar. No AFB_{2a}, AFP_1, or aflatoxicol were found. In the second experiment, AFB_1 was administered twice daily for 14 days to four lactating dairy cows fed concentrate containing either 10, 50, 250, or 1250 ppb AFB_1. Twenty-four hours prior to slaughter, [^3H]AFB_1 was included with the last feeding. Only the animal fed 1250 ppb AFB_1 showed detectable chloroform-soluble metabolites in the edible portions of its carcass. Other than small quantities of AFB_1 and AFM_1 in the kidney and liver, no other identifiable chloroform-soluble metabolites were detectable (<1 ppb) in the edible portions such as brain, heart, and skeletal muscle. On the other hand, based on recoverable radioactivity, skeletal muscle and liver contained approximately 1.7 ppb and 0.3 ppb unidentified, chloroform-soluble metabolites, expressed as "AFB_1 equivalents". Conclusions drawn were (1) that there should be essentially no acute toxicity health hazard for humans who consume meat or edible organs taken from animals fed less than 46 ppb AFB_1. (This latter level was previously reported by this laboratory to be the maximum feed level of AFB_1 giving rise to detectable levels of AFM_1 in milk (Polan et al., 1974); (2) that negligible carcinogenic hazard due to the *chloroform-soluble* AFB_1 metabolites can be assumed from consumption of meat products from animals fed 46 ppb AFB_1; and (3) that more research information is needed before potential hazard due to water-soluble aflatoxin residues in meat products can be assessed.

Aflatoxin B_1 (AFB_1), a potent hepatocarcinogen, is metabolized by the hepatic microsomal mixed function oxidase (MFO) system to a group of hydroxylated derivatives; however, the composition of AFB_1 metabolites is apparently species specific (Masri et al., 1974). For instance, whereas mouse and rat liver produce aflatoxin Q_1 (AFQ_1) (Faris and Hayes, 1975), only rat liver produces significant quantities of aflatoxin M_1 (AFM_1) (Bassir and Emafo, 1970; Faris and Hayes, 1975), and only mouse liver produces significant quantities of aflatoxin P_1 (AFP_1) (Merrill and Campbell, 1968; Faris and Hayes, 1975). Salhab and Hsieh (1975) have shown that monkey and human hepatic microsomal systems produce aflatoxicol H_1 ($AF_{OL}H_1$), which is not produced by either rat or mouse hepatic microsomes.

Another possible source of tissue residues are the water-soluble conjugates of various AFB_1 metabolites. These are a group of poorly defined compounds (Dalezios et al., 1973; Mabee and Chipley, 1973), which may represent a major pathway of AFB_1 metabolism. Since it is possible for these conjugates to be hydrolyzed by enzymes in the digestive tract of humans consuming contaminated tissue, with subsequent absorption of the free aflatoxins, these residues may represent a potential health hazard. Hydrolytic enzymes, such as β-glucuronidase are readily available through gut microflora (Hawksworth et al., 1971).

A third pathway of AFB_1 metabolism may give rise to covalently bound residues. In theory, these may be either the electrophilic aflatoxin 2,3-epoxide, which binds to various nucleophiles (Garner et al., 1972; Swenson et al., 1973), or the aflatoxin hemacetal (AFB_{2a}), which readily forms Schiff bases with the ubiquitous amino group (Patterson and Roberts, 1970; Gurtoo and Campbell, 1974; Ashoor and Chu, 1975).

There are few reports on the significance of tissue levels of aflatoxin residues present in animals ingesting dietary aflatoxins, although the importance of animal products in human diets deems it essential that more information be gained on possible tissue contamination. Allcroft et al. (1966) found that the blood, liver, and kidneys of sheep fed AFB_1 contained AFM_1. Allcroft and Carnaghan (1963) reported that the liver removed from a cow fed a diet containing 2 ppm aflatoxin for 6 days, elicited no toxic response when fed to ducks. (The general term "aflatoxin" will be used when reference to the literature is made, since in many of the earlier papers it is difficult to know the purity of the aflatoxin preparations utilized.) These workers (Allcroft and Carnaghan, 1963) also showed the presence of AFM_1 in milk, an observation later confirmed by Masri et al. (1969). Purchase (1972) calculated, from Allcroft and Carnaghan's data, that the liver contained less than 40 ppb aflatoxin. Allcroft and Carnaghan (1963)

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