IN VITRO METABOLISM OF N-NITRODIALKYLAMINES

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The in vitro metabolism of N-nitramines was investigated in order to compare it with that of N-nitrosamines and to elucidate the mode of mutagenic action. N-Nitrodibutylamine (NO$_2$DBA) and N-nitrodiethylamine (NO$_2$DEA) were incubated with liver microsomes and hepatocytes prepared from rats treated with phenobarbital, and the products were analyzed by high-performance liquid chromatography and gas-liquid chromatography. The in vitro metabolic pattern of these nitramines was similar to that of the corresponding nitrosamines, except that N-nitro-N-alkylamines (produced via $\alpha$-hydroxylation) were identified after incubation of the nitrodialkylamines. In the case of NO$_2$DBA, besides N-nitro-N-butylamine, several nitramines produced by $\omega$, $\omega$-1, and $\omega$-2 oxidations were identified as metabolites. NO$_2$DBA and NO$_2$DEA were mutagenic to Escherichia coli WP2 hr$^-$ but not to Salmonella typhimurium TA1535. They were mutagenic only in the presence of hepatic microsomes, whereas their metabolites, N-nitro-N-butylamine and N-nitro-N-ethylamine, were direct mutagens. Thus, N-nitrodialkylamines are also metabolically activated to mutagens through $\alpha$-hydroxylation.

Key words: Nitramine synthesis — Metabolic activation — Metabolism by microsomes and hepatocytes — Mutagenicity

N-Nitramines can be produced as atmospheric pollutants together with N-nitrosamines when nitrogen oxides react with secondary amines.$^1$ Their mutagenic,$^7, 12$ carcinogenic,$^3, 8, 15$ and other biological activities$^{14}$ were reported to be much lower than those of the corresponding nitrosamines. $\alpha$-Hydroxylation has been shown to be a key step in the metabolic activation of N-nitrosamines,$^3, 11$ but little is known about the metabolism and metabolic activation of N-nitramines. In the present work, we have investigated the in vitro metabolism of NO$_2$DBA$^*$ and NO$_2$DEA by rat liver microsomes, with regard to their metabolic activation to mutagens and their metabolism by rat hepatocytes.

MATERIALS AND METHODS

Instrumental Analyses Ultraviolet (UV) spectra were measured in 99.5% ethanol solution on a Hitachi EPS-3T spectrometer. Infrared (IR) spectra were determined for liquid films with a Hitachi EPI-S2 spectrometer. Nuclear magnetic resonance (NMR) spectra were taken in CDCl$_3$ or CCl$_4$ solution at 60 MHz on a Hitachi R-20A spectrometer. Chemical shifts are expressed in $\delta$ with tetramethylsilane as an IS. The coupling constants ($J$) are expressed in Hz: $s$, singlet; $t$, triplet; $q$, quartet.

Chemicals NDBA, NDEA, and 7,8-benzo-flavone were purchased from Tokyo Kasei Kogyo Co., Tokyo, and NADP, NADPH, NAD and NADH were purchased from Oriental Yeast Co., Ltd., Tokyo. Glucose 6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and collagenase (type I, EC 3.4.24.3) were obtained from

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Sigma Chemical Co., St. Louis, Mo. SKF 525-A (2-diethylaminoethyl 2,2-diphenylpentanoate hydrochloride) was a gift from Smith, Kline & French Laboratories, Philadelphia, Pa. NO2DBA and NO2DEA were synthesized by the oxidation of NDBA and NDEA, respectively, with trifluoroacetic acid and hydrogen peroxide.4) NO2BA was prepared by the method described previously.5) NO2HEBA-2: Trifluoroacetic acid (25 g, 0.2 mol) was added at room temperature to 5.1 ml of 30% hydrogen peroxide, and 1.46 g (0.01 mol) of N-nitroso-N-(2-hydroxyethyl)butylamine13) was added to this solution. The resulting solution was warmed at 50° for 1.5 hr. The mixture was then poured into water (100 ml) and extracted with dichloromethane (100 ml × 4). The organic layer was washed with 10% NaOH and water, and dried over anhydrous Na2SO4. The residue obtained after evaporation of the solvent under reduced pressure gave two spots (Rf = 0.8 and 0.3) on TLC with hexane:ether:dichloromethane = 1:1:1. The compound with Rf = 0.8 was considered to be the trifluoroacetate of NO2HEBA-2, since it gave NO2HEBA-2 (Rf = 0.3) on alkaline hydrolysis. The residue was dissolved in a mixture of methanol (30 ml) and 40% NaOH (1 ml). After standing for several minutes, the resulting solution was neutralized with 12N HCl, then methanol was removed in vacuo, and the residue was extracted with ethyl acetate (200 ml). The ethyl acetate solution was washed with water and dried over anhyd. Na2SO4. The residue (1.5 g) obtained after evaporation of the solvent was subjected to column chromatography with hexane:ether:dichloromethane = 1:1:1 to give NO2HEBA-2 (1.28 g, 79% yield) as a colorless oil.

NO2HBB-2: The acetate of N-nitroso-N-[2-hydroxybutyl]butylamine13) (1.0 g) was oxidized with hydrogen peroxide in trifluoroacetic acid as described above for NO2HEBA-2. The product was purified by column chromatography with hexane:ether:dichloromethane = 8:3:2 to afford the acetate of NO2HBB-2 (950 mg, 96% yield) as a colorless oil. Alkaline hydrolysis of this acetate (560 mg) as described above followed by purification of the product by column chromatography (solvent, hexane:ether:dichloromethane = 5:3:2) gave NO2HBB-2 (430 mg, 94% yield).

NO2HBB-3: A solution of the acetate of N-nitroso-N-(3-hydroxybutyl)butylamine13) (707 mg) in hexane (780 ml) was irradiated with a 100 W high-pressure mercury lamp under bubbling of oxygen at 25°. After 70 min of irradiation, the mixture was removed by evaporation in vacuo to give a pale yellow residue, which was subjected to column chromatography. Elution with hexane:ether:dichloromethane = 8:3:2 afforded the acetate of NO2HBB-3 (480 mg, 59% yield) as a colorless oil. NO2HBB-3 (314 mg, 89% yield) was obtained after alkaline hydrolysis of the acetate (418 mg).

NO2HBB-4: N-Nitroso-N-(4-hydroxybutyl)-butylamine13) (1.74 g, 0.01 mol) was oxidized as described above for NO2HEBA-2 to give NO2HBB-4 (1.31 g, 69% yield) as a colorless oil.

Acetate of NO2HBB-1: A solution of N-nitroso-N-(1-acetoxybutyl)butylamine5) (470 mg) in hexane (400 ml) was irradiated for 30-45 min as described for the acetate of NO2HBB-3. The product was subjected to column chromatography, and elution with hexane:ether:dichloromethane (15:3:2) gave the acetate of NO2HBB-1 (286 mg, 53% yield) and NO2BA (18 mg, 6% yield), each as a colorless oil.

Acetate of NO2HEEA-1 and NO2EA: A solution of N-nitroso-N-(1-acetoxyethyl)ethylamine (3.8 g) in hexane (3.1 liter) was irradiated for 45 min as described above. The product was subjected to column chromatography, and elution with hexane:ether:dichloromethane (8:3:2) gave the acetate of NO2HEEA-1 (965 mg, 26% yield) and NO2EA (598 mg, 25% yield), each as a colorless oil.

NO2CPBA: Oxidation of the methyl ester of N-nitroso-N-(3-carboxypropyl)butylamine18) (1.12 g) with hydrogen peroxide and trifluoroacetic acid as described above gave the methyl ester of NO2CPBA (713 mg, 59% yield) as a colorless oil. The methyl ester (1.1 g) was dissolved in a mixture of methanol (10 ml) and 2 N NaOH (2.7 ml). After standing for 2 hr at room temperature, the solution was neutralized with 2 N HCl, then methanol was removed under reduced pressure, and the product was extracted with dichloromethane. The organic layer was washed with water and dried over anhyd. Na2SO4. The residue obtained after evaporation of the solvent was subjected to column chromatography with hexane:ether:dichloromethane = 1:1:1 to give NO2HBB-1 (0.5 g, 79% yield) as a colorless oil.

Elemental analysis results and spectral (UV, IR and NMR) data for the newly synthesized compounds are given in Tables I and II.

Chromatography TLC was performed on plates coated with a 0.25 mm layer of silica gel HF254 (E. Merck AG, Darmstadt). Spots were visualized with UV light (254 nm) and the reagent reported earlier.13) For column chromatography, silica gel (230-400 mesh, E. Merck AG) was used.

HPLC was run with a mini-pump model 800-9 (Nihon Seimitsu Kagaku Co., Ltd., Tokyo), equipped with a JF 1205A ultraviolet detector (Atto Corporation, Tokyo) operated at 254 nm. The column used (30 cm × 3.7 mm ID) was a reversed-phase LiChrosorb RP-8 (5 μm; E. Merck AG). When 40% acetonitrile was used as
the eluent, the retention times (min) for NO₂HEBA-2, N-nitroso-N-(4-hydroxybutyl)propylamine (as an IS), NO₂HBBA-2, NO₂BA, NO₂HBBA-3, NO₂HBBA-4, and NO₂HBBA-5 were 6.5, 11.7, 16.0, 18.5, 19.7, 21.0 and 26.5, and those for NO₂EA, N-nitroso-N-(4-hydroxybutyl)butylamine (IS) and NO₂DEA were 10.0, 14.7 and 18.5, respectively.

For the analysis by GLC, a Shimadzu GC-7AG gas chromatograph equipped with a flame ionization detector (FID) was used with a glass column (2.5 m × 3 mm ID) packed with 1.5% OV-17 on Chromosorb W AW-DMCS (60–80 mesh). The operating conditions were as follows: injector and detector temperatures, 220°; carrier gas (N₂) flow rate, 40 ml/min; column temperature, isothermal at 90° for the first 4 min, then programmed from 90° to 190° at 8°/min; detection by FID. Under these conditions, the retention times (min) for isobutyl benzoate (IS) and NO₂DBA were 12.9 and 14.4, respectively.

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**Table I. N-Nitrodialkylamines and Their Derivatives**

<table>
<thead>
<tr>
<th>Compound</th>
<th>UVλmax (nm)</th>
<th>IRλmax cm⁻¹</th>
<th>Formula</th>
<th>Analysis (%) Clad.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO₂HEBA-2</td>
<td>244 (6400)</td>
<td>3430, 1505</td>
<td>C₆H₁₄N₂O₄</td>
<td>44.43  8.70  17.27</td>
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<tr>
<td>NO₂HBBA-2</td>
<td>245 (6800)</td>
<td>3430, 1505</td>
<td>C₆H₁₅N₂O₃</td>
<td>(44.38) 8.76  16.90</td>
</tr>
<tr>
<td>NO₂HBBA-3</td>
<td>244 (7100)</td>
<td>3410, 1505</td>
<td>C₆H₁₅N₂O₃</td>
<td>50.50  9.54  14.73</td>
</tr>
<tr>
<td>NO₂HBBA-4</td>
<td>244 (6900)</td>
<td>3390, 1505</td>
<td>C₆H₁₅N₂O₃</td>
<td>(50.60) 9.68  14.68</td>
</tr>
<tr>
<td>Acetate of NO₂HBBA-1</td>
<td>238 (5900)</td>
<td>1755, 1535</td>
<td>C₁₀H₂₆N₂O₄</td>
<td>51.71  8.68  12.06</td>
</tr>
<tr>
<td>Acetate of NO₂HEBA-1</td>
<td>236 (5800)</td>
<td>1745, 1530</td>
<td>C₆H₁₂N₂O₄</td>
<td>(51.62) 8.80  11.91</td>
</tr>
<tr>
<td>NO₂CPBA</td>
<td>244 (6900)</td>
<td>1690, 1500</td>
<td>C₆H₁₆N₂O₄</td>
<td>47.05  7.90  13.72</td>
</tr>
</tbody>
</table>

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**Table II. NMR Spectral Data for N-Nitrodialkylamine Metabolites and Their Derivatives**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift,δ</th>
<th>δ</th>
</tr>
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<tbody>
<tr>
<td>O₂N-N-C₆H₄CH₂CH₂CH₂OH</td>
<td>&gt;N-CH₂ &gt;N-CH</td>
<td>3.6-4.1</td>
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<tr>
<td>O₂N-N-C₆H₄CH₂CH₂CH₃OH</td>
<td>&gt;N-CH₂ &gt;N-CH</td>
<td>3.4-4.2</td>
</tr>
<tr>
<td>O₂N-N-C₆H₄CH₂CH₃OH</td>
<td>&gt;N-CH₂ &gt;N-CH</td>
<td>3.6-4.0</td>
</tr>
<tr>
<td>O₂N-N-C₆H₄CH₂CH₃OH</td>
<td>&gt;N-CH₂ &gt;N-CH</td>
<td>3.5-3.9</td>
</tr>
<tr>
<td>O₂N-N-C₆H₄CH₂CH₃OH</td>
<td>&gt;N-CH₂ &gt;N-CH</td>
<td>3.57 (t)</td>
</tr>
<tr>
<td>O₂N-N-C₆H₄CH₂CH₂COOH</td>
<td>&gt;N-CH₂ &gt;N-CH</td>
<td>3.75 (q)</td>
</tr>
<tr>
<td>O₂N-N-C₆H₄CH₂CH₂COOH</td>
<td>&gt;N-CH₂ &gt;N-CH</td>
<td>3.5-4.0</td>
</tr>
</tbody>
</table>

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a) J=6.0-7.0 Hz.
b) The NMR spectrum was taken in CDCl₃ solution.
c) The NMR spectrum was taken in CCl₄ solution.
Bacterial Strains  
*Salmonella typhimurium* strain TA1535 was kindly provided by Dr. B. N. Ames, University of California, Berkeley, Calif. *Escherichia coli* WP2 try*−* his*−* was kindly donated by Dr. S. Iwahara, Food and Drug Center, Hadano, Kanagawa-ken.

Assay for Metabolism of N-Nitramines by Rat Liver Microsomes  
Microsomes were prepared from the liver of male Sprague-Dawley rats (6 weeks old, weighing 140–180 g) treated with 0.1% PB in the drinking water for 1 week as described earlier. The incubation mixture contained 5–6 mg of microsomal protein, 0.1M sodium phosphate buffer (pH 7.4), 33mM KCl and an NADPH-generating system (4mM NADP, 5mM glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase) in a final volume of 2.0 ml. The reaction was initiated by the addition of NO2DBA (2mM) in DMSO (40 µl) or NO2DEA (2mM) in DMSO (20 µl). Incubation was carried out in a brown 25-ml test tube for 1 hr at 37° under an atmosphere of oxygen with shaking (150 strokes/min). The reaction was terminated by chilling the mixture on ice. One ml of 20% ZnSO4 containing 0.01% N-nitroso-N-(4-hydroxybutyl)propylamine for NO2-DBA or 0.02% N-nitroso-N-(4-hydroxybutyl)butylamine for NO2DEA, as an IS, and saturated Ba(OH)2 (1.0 ml) were added to the incubation mixture and the precipitate was removed by centrifugation at 4°. For the analysis of remaining NO2DBA, the aqueous supernatant (0.25 ml) was extracted with ethyl acetate (0.5 ml) containing isobutyl benzoate (0.1mM) as an IS, and the organic phase was separated by centrifugation at 4°, dried over anhyd. Na2SO4 and subjected to GLC. N-Nitramines produced from NO2DBA and NO2DEA by the incubation, on the other hand, were analyzed by HPLC by direct injection of the aqueous supernatant (10 µl). For the identification of NO2CPBA, an acidic metabolite, the aqueous supernatant was made acidic by the addition of 2N HCl and extracted with dichloromethane, and the organic layer was subjected to HPLC. Determination of aldehydes was performed by HPLC as described earlier using 0.5 ml of the supernatant.

NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were omitted from the incubation mixture when a cofactor NADPH, NADP, NADH, or NAD (4 mM) was used instead of the NADPH-generating system. SKF 525-A (1mM) was added as an aqueous solution and 7,8-benzoflavone (1mM) was added as a DMSO solution. The amount of DMSO in the incubation mixture (2.0 ml) was adjusted to 40 µl. Incubation under an atmosphere of oxygen with shaking (150 strokes/min). The reaction was terminated by chilling the mixture on ice. One ml of 20% ZnSO4 containing 0.01% N-nitroso-N-(4-hydroxybutyl)propylamine for NO2DBA or 0.02% N-nitroso-N-(4-hydroxybutyl)butylamine for NO2DEA, as an IS, and saturated Ba(OH)2 (1.0 ml) were added to the incubation mixture and the precipitate was removed by centrifugation at 4°. For the analysis of remaining NO2DBA, the aqueous supernatant (0.25 ml) was extracted with ethyl acetate (0.5 ml) containing isobutyl benzoate (0.1mM) as an IS, and the organic phase was separated by centrifugation at 4°, dried over anhyd. Na2SO4 and subjected to GLC. N-Nitramines produced from NO2DBA and NO2DEA by the incubation, on the other hand, were analyzed by HPLC by direct injection of the aqueous supernatant (10 µl). For the identification of NO2CPBA, an acidic metabolite, the aqueous supernatant was made acidic by the addition of 2N HCl and extracted with dichloromethane, and the organic layer was subjected to HPLC. Determination of aldehydes was performed by HPLC as described earlier using 0.5 ml of the supernatant.

RESULTS  
Metabolism of NO2DBA by Microsomes  
NO2DBA (2mM) was incubated at 37° for 1 hr with microsomes because these conditions were found to be optimum in the case of NDBA. NO2BA and NO2HBBA-3 were identified as major metabolites and NO2HBBA-4 and NO2HBBA-2 were identified as minor metabolites by HPLC. Butyraldehyde was identified and determined as its 2,4-dinitrophenylhydrazone by HPLC. The amounts of NO2BA, NO2HBBA-3 and butyraldehyde produced by incubation of 2mM NO2DBA with microsomes prepared from untreated and PB-treated rats are given in Table III together with the amount of unchanged NO2DBA. When NO2DBA or the NADPH-generating system was omitted from the incubation mixture, no product formation was observed. Incubation of the acetate of NO2HBBA-1 (1mM) with the PB-treated microsomes, on the other hand, gave NO2BA and butyraldehyde in 96% and 104% yields, respectively.

Among the cofactors examined, the NADPH-generating system was the most effective
for producing NO₂BA and NO₂HBBA-3. The formation of NO₂BA was reduced to 90% and 70%, respectively, when NADPH or NADH was used as a cofactor instead of the NADPH-generating system, while NADP and NAD had no effect.

The effect of SKF 525-A (1mM) on the formation of NO₂BA and NO₂HBBA-3 was investigated, and the results showed that this compound inhibited the formation of NO₂BA (55% of the control), but not the production of NO₂HBBA-3. 7,8-Benzoflavone (1mM) showed a slight inhibitory effect on the formation of both products (94% and 83% of the controls, respectively), while carbon monoxide strongly inhibited the microsomal oxidation (data not shown).

Metabolism of NO₂DBA by Hepatocytes

Incubation of NO₂DBA (1mM, 2mM, and 4mM) for 1 hr at 37°C with hepatocytes (0.5, 1.0, 1.5, and 2.0×10⁷ cells/ml) showed that the amount of NO₂BA or NO₂HBBA-3 produced was the highest with 2mM NO₂DBA and 1.0×10⁷ cells/ml (data not shown). Thus 2mM NO₂DBA was incubated with 1.0×10⁷ cells/ml of hepatocytes. NO₂BA, NO₂HBBA-2, NO₂HBBA-3, NO₂HEBA-2, NO₂CPBA and compound A were identified as metabolites by HPLC. NO₂HBBA-4 could not be detected. Compound A was considered to be N-nitro-N-(3-oxobutyl)butylamine, because incubation of NO₂HBBA-3 under the same conditions as NO₂DBA gave NO₂HEBA-2 and compound A as metabolites. The presence of butyraldehyde in the aqueous supernatant could not be detected under these experimental conditions. The amounts of the major metabolites, NO₂BA and NO₂HBBA-3, are given in Table III together with the amount of NO₂DBA recovered. The amounts of the minor metabolites NO₂HEBA-2, NO₂HBBA-2 and NO₂CPBA were 2.7%, 2.0% and 1.3%, respectively, with hepatocytes obtained from PB-treated rats (not included in the table). No products were detected when NO₂DBA or hepatocytes were omitted from the incubation mixture.

Metabolism of NO₂DEA by Microsomes and Hepatocytes

Incubation of NO₂DEA (2mM) with microsomes at 37°C for 1 hr gave NO₂EA as a major metabolite, which was identified by HPLC. Acetaldehyde was determined by HPLC after derivatization to 2,4-dinitrophenylhydrazone. Incubation of NDEA (2 mM) with PB-induced rat-liver microsomes gave only acetaldehyde (5.5%) as a major metabolite. Nearly quantitative production of NO₂EA (96%) and acetaldehyde (96%) was observed in the incubation of the acetate of NO₂HEEA-1 (1mM) with the PB-treated microsomes.

Incubation of NO₂DEA (2mM) with hepatocytes in the same way as described above for NO₂DBA gave NO₂EA as a principal metabolite, which was determined by HPLC. The formation of acetaldehyde, however, could not be detected by HPLC.

**Mutagenicity of N-Nitramines**

Fig. 1 illustrates the mutagenicities of NO₂DBA, NO₂BA, NO₂DEA, and NO₂EA tested on...
IN VITRO METABOLISM OF NITRAMINES

Table IV. Analysis of Products Formed after Incubation of NO₂DEA (2mM) with Microsomes and Hepatocytes

<table>
<thead>
<tr>
<th>Oxidation system</th>
<th>Treatment</th>
<th>Incubation time (hr)</th>
<th>Unchanged NO₂DEA (%a)</th>
<th>Products (%a)</th>
<th>Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NO₂EA</td>
<td>Acetaldehyde</td>
</tr>
<tr>
<td>Microsomes</td>
<td>Untreated</td>
<td>1</td>
<td>97.5±0.9d</td>
<td>1.8±0.2</td>
<td>2.9±0.6</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>1</td>
<td>85.7±0.4</td>
<td>12.2±0.7</td>
<td>12.0±0.5</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Untreated</td>
<td>0.5</td>
<td>101.5±0.3</td>
<td>3.2±0.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>94.7±1.4</td>
<td></td>
<td>6.5±0.2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0.5</td>
<td>89.3±4.4</td>
<td>11.2±0.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>71.6±1.5</td>
<td></td>
<td>19.3±0.8</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Percentage of NO₂DEA incubated (2mM).
b) Unchanged NO₂DEA + NO₂EA.
c) Unchanged NO₂DEA + acetaldehyde.
d) Means±SD.

Fig. 1. Mutagenicity of NO₂DBA, NO₂DEA and related compounds tested on E. coli WP2 her⁻ in the presence (●) and absence (○) of rat-liver microsomes. 1, NO₂DBA; 2, NDBA; 3, NO₂BA; 4, Acetate of NO₂HBBA-1; 5, NO₂DEA; 6, NDEA; 7, NO₂EA; 8, Acetate of NO₂HEEA-1.

E. coli WP2 her⁻ in comparison with those of NDBA and NDEA. These nitramines were inactive toward S. typhimurium TA1535 (data not shown). NO₂DBA and NO₂DEA were found to be far less mutagenic than the corresponding nitrosamines (NDBA and NDEA) in the presence of microsomes, while NO₂BA and NO₂EA were found to be active in the absence of microsomes. The acetates of NO₂HBBA-1 and NO₂HEEA-1, model compounds of the unstable α-hydroxy NO₂DBA and NO₂DEA, were also active in the absence of microsomes.
DISCUSSION

Based on the metabolites identified, the in vitro metabolic pathways of NO$_2$DBA and NO$_2$DEA in hepatic microsomes and hepatocytes are shown in Fig. 2.

NO$_2$DBA was metabolized mainly through $\alpha$ and $\omega$-1 oxidations, while $\omega$ and $\omega$-2 oxidation pathways were also observed in vitro as minor metabolic pathways. The metabolic pattern and the extent of these oxidations of NO$_2$DBA on incubation with treated and untreated rat-liver microsomes were similar to those of NDBA.\(^{19}\) A marked difference, however, is that the identity of the intermediary metabolite in the case of NO$_2$DBA has been established. Thus, NO$_2$BA has been separated and identified, while the identification of N-nitroso-N-butylamine as such, following incubation of NDBA, has not yet been accomplished, due to the extreme instability of the N-nitroso-N-alkylamine. The metabolic formation of NO$_2$BA through $\alpha$-hydroxylation of NO$_2$DBA has been confirmed by the production of NO$_2$BA from the acetate of NO$_2$HBBA-1 (a model compound for $\alpha$-hydroxy NO$_2$DBA) on incubation with treated liver microsomes. The enzyme involved in the $\alpha$-hydroxylation of NO$_2$DBA

![Diagram of metabolic pathways](image-url)

**Fig. 2.** In vitro metabolism of NO$_2$DBA and NO$_2$DEA. *Compounds in parentheses have not yet been identified.
is considered to be cytochrome P-450, on the basis of the requirements for NADPH and oxygen, and responses to SKF 525-A and 7,8-benzoﬂavone. NO₂DBA was metabolized through α, ω, ω-1, and ω-2 oxidations on incubation with hepatocytes, with the α and ω-1 oxidations being the principal pathways. The formation of NO₂BA was again demonstrated, but the formation of butyraldehyde could not be detected in this oxidation.

NO₂EA was identiﬁed as a major metabolite of NO₂DEA after incubation with microsomes and hepatocytes. Thus, α-hydroxylation was also a principal metabolic pathway of this nitramine. The metabolic formation of NO₂EA through α-hydroxylation (NO₂HEEA-1) has also been conﬁrmed by the formation of NO₂EA from the acetate of NO₂HEEA-1.

In contrast to the microsomal systems, no aldehyde (butyraldehyde or acetaldehyde) could be detected with hepatocytes on incubation. It is possible that the aldehydes, once formed, undergo further metabolism with hepatocytes, but are not metabolized further by liver microsomes to any signiﬁcant extent.

It has been shown that E. coli WP2 her is far more sensitive than S. typhimurium TA 1535 to NDBA and NDEA in the presence of a metabolic activation system. No appreciable mutagenicity of NO₂DEA was observed with S. typhimurium TA1535 or TA100. In accordance with these findings, NO₂DBA and NO₂DEA did not show mutagenic activity toward S. typhimurium TA 1535. They showed a weak mutagenic activity toward E. coli WP2 her in the presence of microsomes, while NO₂BA and NO₂EA were directly mutagenic to this bacterial strain. As expected, the acetates of NO₂HBBA-1 and NO₂HEEA-1 were found to be mutagenic in the absence of microsomes. Thus, the weak mutagenicity of the N-nitrodialkylamines (NO₂DBA, NO₂DEA) may be due to the metabolic formation of the N-nitro-N-alkylamines (NO₂BA, NO₂EA) through α-hydroxylation. Further biochemical transformations of the N-nitro-N-alkylamines might be involved in the manifestation of mutagenicity, and this possibility should be explored in future studies.

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