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Characterization of Directly Acting Mutagens Produced from *N*-Nitroso-*N*-(formylmethyl)alkylamines: Their Possible Involvement in the Carcinogenesis of *N*-Nitrosamines Having a 2-Hydroxyethyl Group

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Directly acting mutagens formed from *N*-nitroso-*N*-(formylmethyl)alkylamines (**3**) were isolated and identified as *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoethylamines (**4**). Their structures were elucidated on the basis of nuclear magnetic resonance spectra and confirmed by derivatization to the crystalline 2,4-dinitrophenylhydrazones (**5**). Compounds **4** (alkyl=ethyl and butyl) were strongly mutagenic to *Salmonella typhimurium* TA1535 and *Escherichia coli* WP2 *hcr*⁻ without metabolic activation, while **4** with a *tert*-butyl group was not mutagenic. The formation of **4** is considered to proceed by the nitrosation of **3**, indicating a possible involvement of the formylmethyl metabolite in carcinogenesis by *N*-nitrosamines with a 2-hydroxyethyl group.

Keywords—*N*-nitroso-*N*-(2-hydroxyethyl)alkylamine; *N*-nitroso-*N*-(formylmethyl)alkylamine; *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoethylamine; *N*-nitroso-*N*-(oxoalkyl)alkylamine; directly acting mutagen; β -hydroxylation activation; nitrosamine activation; mutagenicity

N-Nitroso-*N*-(2-hydroxyethyl)alkylamines (**1**) (alkyl=ethyl and butyl) are potent carcinogens which induce tumors of the liver and esophagus in rats.^{2,3)} Since their principal urinary metabolites, *N*-nitroso-*N*-(carboxymethyl)alkylamines (**2**) (alkyl=ethyl and butyl), are not carcinogenic,⁴⁾ *N*-nitroso-*N*-(formylmethyl)alkylamines (**3**), the obligatory metabolic intermediates between **1** and **2**, are suspected to be involved in carcinogenesis by **1**. Previously⁵⁾ we prepared *N*-nitroso-*N*-(formylmethyl)butylamine (**3b**), *N*-nitroso-*N*-(formylethyl)butylamine, and *N*-nitroso-*N*-(formylpropyl)butylamine in connection with our studies on the correlation of chemical structure and *in vivo* metabolism with urinary bladder carcinogenicity of *N*-nitrosodialkylamines. They were all mutagenic in the presence of rat-liver 9000 $\times$ *g* supernatant toward *Salmonella typhimurium* TA1535, *Escherichia coli* WP2

Chart 1

hcr⁻ and *E. coli* WP2.⁶⁾ Among these three formylalkyl compounds only the formylmethyl compound (**3b**) was chemically unstable, and produced directly acting mutagen(s) upon standing at room temperature.⁵⁾ This paper deals with the isolation and characterization of the directly acting mutagens formed from **3b** and its homolog, *N*-nitroso-*N*-(formylmethyl)ethylamine (**3a**) and a nonmutagen formed from *N*-nitroso-*N*-(formylmethyl)-*tert*-butylamine (**3c**). Their possible role in carcinogenesis by **1** is discussed.

Experimental

Instrumental Analyses—Ultraviolet (UV) spectra were measured in CH₃CN solution with a Hitachi 200-20 spectrophotometer. Infrared (IR) spectra were determined on a Hitachi EPI-G3 spectrometer. Nuclear magnetic resonance (NMR) spectra were taken at 60 MHz on a Hitachi R-24B spectrometer. Chemical shifts are expressed in δ with tetramethylsilane as an internal standard; s=singlet, d=doublet, t=triplet, q=quartet, and br s=broad singlet. Melting points were determined on a micro hot-stage apparatus and are uncorrected.

Chromatography—Thin-layer chromatography (TLC) was performed on pre-coated TLC plates with a 0.25 mm layer of Silica gel 60 F₂₅₄ (E. Merck AG, Darmstadt, G.F.R.). Spots were visualized with UV light (254 nm) and the reagent reported earlier⁷⁾ or with 2,4-dinitrophenylhydrazine solution [dissolve 2,4-dinitrophenylhydrazine (400 mg) in a mixture of 95% H₂SO₄ (2 ml), H₂O (3 ml) and ethanol (10 ml)]. For column chromatography, silica gel (230–400 mesh, E. Merck AG.) and Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were used. The following solvent systems were used: S₁, hexane–ether–methylene chloride (8:3:2); S₂, hexane–ether–chloroform (5:3:2); S₃, hexane–ether–chloroform (4:3:2); S₄, hexane–ether–chloroform (3:3:2); S₅, chloroform–acetonitrile (9:1).

***N*-Nitroso-*N*-(formylmethyl)butylamine (**3b**)**—**3b** diethyl acetal was synthesized according to the method reported earlier,⁵⁾ and **3b** was prepared from the diethyl acetal with some modifications of the previous procedure.⁵⁾ Thus, **3b** diethyl acetal (1.5 g) was added to 1 N HCl (300 ml) and the mixture was stirred at room temperature for 1 h and then extracted with ethyl acetate (300 ml $\times$ 3). The organic layer was washed with water and dried over anhydrous Na₂SO₄. The solvent was evaporated off under reduced pressure below 25 °C and the residue (953 mg, 96% yield) was used without purification for further experiment. A part of the residue was treated with 2,4-dinitrophenylhydrazine to give the hydrazone as described previously.⁵⁾

***N*-Nitroso-*N*-(formylmethyl)ethylamine (**3a**)**—Bromoacetaldehyde diethyl acetal (94% purity, 25 g) was added to 70% ethylamine aqueous solution (50 ml), and the mixture was left at room temperature with occasional stirring for 2 d. Then it was heated at 65 °C in a sealed glass tube for 6 h. After cooling of the reaction mixture, excess of ethylamine was evaporated off. After usual work-up,⁵⁾ *N*-(formylmethyl)ethylamine diethyl acetal was obtained as a colorless oil (13 g, 60% yield), bp 76.5–78 °C (19.5 mmHg). Nitrosation of this diethyl acetal with sodium nitrite and acetic acid was carried out in the usual way and the reaction mixture was extracted with hexane. The residue obtained after evaporation of hexane was subjected to silica gel column chromatography and elution with S₁ to give **3a** diethyl acetal as a pale yellow oil (97% yield). *Anal.* Calcd for C₈H₁₈N₂O₃: C, 50.50; H, 9.54; N, 14.73. Found: C, 50.60; H, 9.55; N, 14.71.

3a diethyl acetal (2.0 g) was added to 1 N HCl (60 ml) and the mixture was stirred at room temperature for 1 h, then processed as described above for **3b**. **3a** (1.05 g, 86% yield) obtained was used without further purification: ¹H-NMR (CDCl₃) δ : 1.08, 1.41⁸⁾ (3H, t, *J*=7 Hz, CH₃), 3.64, 4.28 (2H, q, *J*=7 Hz, CH₂CH₃), 4.21, 4.97 (2H, s, CH₂CHO), 9.40, 9.78 (1H, s, CHO). Treatment of **3a** (70 mg) with 2,4-dinitrophenylhydrazine reagent gave **3a** 2,4-dinitrophenylhydrazone as yellow needles (128 mg, 82% yield), mp 151–152 °C (from hexane–methylene chloride). *Anal.* Calcd for C₁₀H₁₂N₆O₅: C, 40.54; H, 4.08; N, 28.37. Found: C, 40.43; H, 4.05; N, 28.13. ¹H-NMR (CDCl₃) δ : 1.13, 1.48 (3H, t, *J*=7 Hz, CH₃), 3.67, 4.30 (2H, q, *J*=7 Hz, CH₂CH₃), 4.42, 5.03 (2H, d, *J*=5 Hz, CH₂CH), 7.40, 7.64 (1H, t, *J*=5 Hz, CH), 7.8–9.2 (3H, aromatic H).

***N*-Nitroso-*N*-(formylmethyl)*tert*-butylamine (**3c**)**—*tert*-Butylamine (58.4 g) was added in portions to the bromoacetaldehyde diethyl acetal (94% purity, 64 g). The mixture was left at room temperature for 3 d with occasional stirring and then refluxed for 4 h in an oil bath (95 °C). After usual work-up, the residue was distilled to give a colorless oil, bp 67–72 °C (12 mmHg), which was nitrosated with sodium nitrite and acetic acid in the usual manner to give the product as a pale yellow oil. It was purified on a column of silica gel with S₁ to give **3c** diethyl acetal (overall yield was 24% starting from bromoacetaldehyde diethyl acetal). *Anal.* Calcd for C₁₀H₂₂N₂O₃: C, 55.02; H, 10.10; N, 12.83. Found: C, 54.91; H, 10.24; N, 12.97.

3c diethyl acetal (1.0 g) was added to 0.1 N HCl (200 ml) and the mixture was stirred at room temperature for 25 min. After processing as described above, **3c** was obtained as a pale yellow oil (522 mg, 79% yield), which crystallized in a freezer (–20 °C). ¹H-NMR (CDCl₃) δ : 1.57 (9H, s, C(CH₃)₃), 4.09 (2H, s, CH₂), 9.31 (1H, s, CH). Treatment of **3c** (300 mg) with 2,4-dinitrophenylhydrazine reagent gave the 2,4-dinitrophenylhydrazone as yellow needles (206 mg, 46% yield), mp 162 °C (from methanol–chloroform). *Anal.* Calcd for C₁₂H₁₆N₆O₅: C, 44.44; H, 4.97;

N, 25.92. Found: C, 44.35; H, 5.00; N, 26.06. $^1\text{H-NMR}$ (CDCl_3) δ : 1.63 (9H, s, $\text{C}(\text{CH}_3)_3$), 4.38 (2H, d, $J=5$ Hz, CH_2), 7.46 (1H, t, $J=5$ Hz, CH).

2,4-Dinitrophenylhydrazine Reagent—2,4-Dinitrophenylhydrazine (1 g) was dissolved in acetonitrile (450 ml) and 0.1 N HCl (1 ml) was added to the solution (9 ml) before use.

Isolation and Identification of Directly Acting Mutagens (4)—A solution (100 mg/ml) of *N*-nitroso-*N*-(formylmethyl)alkylamine (3) in methyl acetate was left at room temperature for several days with occasional monitoring by TLC. Several spots besides that of 3 were observed on thin-layer chromatograms, using S_3 as the solvent. When a product having $R_f=0.4$, 0.5 or 0.5, which is less polar than the starting material (3), reached its maximal amount in the methyl acetate solution of 3a, 3b, or 3c, respectively, the residue obtained after evaporation of the solvent was chromatographed on a silica gel column using S_4 for 3a and S_3 for 3b and 3c as eluting solvents. The fraction containing the product having $R_f=0.4$, 0.5 or 0.5, respectively, was collected in a flask containing acetonitrile. Each fraction was concentrated under reduced pressure below 20°C and the concentrate (acetonitrile solution) was chromatographed on a Sephadex LH-20 (20 g) column with S_5 . The concentrated fraction containing the product (4) was used for mutation assay. 4a (8% yield). $^1\text{H-NMR}$ (CD_3CN) δ : 0.98, 1.28 (3H, t, $J=7$ Hz, CH_3), 3.71, 4.33 (2H, q, $J=7$ Hz, CH_2), 9.26, 9.54 (1H, s, CHO). 4b (11% yield). $^1\text{H-NMR}$ (CD_3CN) δ : 0.90 (3H, t, CH_3), 1.1–1.7 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 3.70, 4.26 (2H, t, $J=7$ Hz, NCH_2), 9.31, 9.60 (1H, s, CHO). Of these three compounds, 4c was much more stable than the others and could be concentrated to dryness without decomposition to give colorless crystals (18% yield), mp 104°C (dec.). Anal. Calcd for $\text{C}_6\text{H}_{11}\text{N}_3\text{O}_3$: C, 41.61; H, 6.40; N, 24.27. Found: C, 41.77; H, 6.36; N, 24.04. UV $\lambda_{\text{max}}^{\text{CH}_3\text{CN}}$ nm (ϵ): 223 (12000), 363 (77), 375 (84), 385 (65).⁹ IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3100 (OH), 2800 (CHO), 1695 (C=O), 1465 (N=O), 1035 (C–O). $^1\text{H-NMR}$ (CD_3CN) δ : 1.52 (9H, s, $\text{C}(\text{CH}_3)_3$), 9.40 (1H, s, CHO).

4a, 4b and 4c in acetonitrile were treated with 2,4-dinitrophenylhydrazine reagent and kept at room temperature for 1 h. After evaporation of the solvent, the yellow crystalline products were dissolved in ethyl acetate. Each ethyl acetate solution was washed with water, and dried over anhydrous Na_2SO_4 . After evaporation of the solvent, the residue was purified on a column of silica gel with S_3 or S_4 as the solvent in the usual way to give the respective hydrazones, 5a, 5b and 5c, as yellow needles. 5a, mp $132\text{--}133^\circ\text{C}$ (dec.) (from chloroform–methanol). Anal. Calcd for $\text{C}_{10}\text{H}_{11}\text{N}_7\text{O}_6$: C, 36.93; H, 3.41; N, 30.15. Found: C, 37.07; H, 3.43; N, 30.31. UV $\lambda_{\text{max}}^{\text{CH}_3\text{CN}}$ nm (ϵ): 365 (33000).⁹ IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3350 (OH), 3250 (NH), 1610 (C=N). $^1\text{H-NMR}$ (CD_3COCD_3) δ : 1.14, 1.46 (3H, t, $J=7$ Hz, CH_3), 3.88, 4.51 (2H, q, $J=7$ Hz, CH_2), 8.34, 8.59 (1H, s, CH), 7.7–9.0 (3H, aromatic H). 5b, mp 119°C (dec.) (from acetonitrile). Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{N}_7\text{O}_6$: C, 40.80; H, 4.28; N, 27.75. Found: C, 40.84; H, 4.23; N, 27.71. UV $\lambda_{\text{max}}^{\text{CH}_3\text{CN}}$ nm (ϵ): 365 (31000).⁹ IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3270 (NH, OH), 1610 (C=N). $^1\text{H-NMR}$ (CD_3COCD_3) δ : 0.96 (3H, t, $J=6$ Hz, CH_3), 1.1–1.9 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 2.87 (1H, br s, NH or OH), 3.90, 4.47 (2H, t, $J=7$ Hz, NCH_2), 8.33, 8.58 (1H, s, CH=N), 7.7–9.0 (3H, aromatic H). 5c, mp 137.5°C (dec.) (from methanol). Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{N}_7\text{O}_6$. CH_3OH : C, 40.51; H, 4.97; N, 25.45. Found: C, 40.46; H, 4.94; N, 25.51. UV $\lambda_{\text{max}}^{\text{CH}_3\text{CN}}$ nm (ϵ): 365 (34000).⁹ IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3270 (NH), 3150 (OH), 1610 (C=N). $^1\text{H-NMR}$ (CD_3OD) δ : 1.68 (9H, s, $\text{C}(\text{CH}_3)_3$), 4.79 (2H, s, NH, OH), 8.07 (1H, s, CH=N), 7.6–9.0 (3H, aromatic H).

Formation of 4c from 3c by Nitrosation—Nitrosation by Sodium Nitrite and HCl: 3c (118 mg, 0.82 mmol) was dissolved in 0.1 N HCl (8.2 ml). After the solution had been made acidic (pH 2–3) with 12 N HCl, sodium nitrite (56.6 mg, 0.82 mmol) was added and the reaction mixture was stirred for 3 h at room temperature, then extracted with ethyl acetate. The organic layer was processed in the usual way to give 4c (9 mg, 6% yield) after chromatographic purification of the product on a silica gel column (42% of 3c was recovered).

Nitrosation by Nitrosyl Chloride: Nitrosyl chloride (1.1 mmol) in chloroform (0.4 ml) was added to 3c (156 mg, 1.1 mmol) in methyl acetate (1.5 ml) and the whole was left for 3 h at room temperature. After evaporation of the solvents, the residue was chromatographed on a column of silica gel to give 4c (17 mg, 9% yield) (42% of 3c was recovered).

Nitrosation by Amyl Nitrite: Amyl nitrite (0.1 ml, 0.1 mmol) was added to 3c (109 mg, 0.8 mmol) in methyl acetate (1.1 ml) and the mixture was stirred for 3 h at room temperature. After usual work-up, 4c (0.3 mg, 0.2% yield) and 3c (96 mg, 88% recovery) were obtained.

Nitrosation of *N*-Nitroso-*N*-(2-oxopropyl)butylamine (6), *N*-Nitroso-*N*-(2-oxobutyl)butylamine (7) and *N*-Nitroso-*N*-(3-oxobutyl)butylamine (8)—6, 7 and 8 were synthesized according to the procedure described previously.⁷ A methyl acetate solution of these compounds (100 mg/ml) was treated at room temperature with nitrosyl chloride (1–1.5 mol eq) in chloroform. After the brown color of nitrosyl chloride had disappeared, the organic solvents were evaporated off and the residue was chromatographed on a column of silica gel to isolate the products.

Products Formed from 6 by Nitrosation: Thin-layer chromatographic examination with S_3 of the products obtained from 6 (2.26 g) by nitrosation showed the presence of three products (9, R_f 0.6; compound X, R_f 0.41; 10, R_f 0.38) besides 6 (R_f 0.27). 9 (401 mg, 20% yield) was obtained from the first fraction after chromatographic separation on a column of silica gel (120 g) with S_2 . It was identified as *N*-butyl-1,2-dioxopropylamine (9) on the basis of IR and $^1\text{H-NMR}$ spectral data. IR $\nu_{\text{max}}^{\text{film}}$ cm^{-1} : 3320 (NH), 1725 (C=O), 1675, 1530 (CONH), 1170 (C–O). $^1\text{H-NMR}$ (CD_3CN) δ : 0.88 (3H, t, $J=6$ Hz, CH_2CH_3), 1.2–1.8 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 2.33 (3H, s, COCH_3), 3.17 (2H, q, $J=6$ Hz, NCH_2), 7.4 (1H, br s, NH). 9 was derivatized to the 2,4-dinitrophenylhydrazone in the usual way to give yellow

plates (61% yield); mp 150–151 °C (from ethyl acetate). *Anal.* Calcd for $C_{13}H_{17}N_5O_5$: C, 48.29; H, 5.30; N, 21.66. Found: C, 48.36; H, 5.16; N, 21.65. UV $\lambda_{\text{max}}^{\text{CH}_3\text{CN}}$ nm (ϵ): 356 (23000).⁹⁾ IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3325, 3290 (NH), 1650, 1540 (CONH), 1610 (C=N). $^1\text{H-NMR}$ (CD_3COCD_3) δ : 0.92 (3H, t, $J=6$ Hz, CH_2CH_3), 1.1–1.9 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 2.27 (3H, s, C- CH_3), 3.30 (2H, q, $J=6$ Hz, CH_2NH), 8.0 (1H, brs, NH), 8.2–9.1 (3H, aromatic H).

The fraction containing compound X and **10** was further subjected to LH-20 column chromatography with S_2 to give compound X (68 mg) and **10** (64 mg, 2% yield). The latter compound was identified as *N*-nitroso-*N*-butyl-2-oxo-3-hydroxyiminopropylamine (**10**) based on the following data. *Anal.* Calcd for $C_7H_{13}N_3O_3$: C, 44.91; H, 7.00; N, 22.45. Found: C, 44.86; H, 7.02; N, 22.61. UV $\lambda_{\text{max}}^{\text{CH}_3\text{CN}}$ nm (ϵ): 225 (18000), 353 (145). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3150 (OH), 1710 (C=O), 1620 (C=N), 1415 (N=O), 1020 (C-O). $^1\text{H-NMR}$ (CD_3CN) δ : 0.94 (3H, t, $J=6$ Hz, CH_3), 1.1–1.8 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 3.52, 4.18 (2H, t, $J=7$ Hz, N- CH_2CH_2), 4.60, 5.34 (2H, s, CH_2CO), 7.50, 7.60 (1H, s, CH), 10.0 (1H, brs, N-OH). The structure of compound X has not yet been elucidated. IR $\nu_{\text{max}}^{\text{film}}$ cm^{-1} : 2950, 1745, 1690, 1450, 1425. $^1\text{H-NMR}$ (CD_3CN) δ : 0.94 (3H, t, $J=6$ Hz, CH_3), 1.1–1.8 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 4.17 (2H, t, $J=7$ Hz, N- CH_2), 4.33, 4.34 (3H, s). Approximately 10% of **6** was recovered unchanged after the chromatographic separation on a silica gel column.

Products Formed from 7 by Nitrosation: Examination by TLC with solvent S_3 of the reaction products obtained from **7** (1.35 g) by nitrosation revealed the presence of three main products (**11**, R_f 0.69; compound Y, R_f 0.53; **12**, R_f 0.37). Chromatography on a column of silica gel (120 g) with S_2 gave three fractions containing **11**, compound Y, and **12**. Each fraction was further purified on a column of LH-20 with S_5 to give **11**, compound Y, and **12**. **11** (158 mg, 13% yield) was identified as *N*-butyl-1,2-dioxobutylamine. IR $\nu_{\text{max}}^{\text{film}}$ cm^{-1} : 3310 (NH), 1720 (C=O), 1675, 1525 (CONH), 1130 (C-O). $^1\text{H-NMR}$ (CD_3CN) δ : 1.0 (6H, t, $J=7$ Hz, $\text{CH}_3 \times 2$), 1.2–1.8 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 2.86 (2H, q, $J=7$ Hz, COCH_2CH_3), 3.19 (2H, q, $J=7$ Hz, N- CH_2), 7.32 (1H, brs, NH). For confirmation of the structure of **11**, it was derivatized quantitatively to its 2,4-dinitrophenylhydrazone; yellow needles, mp 173 °C (from ethyl acetate). *Anal.* Calcd for $C_{14}H_{19}N_5O_5$: C, 49.84; H, 5.68; N, 20.76. Found: C, 49.62; H, 5.55; N, 20.75. UV $\lambda_{\text{max}}^{\text{CH}_3\text{CN}}$ nm (ϵ): 371 (30000).⁹⁾ IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400 (NH), 1640, 1580 (CONH), 1615 (C=N). $^1\text{H-NMR}$ (CDCl_3) δ : 0.95 (3H, t, $J=6$ Hz, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.1–1.8 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.29 (3H, t, $J=7$ Hz, C- CH_2CH_3), 2.58 (2H, q, $J=7$ Hz, CCH_2CH_3), 3.43 (2H, q, $J=7$ Hz, N- CH_2), 6.0 (1H, brs, NH), 7.9–9.1 (3H, aromatic H).

12 (360 mg, 23% yield) was obtained as colorless needles, mp 104–105.5 °C (from acetonitrile). It was identified as *N*-nitroso-*N*-butyl-2-oxo-3-hydroxyiminobutylamine (**12**) on the basis of $^1\text{H-NMR}$ spectral and other data. *Anal.* Calcd for $C_8H_{15}N_3O_3$: C, 47.75; H, 7.51; N, 20.88. Found: C, 47.73; H, 7.53; N, 20.86. UV $\lambda_{\text{max}}^{\text{CH}_3\text{CN}}$ nm (ϵ): 228 (17000), 355 (120). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3150 (OH), 1700 (C=O), 1630 (C=N), 1415 (N=O), 1030 (C-O). $^1\text{H-NMR}$ (CD_3CN) δ : 0.94 (3H, t, $J=7$ Hz, CH_2CH_3), 1.1–1.8 (4H, m, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.84 (3H, s, C- CH_3), 4.12 (2H, t, $J=7$ Hz, N- CH_2CH_2), 4.63, 5.30 (2H, s, N- CH_2CO), 10.00 (1H, s, N-OH). Compound Y (14 mg, 1.5% yield) was tentatively identified as 2-oxo-3-hydroxyiminobutanol based on its $^1\text{H-NMR}$ spectrum. $^1\text{H-NMR}$ (CD_3CN) δ : 1.91 (3H, s, CH_3), 2.30 (2H, s, CH_2OH), 3.80 (2H, s, CH_2), 10.57 (1H, N-OH). No further study has been done on this compound.

Products Formed from 8 by Nitrosation: Thin-layer chromatographic examination with S_3 of the products formed from **8** (150 mg) by nitrosation showed the presence of a single product (**13**), which was purified by chromatography on a column of silica gel (30 g) with S_3 as a solvent. **13** (101 mg, 66% yield) was obtained as colorless crystals, mp 62–63 °C. This compound was identified as *N*-nitroso-*N*-butyl-2-hydroxyimino-3-oxobutylamine (**13**)

Chart 2. Products Formed from *N*-Nitroso-*N*-(oxoalkyl)butylamines by Treatment with Nitrosyl Chloride

on the basis of the following data. *Anal.* Calcd for $C_8H_{15}N_3O_3$: C, 47.75; H, 7.51; N, 20.88. Found: C, 47.40; H, 7.50; N, 21.03. UV $\lambda_{\max}^{CH_3CN}$ nm (ϵ): 223 (14500), 356 (100). IR $\nu_{\max}^{KBr}$ cm^{-1} : 3120 (OH), 1690 (C=O), 1625 (C=N), 1450 (N=O), 1000 (C-O). 1H -NMR (CD_3CN) δ : 0.98 (3H, t, CH_2CH_3), 1.1–1.9 (4H, m, $CH_2CH_2CH_3$), 2.27, 2.33 (3H, s, $COCH_3$), 3.46, 4.12 (2H, t, $J=7$ Hz, $N-CH_2CH_2$), 4.34, 4.97 (2H, s, $N-CH_2C$), 10.06, 10.4 (1H, s, $N-OH$).

Nitrosation of 8 with 3c—Treatment of **8** with **3c** Alone: **8** (86 mg, 0.5 mmol) and **3c** (144 mg, 1 mmol) were dissolved in methyl acetate (1.6 ml) and the solution was left at room temperature for 5 d. After evaporation of the solvent, the residue was chromatographed on a column of silica gel (60 g) with S_3 as a solvent to give **4c** (18 mg, 20% yield) after usual work-up. No **13** was produced, and **8** was mostly recovered unchanged.

Treatment of **8** with **3c** and Nitrosyl Chloride: **8** (172 mg, 1 mmol) and **3c** (144 mg, 1 mmol) were dissolved in methyl acetate (1.5 ml). Nitrosyl chloride (1 mmol) in chloroform (350 μ l) was added to this solution and the mixture was left at room temperature for 85 min. After evaporation of the solvents, the residue was subjected to column chromatography on silica gel (70 g) to give **4c** (3 mg, 2% yield) and **13** (100 mg, 50% yield), recovering **8** (53 mg, 31%) and **3c** (51 mg, 35%).

Mutation Assay—The mutation assay using *S. typhimurium* TA1535 and TA1538, and *E. coli* WP2 *her*[−] was carried out as described previously in the absence⁽¹⁰⁾ and in the presence of rat-liver 9000 $\times$ g supernatant.⁽¹¹⁾

Results

The IR spectrum of **4c** showed a strong absorption band at 3100 cm^{-1} due to OH, bands at 2800, 1695 and 1035 cm^{-1} ascribable to the aldehyde group and a band at 1465 cm^{-1} due to the N=O group. The UV spectrum of **4c** with absorption at 223 nm ($\epsilon=12000$) also indicated the presence of the *N*-nitroso group. The NMR spectrum of **4a** taken in CD_3CN showed two sets of signals indicating the existence of a mixture of *E*- and *Z*-isomers because of the partial double bond character of the N–N bond^(8a): signals at 0.98 and 3.71 ppm for the *E*-form and 1.28 and 4.33 ppm for the *Z*-form were assignable to the ethylnitrosamino protons. A similar spectral characteristic was noticed with **3a** (in $CDCl_3$): 1.08 and 3.64 ppm for *E*-form and 1.41 and 4.28 ppm for *Z*-form. Singlet peaks assignable to the aldehyde proton were also observed at 9.26 (*Z*-form) and 9.54 (*E*-form) ppm for **4a** and at 9.40 (*Z*-form) and 9.78 (*E*-form) ppm for **3a**. However, singlet peaks observed with **3a** at 4.21 (*Z*-form) and 4.97 (*E*-form) ppm due to two protons adjacent to the aldehyde group disappeared in **4a**, indicating substitution at this position.

Quite similar NMR spectral results were observed with **4b** and **3b**. On the other hand, **4c** and **3c** exist as *Z*- and *E*-form exclusively, respectively, because of the bulkiness of the *tert*-butyl group.^(8b) Besides these spectral data, the result of elemental analysis of **4c** supported its identification as *N*-nitroso-*N*-*tert*-butyl-1-hydroxyimino-2-oxoethylamine. Analogously the structure of **4a** and **4b** could be assigned as indicated in Chart 1, although they were not stable enough for elemental analysis. For further confirmation of their structures, **4a**, **4b** and **4c** were derivatized into their crystalline 2,4-dinitrophenylhydrazones (**5**). The NMR spectrum of **5a** taken in CD_3COCD_3 also showed two sets of signals assignable to the ethylnitrosamino protons, indicating the presence of *E*- and *Z*-isomers due to the *N*-nitroso group; 1.14 and 3.88 ppm for *E*-form and 1.46 and 4.51 ppm for *Z*-form. The aldehyde proton signals observed in **4a** disappeared in **5a** while new singlet peaks appeared at 8.34 (*Z*-form) and 8.59 (*E*-form) in addition to peaks due to phenyl protons, indicating that **4a** is derivatized to the 2,4-dinitrophenylhydrazone (**5a**) with retention of its structure. A similar conclusion was reached when the NMR spectra of **4b** and **5b** were compared. The NMR spectrum of **5c** indicated that it exists as the *Z*-form exclusively. Based on these spectral and elemental analysis data for **5a**, **5b** and **5c**, their structures were elucidated to be as shown in Chart 1.

The IR spectrum of **9** showed absorption bands at 3320, 1675 and 1530 cm^{-1} attributable to a secondary amido group and at 1725 and 1170 cm^{-1} due to a carbonyl group. The NMR spectrum of **9** taken in CD_3CN showed peaks at 0.88, 1.2–1.8 and 3.17 ppm assignable to a butylamino group. No indication of the presence of *E*- and *Z*-isomers due to the *N*-nitroso group was observed. On derivatization of **9** to its 2,4-dinitrophenylhydrazone, the absorption

bands due to a carbonyl group observed in the IR spectrum disappeared, while a new absorption band due to $C=N$ appeared at 1610 cm^{-1} . The NMR spectrum indicated that the butylamino group remained intact (0.92, 1.1–1.9 and 3.30 ppm) and a singlet peak assignable to $C-CH_3$ appeared at 2.27 ppm instead of that at 2.33 ppm assignable to $COCH_3$. Based on these spectral findings, the structure of **9** was assigned as *N*-butyl-1,2-dioxopropylamine. The elemental analysis data for its 2,4-dinitrophenylhydrazone also supported this structure.

The IR spectrum of **10** exhibited absorptions at 3150 and 1620 cm^{-1} , 1710 and 1020 cm^{-1} , and 1415 cm^{-1} due to hydroxyimino, carbonyl, and nitroso groups, respectively. The presence of the *N*-nitroso group was also apparent from the UV spectrum. The NMR spectrum of **10** taken in CD_3CN showed the existence of *E*- and *Z*-isomers arising from the *N*-nitroso group. The presence of a butylnitrosamino grouping was presumed on the basis of the observed peaks at 0.94, 1.1–1.8, and 3.52 (*E*-form) & 4.18 (*Z*-form) ppm. When the NMR spectra of **10** and **6** taken in CD_3CN were compared, the singlet peaks at 2.07 (*Z*-form) and 2.18 (*E*-form) ppm observed in **6** due to $COCH_3$ had disappeared in the spectrum of **10**, while new singlet peaks appeared at 7.50 (*Z*-form) and 7.60 (*E*-form) ppm. The singlet peaks at 4.27 (*Z*-form) and 4.98 (*E*-form) ppm of **6**, assignable to methylene protons adjacent to the oxo group, were shifted to 4.60 (*Z*-form) and 5.34 (*E*-form) ppm in **10**, indicating substitution of two protons of $COCH_3$ with an electron-withdrawing group. Based on these spectral and elemental analysis data, the structure of **10** was determined to be *N*-nitroso-*N*-butyl-2-oxo-3-hydroxyiminopropylamine.

Compound **11** was considered to be a homolog of **9** from a comparison of their IR, UV and NMR spectral data. Since **11** showed triplet and quartet peaks at 1.0 and 2.86 ppm, respectively, an ethyl group adjacent to the carbonyl group remained intact, thus identifying **11** as *N*-butyl-1,2-dioxobutylamine. Compound **11** was derivatized to its 2,4-dinitrophenylhydrazone, whose IR, UV, NMR and elemental analysis data also supported the assigned structure.

Quite similar IR and UV spectra were obtained with **10** and **12**. However, instead of a singlet peak observed in the 1H -NMR spectrum of **10** due to CH , a singlet peak assignable to $C-CH_3$ appeared in that of **12** at 1.84 ppm. Thus **12** was identified as *N*-nitroso-*N*-butyl-2-oxo-3-hydroxyiminobutylamine.

Comparison of the IR, UV and NMR spectral data of **13** with those of **8** indicated that methylene protons of **8** adjacent to a carbonyl group were replaced with a hydroxyimino group. The structure of **13** was thus assigned as *N*-nitroso-*N*-butyl-2-hydroxyimino-3-oxobutylamine, which was supported by elemental analysis data.

Compounds **4a**, **4b** and **4c** were tested for mutagenicity using acetonitrile as a solvent.

Fig. 1. Mutagenicity of **4a** and **4b** without Metabolic Activation

S. typhimurium TA1535 (○); *E. coli* WP2 *her*⁻ (●). Data from three different experiments are shown.

Since **4a** and **4b** were unstable in the absence of acetonitrile, their content in the solution was determined by HPLC after conversion to the 2,4-dinitrophenylhydrazones (**5a** and **5b**). The formation of **5c** from **4c** was quantitative. The retention times (min) of **5a**, **5b** and **5c** were 6.5 & 6.8,¹²⁾ 12.0 & 13.0 and 10.0, respectively.

Compounds **4a** and **4b** were strongly mutagenic to *S. typhimurium* TA1535 and *E. coli* WP2 *hcr*⁻ without metabolic activation (Fig. 1), while **4c** was not mutagenic to either of the bacterial strains with or without metabolic activation. These three compounds were not mutagenic to *S. typhimurium* TA1538.

The mutagenic activities of **9**—**13** and compound X were tested without metabolic activation. None of them was mutagenic to *E. coli* WP2 *hcr*⁻ and compound X alone was mutagenic to *S. typhimurium* TA1535, its potency being about one-third of that of **4b**.

Discussion

N-Nitrosoformylmethyl compounds (**3**) are chemically unstable and, upon standing at room temperature, they (**3a** and **3b**) produced directly acting mutagens in the Ames assay.⁵⁾ We tried to isolate and characterize those mutagens since they are presumably relevant to carcinogenesis by *N*-nitrosodialkylamines (**1**) containing a 2-hydroxyethyl group, which would form **3**.

By following the mutagenic activity of a methyl acetate solution of **3** upon standing, we were able to isolate a new type of *N*-nitroso compounds and identify them as **4** (Chart 1). Compounds **4a** and **4b** showed potent direct mutagenicity toward *S. typhimurium* TA1535 and *E. coli* WP2 *hcr*⁻ (Fig. 1), while **4c** did not show any mutagenic activity with or without metabolic activation. Most of the mutagenic activity (99%) toward *S. typhimurium* TA1535 of the methyl acetate solution containing **3b** could be accounted for by **4b**, while about half of the mutagenic activity of the solution against *E. coli* WP2 *hcr*⁻ was explainable in terms of **4b**, indicating the formation of unidentified mutagenic products.

The potent direct mutagenic activities of **4a** and **4b** are evident from the finding that their mutagenic activities are ten times more potent than those of the corresponding α -hydroxy *N*-nitroso compounds, that is *N*-nitroso-*N*-(hydroxymethyl)alkylamines (alkyl=ethyl and butyl).¹³⁾ Compounds **4a** and **4b** were not mutagenic to *S. typhimurium* TA1538, indicating that they act as alkylating agents.

Loeppky *et al.*¹⁴⁾ reported that *N*-nitrosoformylmethylamines readily transfer their nitroso group to an acceptor such as dimethylamine to give *N*-nitrosodimethylamine. There are reports that nitrosation of ketones lead to the formation of oximes *via* addition of NO⁺ to an enol tautomer.¹⁵⁾ Thus, it is conceivable that upon standing of **3** at room temperature, a nitrosating agent(s) is produced slowly, which attacks **3**, followed by isomerization to oximes (**4**). In order to demonstrate the formation of NO⁺ during storage of **3**, the effect of three nitrosating agents on the formation of **4c** was studied, since **4c** is stable enough for isolation. The formation of **4c** was greatly accelerated by addition of nitrosyl chloride or nitrite under acidic conditions to a solution of **3c**, while little effect was observed with amyl nitrite under neutral conditions in accordance with the order of easiness of NO⁺ formation. Compound **4c** was not formed within 3 h in the absence of a nitrosating agent.

Compounds **6**, **7** and **8** are also potent carcinogens that induce tumors of the liver and esophagus in rats.^{3,4)} Although these compounds are not considered to produce nitrosating agents by themselves upon standing at room temperature, they might give *N*-nitroso-*N*-alkyl-1-hydroxyiminoalkylamine-type compounds by addition of nitrosating agents. Addition of nitrosyl chloride to methyl acetate solutions of **6**, **7** and **8** gave **9** and **10**, **11** and **12**, and **13**, respectively (Chart 2). The formation of **9** and **10** is explainable as follows. After acid-catalyzed enolization of **6**, addition of NO⁺ to enol tautomers occurs,^{15b)} followed by

isomerization to form oximes, namely *N*-nitroso-*N*-butyl-1-hydroxyimino-2-oxopropylamine and **10**. The former oxime would be further changed to *N*-nitroso-*N*-butyl-1,2-dioxopropylamine by the action of excess nitrosyl chloride to afford *N*-nitroso-*N*-butyl-2-oxopropamide,¹⁶⁾ which would not be stable in an acidic solution due to the electron-withdrawing effect of the 1,2-dioxo group, resulting in cleavage of the *N*-nitroso bond to give **9**.¹⁷⁾ The production of **11** and **12** from **7** would occur through a similar mechanism. Compound **8** gave only **13** in a good yield.

These compounds (**9**, **10**, **11**, **12** and **13**) are not 1-hydroxyimino-type compounds, and showed no mutagenic activity toward *S. typhimurium* TA1535 or *E. coli* WP2 *hcr*⁻. Compound X (obtained from **6**) was mutagenic to *S. typhimurium* TA1535. Although its structure has not been elucidated yet, it has no hydroxyimino group based on its IR spectrum. The mutagenic activation of **6**, **7** and **8** might be different from that of **1** or **3**.

In order to study the ability of **3c** to produce nitrosating agents during storage in methyl acetate, nitrosation of **3c** was investigated in the presence of **8**. Compound **4c** was obtained in 20 or 18% yield with or without **8**, while **13** was not produced and **8** was recovered unchanged. On the other hand, **13** was produced in 50% yield when nitrosyl chloride was added as a nitrosating agent to a methyl acetate solution of **3c** and **8**. These results showed that **3** is much more easily nitrosated than **8** by nitrosating agents formed from itself.

In addition to the generally accepted α -hydroxylation pathway for the metabolic activation of *N*-nitrosamines, an activation through β -hydroxylation has been proposed especially for *N*-nitrosamines with a 2-hydroxyethyl group.^{14,18)} The formation of a directly acting mutagen with a unique structure from the formylmethyl compound may be another possible pathway for activation through β -hydroxylation. To determine which of these pathways is mostly involved in the initiation of carcinogenesis, further work will be necessary.

Since **4c** is formed even in aqueous media with nitrite and hydrochloric acid, it may be expected that **4** is formed *in vivo* in the stomach from **3** and nitrite.

References and Notes

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