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Metabolic α-Hydroxylation of *N*-Nitrosomorpholine and 3,3,5,5-Tetradeutero-*N*-nitrosomorpholine in the F344 Rat[1, 2]

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ABSTRACT

We studied the metabolism in the male F344 rat of *N*-nitrosomorpholine and of 3,3,5,5-tetradeutero-*N*-nitrosomorpholine; the latter is less carcinogenic and less mutagenic than is *N*-nitrosomorpholine. α-Hydroxylation (3- or 5-hydroxylation) of *N*-nitrosomorpholine by liver microsomes and a reduced nicotinamide adenine dinucleotide phosphate-generating system produced (2-hydroxyethoxy)acetaldehyde, which was identified as its 2,4-dinitrophenylhydrazone derivative. When we administered *N*-nitrosomorpholine to rats i.p., we did not detect (2-hydroxyethoxy)acetaldehyde in the urine, but we did identify (2-hydroxyethoxy)acetic acid (16% of the dose). We also identified *N*-nitroso(2-hydroxyethyl)glycine (33% of the dose) from β-hydroxylation (2- or 6-hydroxylation), *N*-nitrosodiethanolamine (12%), and unchanged *N*-nitrosomorpholine (1.5%) in the urine. The deuterated analogs of the above metabolites were isolated from the urine of rats treated with 3,3,5,5-tetradeutero-*N*-nitrosomorpholine in yields as follows: (2-hydroxyethoxy)acetic acid (3.4%); *N*-nitroso(2-hydroxyethyl)glycine (37%); *N*-nitrosodiethanolamine (12%); *N*-nitrosomorpholine (0.4%). These data demonstrate that deuterium substitution in the α-positions of *N*-nitrosomorpholine caused a decrease in the extent of α-hydroxylation and indicate that α-hydroxylation is the mechanism of activation of *N*-nitrosomorpholine.

INTRODUCTION

NMOR[4] (see Chart 1) is a powerful carcinogen which induces liver and kidney tumors in rats, tracheal and nasal cavity tumors in Syrian golden hamsters, and liver and lung tumors in mice (2, 8, 9, 16, 23). Its potency is indicated by the observation that a single dose of only 0.075 of the 50% lethal dose (approximately 4 mg/animal) induced tumors in approximately 20% of hamsters treated with NMOR (23). NMOR has been detected as a contaminant in crankcase emissions from diesel engines and in rubber manufacturing (11, 12). It is readily formed by nitrosation of morpholine, *in vitro* and *in vivo* (13, 16, 22, 25).

The mechanism of metabolic activation of NMOR is believed to involve breaking of an α (positions 3 and 5) C—H bond since 3,3,5,5-tetradeutero-NMOR is significantly less carcinogenic than NMOR in rats and less mutagenic than NMOR toward *Salmonella typhimurium* TA1535 and *Escherichia coli* WU 3610 (5, 10, 19). By analogy to studies on other nitrosamines, α-hydroxylation has been assumed to be the metabolic step leading to reactive intermediates which would be ultimate carcinogens or mutagens of NMOR (8, 15, 20). However, metabolic α-hydroxylation of NMOR has not been demonstrated previously. Stewart *et al.* (27) identified *N*-nitrosodiethanolamine as a urinary metabolite of NMOR in the rat. Manson *et al.* (21) confirmed these observations and also identified 2-hydroxy-NMOR as a microsomal metabolite. Denitrosation and reduction of NMOR have also been observed in microsomal systems (1, 30). In the present study, we have identified urinary and microsomal metabolites that resulted from α-hydroxylation of NMOR in the rat. The extents of formation of urinary metabolites resulting from α-hydroxylation and β-hydroxylation of NMOR and of 3,3,5,5-tetradeutero-NMOR were compared, and the results support the hypothesis that NMOR is activated by α-hydroxylation.

MATERIALS AND METHODS

Apparatus

Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were determined with a Hitachi Perkin-Elmer Model R-124 spectrometer in $CDCl_3$ solution and are reported as ppm downfield from tetramethylsilane as internal reference. Mass spectra and GLC-MS were run with a Hewlett-Packard Model 5982A dual-source instrument with a membrane separator. GLC was done on a Hewlett-Packard Model 5830A instrument equipped with a flame ionization detector and the following columns: Column A, 6-ft x 0.125-inch 10% UCW98 on WHP. 7620; Column B, 6-ft x 0.25-inch (glass) 10% Carbowax 20M-TPA on Gas-chrom Q, with helium as carrier gas (50 ml/min). Thin-layer chromatography was performed with Silica Gel 60 F glass plates (EM Laboratories, Elmsford, N.Y.). HPLC was carried out with a Waters Associates Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 660 solvent programmer, a Model U6K septumless injector, a Model 440 UV/visible detector, and two 3.9-mm x 30-cm C_{18}-μBondapak columns in series (Waters Associates, Milford, Mass). Cell disruption was performed with a Polytron homogenizer (Willems type; Kinematic GmbH, Lucerne, Switzerland). Centrifugation was done with a Sorval RC2-B centrifuge and a Spinco Model L ultracentrifuge.

Chemicals

NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo. Aroclor 1254 was obtained from Analabs, Inc., Hamden, Conn. Regisil RC-2 was procured from Regis Chemical Co., Morton Grove, Ill. NMOR (18), 3,3,5,5-tetradeutero-NMOR (19), *N*-nitrosodiethanolamine (26), and *N*-nitroso(2-hydroxyethyl)glycine (28) were prepared essentially as

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[4] The abbreviations used are: NMOR, N-nitrosomorpholine; 3,3,5,5-tetradeutero-NMOR, 3,3,5,5-tetradeutero-*N*-nitrosomorpholine; 2-hydroxy-NMOR, 2-hydroxy-*N*-nitrosomorpholine; GLC-MS, combined gas-liquid chromatography-mass spectrometry; GLC, gas-liquid chromatography; HPLC, high-pressure liquid chromatography; t, triplet; s, singlet.

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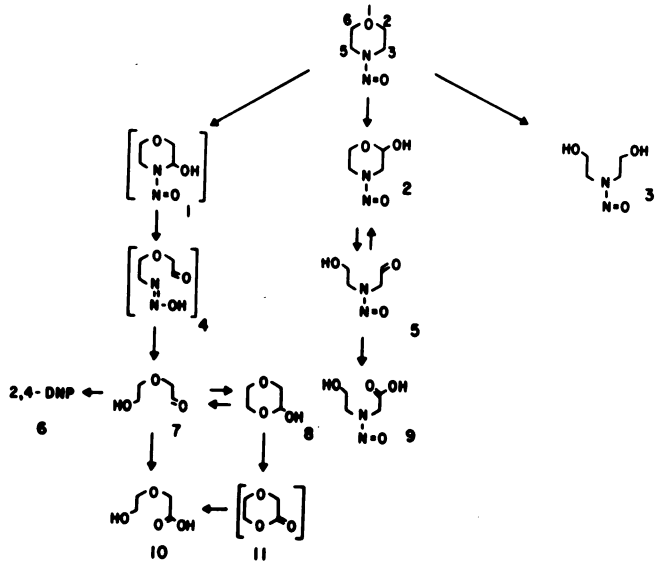

Chart 1. Metabolsim of NMOR in the F344 rat. Compounds in *brackets* represent hypothetical intermediates.

described previously.

(2-Hydroxyethoxy)acetaldehyde-2,4-dinitrophenylhydrazone (Structure *6*). 2,4-Dinitrophenylhydrazine (0.4 g, 2 mmol; Eastman Kodak Co., Rochester, N. Y.) was dissolved in 100 ml of 2 N HCl at 60°. After cooling to room temperature, 40 ml of a 0.67% aqueous solution of 2,3-dioxene (3.1 mmol) (29) were added. The resulting mixture was cooled to 0° for 30 min and filtered, and the solid Structure *6* was rinsed with 20 ml of 2 N HCL and 40 ml of H_2O, and recrystallized from ethanol, m.p. 138–140°; literature (24) m.p. 136–138°.

Dioxane-2-one (Structure *11*). The corresponding polymer (4) was obtained from Aldrich Chemical Co., Milwaukee, Wis., and was depolymerized by microdistillation at 70° under reduced pressure (aspirator). Mass spectrum: *m/e* 102 (M^+, 19); 90 (24); 60 (39); 61 (73); 60 (100). Nuclear magnetic resonance spectrum; 3.85 ppm, 2H, t; 4.35 ppm, 2H, 2; 4.49 ppm, 3H, t.

(2-Hydroxyethoxy)acetic Acid (Structure *10*). Dioxane-2-one polymer (42.9 mg) was heated under reflux in 2 ml of 1 N NaOH for 1 hr (4). The mixture was neutralized with HCl, filtered, and washed with ether, and the solvent was removed under reduced pressure to give Structure *10* (18.1 mg). An aliquot was silylated and analyzed by GLC-MS on Column A, under conditions described below. A single major peak, other than Regisil RC-2 was observed; for mass spectrum, see Chart 4. The mass spectrum of Structure *10*, obtained by direct introduction without silylation, was identical to that of Structure *11*, owing to thermal cyclization (3).

Metabolism of NMOR *in Vitro*

Isolation of (2-Hydroxyethoxy)acetaldehyde-2,4-dinitro-phenylhydrazone (*6*). Liver microsomes were obtained from male F344 rats which had been given Aroclor 1254 (500 mg/kg i.p.) 4 days prior to sacrifice. Microsomes were prepared as described previously (15). For all incubations, a stock solution was prepared containing 0.1 M Tris-base, 0.1 M glucose 6-phosphate, and 0.1 M $MgCl_2$. The pH of this stock was adjusted to 7.6 with HCl. Incubations were carried out for 20 min at 37° in two 50-ml Erlenmeyer flasks. Each flask contained 5 ml of stock solution, 23.2 mg (0.2 mmol) NMOR, 2.5 mg (3.0 μmol) NADPH, 50 units glucose-6-phosphate dehydrogenase, and 1.0 ml microsomal suspension (13.1 mg/ml protein). The total volume was brought to 10 ml with distilled H_2O. At the end of the incubation, 10 ml of cold ethanol were added, the mixture was centrifuged at 2500 rpm at 10° to remove protein, and the supernatants were decanted and combined. 2,4-Dinitrophenylhydrazine reagent (2.5 ml, 0.15 M) was added to the combined supernatants, and the mixture was allowed to stand overnight. It was then extracted 3 times with 50 ml of $CHCl_3$, and the combined $CHCl_3$ layers were dried ($MgSO_4$) and concentrated. The residue was applied to 0.5-mm silica gel preparative thin-layer chromatography plates and eluted with $CHCl_3/CH_3OH$ (15/1). The band corresponding to (2-hydroxyethoxy)acetaldehyde-2,4-dinitrophenylhydrazone (Structure *6*) was isolated and purified further by HPLC using Column A with a gradient as follows: 30% CH_3OH in H_2O to 60% CH_3OH in H_2O in 1 hr at 1.8 ml/min. Detection was by absorbance at 340 nm. The retention volume of Structure *6* was 76 ml. The peak corresponding to Structure *6* was collected and concentrated; its mass spectrum was determined by direct introduction.

Analysis of Incubation Mixtures for Structure *6*. Incubations were carried out in 25-ml Erlenmeyer flasks, on one-fifth the scale described above. After quenching with 2.0 ml cold ethanol, the resulting mixtures were centrifuged at 2500 rpm at 10° to remove protein. Three ml of the supernatant were treated with 0.2 ml of 0.15 M 2,4-dinitrophenylhydrazine reagent and allowed to stand overnight. For HPLC analysis, 0.5 ml was neutralized with 14 μl 10 N NaOH, and 50 μl were injected. HPLC conditions were exactly as described above. The formation of Structure *6* was quantified by comparison of peak height to that of Reference Structure *6*.

Metabolism of NMOR and 3,3,5,5-Tetradeutero-NMOR *in Vivo*

Analysis for (2-Hydroxyethoxy)acetaldehyde-2,4-dinitrophenylhydrazone (Structure *6*). Two male F344 rats were each given i.p. injections of NMOR (150 mg/kg body weight) in 0.9% NaCl solution. The 48-hr urine was collected over 20 ml of 0.15 M 2,4-dinitrophenylhydrazine reagent. The urine was combined, neutralized with NaOH, and extracted with CH_2Cl_2. The CH_2Cl_2 extracts were dried (Na_2CO_3), concentrated, and analyzed essentially as described above for the isolation of Structure *6 in vitro*.

Analysis for (2-Hydroxyethoxy)acetic acid (Structure *10*), *N*-Nitroso(2-hydroxyethyl)glycine (Structure *9*), and *N*-Nitrosodiethanolamine (Structure *3*). Male F344 rats were given i.p. 125-mg/kg body weight injections of NMOR, 3,3,5,5-tetradeutero-NMOR, or *N*-nitrosodiethanolamine in 0.9% NaCl solution. The 48-hr urine was collected at −76° and then lyophilized. The residue from each urine sample was sonically dispersed with CH_3OH, filtered, concentrated, and transferred to a 5-ml volumetric flask. A 0.05-ml aliquot was concentrated under a stream of N_2 in a 1-ml Reacti-vial (Pierce Chemical Co., Rockford, Ill.). The residue was treated with 200 μl Regisil for 20 min at 70°. The samples were analyzed immediately by GLC using Column A with a temperature program as follows: 60° for 8 min, then 4°/min to 240°. Metabolites were identified by coinjection with standards and by GLC-MS (see Charts 3 to 5).

Analysis for Dioxane-2-one (Structure *11*). Urine was extracted 3 times with CH_2Cl_2, and the extracts were analyzed by GLC-MS on Column A with a temperature program of 60° for 8 min and then 4°/min to 240°.

Analysis for NMOR and 3,3,5,5-tetradeutero-NMOR. An aliquot of the CH_3OH extract of the lyophilized urine was analyzed by GLC on Column B using a temperature program of 100° for 8 min, and then 4°/min to 240°.

RESULTS

Based on previous studies of the metabolism of *N*-nitrosopyrrolidine, *N*-nitrosopiperidine, and *N'*-nitrosonornicotine, we expected that α-hydroxylation of NMOR would lead to (2-hydroxyethoxy)acetaldehyde (Chart 1, Structure *7*) via the unstable intermediates Structures *1* and *4* (6, 15, 17). We incubated NMOR with liver microsomes from F344 rats and a NADPH-generating system. At the end of the incubations, we added

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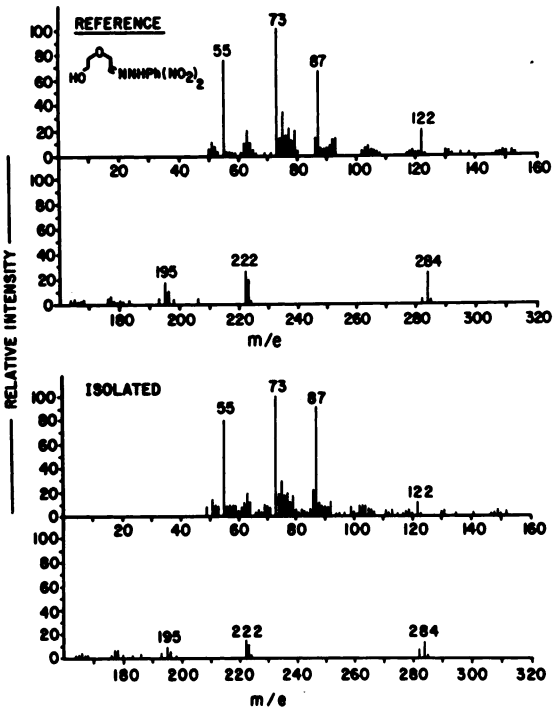

Chart 2. Mass spectra of reference (2-hydroxyethoxy)acetaldehyde 2,4-dinitrophenylhydrazone and the compound isolated upon incubation of NMOR with rat liver microsomes, followed by treatment with 2,4-dinitrophenylhydrazine reagent.

2,4-dinitrophenylhydrazine reagent and analyzed the mixture by HPLC. A peak which coeluted with standard (2-hydroxyethoxy)acetaldehyde-2,4-dinitrophenylhydrazone but which was not present in control incubations was collected, and its mass spectrum was determined. As shown in Chart 2, the mass spectrum was essentially identical to that of the reference standard. (2-Hydroxyethoxy)acetaldehyde, which exists predominantly as 2-hydroxydioxane (Structure *8*) (29), was not formed from NMOR in the absence of NADPH or when heat-deactivated microsomes were used. Its formation under our incubation conditions was linear for at least 20 min. The rate of formation of Structure *7*, measured as its 2,4-dinitrophenylhydrazone derivative, was 2.6 nmol/min/mg protein.

To determine whether Structures *7* and *8* were present as urinary metabolites of NMOR, we treated 2 rats with NMOR (150 mg/kg) and collected the 48-hr urine in vessels containing 2,4-dinitrophenylhydrazine reagent. We did not detect the 2,4-dinitrophenylhydrazone Structure *6* in the urine. We suspected that Structures *7* and *8* might be further oxidized *in vivo*, as has been observed with related metabolites of other cyclic nitrosamines (6, 7). Therefore, we treated another group of rats with NMOR (125 mg/kg). We analyzed the residue of the lyophilized 48-hr urine for lactone Structure *11* and, after silylation, for hydroxy acid Structure *10*. We did not detect Structure *11*, but we did observe Structure *10* as a major urinary metabolite as shown in Chart 3*a* (Peak A) and Table 1. The mass spectra of reference and isolated (2-hydroxyethoxy)acetic acid (Structure *10*), as their bistrimethylsilyl derivatives, are illustrated in Chart 4.

We observed 2 additional major peaks, not present in control samples, in the gas chromatograms of the silylated urine residues of the NMOR-treated rats. These were identified, by

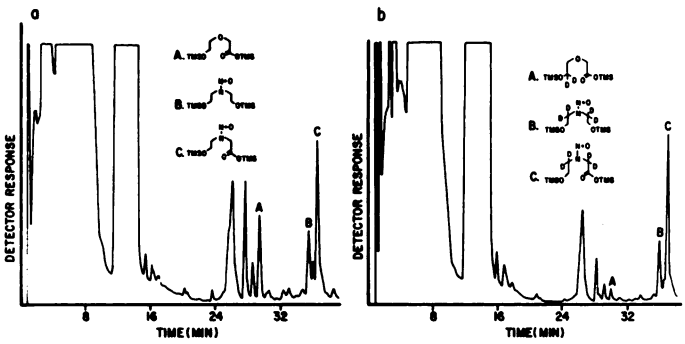

Chart 3. Gas chromatograms of silylated extracts of urine from (*a*) rats treated with NMOR and (*b*) rats treated with 3,3,5,5-tetradeutero-NMOR. Peaks A, B, and C were identified by their mass spectra as the structures indicated. The unlabeled peaks were also observed in the urinary extracts of untreated rats.

Table 1

Urinary metabolites of NMOR and 3,3,5,5-tetradeutero-NMOR

Male F344 rats were given i.p. injections of NMOR or 3,3,5,5-tetradeutero-NMOR (125 mg/kg body weight). The 48-hr urine was analyzed as described in "Materials and Methods."

Metabolite[a]	% of excretion after injection of NMOR	% of excretion after injection of 3,3,5,5-Tetradeutero-NMOR
(2-Hydroxyethoxy)acetic acid (*10*)[b]	16 ± 4[c]	3 ± 2
N-Nitrosodiethanolamine (*3*)	12 ± 3	12 ± 4
N-Nitroso(2-hydroxyethyl)glycine (*9*)	33 ± 5	37 ± 4
Unchanged nitrosamine	1.5 ± 0.5	0.4 ± 0.1

[a] The metabolites from 3,3,5,5-tetradeutero-NMOR contained deuterium atoms as shown in Chart 3*b*.
[b] Numbers in parentheses, structures in Chart 1.
[c] Mean ± S.D. for analyses of urine from 6 rats.

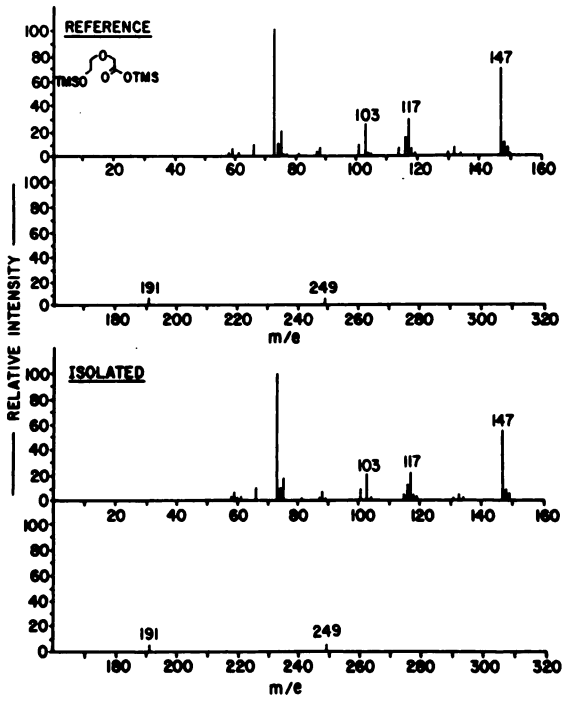

Chart 4. Mass spectra of the bistrimethylsilyl derivative of (2-hydroxyethoxy)acetic acid and a urinary metabolite (Peak A of Chart 3*a*) of NMOR.

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comparison to reference samples, as *N*-nitroso(2-hydroxyethyl)glycine (Structure *9*) and *N*-nitrosodiethanolamine (Structure *3*). The mass spectra of reference and isolated Structure *9*, bis-trimethylsilyl derivatives, are shown in Chart 5. The identification of *N*-nitrosodiethanolamine (Structure *3*) is in agreement with previous studies of NMOR metabolism (21, 27). Less than 1% of *N*-nitrosodiethanolamine was metabolized to Structure *9*.

To determine the effect of deuterium substitution on the metabolism of NMOR and of 3,3,5,5-tetradeutero-NMOR *in vivo*, we compared their urinary metabolites. The gas chromatogram of the silylated urinary metabolites of 3,3,5,5-tetradeutero-NMOR is shown in Chart 3*b*. The indicated peaks were identified by their mass spectra. The spectrum of Peak A was similar to that shown in Chart 4, except that the ions at *m/e* 191 and *m/e* 249 were shifted to *m/e* 193 and *m/e* 251. Ions were also observed at *m/e* 103 and *m/e* 105 and at *m/e* 117 and *m/e* 119. These results indicate the presence of 2 deuterium atoms in the metabolite, as shown in Chart 3*b*. We observed analogous shifts in the mass spectra of Peaks B and C of Chart 3*b*, indicating the presence of 4 deuterium atoms, and we assigned the structures as shown.

Inspection of Chart 3, *a* and *b*, shows that, among the urinary metabolites of 3,3,5,5-tetradeutero-NMOR, Peak A decreased relative to its concentration in the urinary metabolites of NMOR, whereas Peaks B and C, corresponding to *N*-nitrosodiethanolamine and *N*-nitroso(2-hydroxyethyl)glycine remained the same. The effects of deuterium substitution on the formation of the 3 major urinary metabolites of NMOR and on the excretion of unchanged nitrosamine are summarized in Table 1.

DISCUSSION

The formation of (2-hydroxyethoxy)acetaldehyde (Structure *7*) and (2-hydroxyethoxy)acetic acid (Structure *10*) as metabolites of NMOR is most readily explained by an initial α-hydroxylation of NMOR followed by ring opening as shown in Chart 1. This process is analogous to that observed in the metabolism by α-hydroxylation of other cyclic nitrosamines (6, 15, 17). In those cases, the intermediacy of structures such as Structures *1* and *4* has been supported by chemical studies on appropriate model compounds (14). The mechanism for formation of Structure *10* as well as its structural assignment are also supported by the retention of 2 deuterium atoms in the (2-hydroxyethoxy)acetic acid isolated from the urine of rats treated with 3,3,5,5-tetradeutero-NMOR. The detection of Structure *10*, but not lactone Structure *11*, as a urinary metabolite of NMOR is analogous to observations with *N'*-nitrosonornicotine in which the corresponding hydroxy acid was a major urinary metabolite, but the lactone was detected in only trace quantities (6). Both Structures *10* and *11* have been reported as urinary metabolites in rats treated with dioxane (3, 31).

We did not attempt to detect 2-hydroxy-NMOR (Chart 1, Structure *2*) as a microsomal metabolite, because its formation had previously been demonstrated (21). Since Structure *2* is in equilibrium with the hydroxy aldehyde Structure *5* (21), it is reasonable to assume that the urinary metabolite, *N*-nitroso(2-hydroxyethyl)glycine, results from initial β-hydroxylation of NMOR. An alternate mechanism for formation of Structure *9* is metabolic oxidation of *N*-nitrosodiethanolamine (Structure *3*). However, we obtained less than 1% of Structure *9* as a urinary metabolite of Structure *3*. *N*-Nitrosodiethanolamine could be formed by direct cleavage of the C_6—O bond or by reduction of hydroxy aldehyde Structure *5*.

The extents of formation of *N*-nitroso(2-hydroxyethyl)glycine from β-hydroxylation and of *N*-nitrosodiethanolamine were the same in the metabolism of 3,3,5,5-tetradeutero-NMOR as in the metabolism of NMOR. The extent of metabolism of 3,3,5,5-tetradeutero-NMOR to other products, thus far unidentified, was apparently greater than that of NMOR. In contrast, the extent of formation of (2-hydroxyethoxy)acetic acid by α-hydroxylation was approximately one-fifth as great in the metabolism of 3,3,5,5-tetradeutero-NMOR as in the metabolism of NMOR. The incidence of hepatocellular tumors in animals treated with 3,3,5,5-tetradeutero-NMOR was approximately one-third as great as in rats treated with NMOR, and the mutagenicity of 3,3,5,5-tetradeutero-NMOR toward *S. typhimurium* was one-fifth as great as that of NMOR (5, 19). These results strongly indicate that α-hydroxylation is the major activation pathway of NMOR in the rat and suggest that the diazohydroxide Structure *4* is an ultimate carcinogen of NMOR.

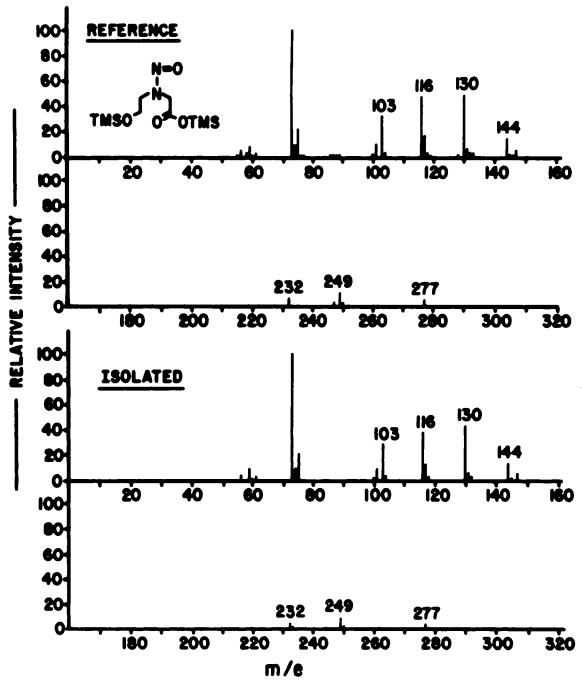

Chart 5. Mass spectra of the bistrimethylsilyl derivative of *N*-nitroso(2-hydroxyethyl)glycine and a urinary metabolite (Peak C of Chart 3*a*) of NMOR.

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