Mutagenicity Studies in *Salmonella typhimurium* on Some Carcinogenic
*N-Nitramines in Vitro* and in the Host-mediated Assay in Rats¹

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**ABSTRACT**

*N-Nitrodimethylamine, N-nitrodiethylamine, N-nitromorpholine and their N-nitroso analogs, N-nitrosodimethylamine, N-nitrosodiethylamine, and N-nitrosomorpholine,* were tested in *Salmonella typhimurium* strains TA100 and TA1530. The mutagenicity of all compounds, except *N*-nitrodiethylamine, was demonstrated in liquid incubation assays in at least one of the tester strains; it required the presence of a postmitochondrial supernatant from the liver of Aroclor-treated rats, reduced nicotinamide adenine dinucleotide phosphate-generating system, and oxygen. When compared on a molar basis with their *N*-nitroso analogs, *N*-nitromorpholine was about 10 times less mutagenic and *N*-nitrosodimethylamine about 70 times less mutagenic. Addition of disulfiram to the assays at a final concentration of 0.1 mM efficiently inhibited mutagenesis by all nitro and nitroso compounds; ascorbic acid at a 7.4 mM concentration produced less inhibition.

Mutagenic activity of the three nitramines was also determined in the host-mediated assay in rats. After p.o. administration of each of the *N*-nitramines, cells of *S. typhimurium* strains TA1530 and TA100 that had been injected i.p. were isolated from the peritoneal liquid after 1, 3, and 6 hr. All three nitramines were found to be mutagenic for strain TA1530 but not for TA100. Mutation frequencies (number of histidine revertants per 10⁶ surviving cells) were in the descending order *N*-nitramines, *N*-nitrosodimethylamine and *N*-nitromorpholine. Inasmuch as the carcinogenic *N*-nitrodiethylamine, an exception, was not found to be mutagenic in this *in vitro* assay, we have extended our studies using a host-mediated mutagenicity assay in rats. Results on the mutagenicity obtained with these nitramines and, for comparative purposes, also those of the respective nitrosamines are reported herein.

**MATERIALS AND METHODS**

**Chemicals**

DMNA, DENA, and *N*-nitrosomorpholine were purchased from Merck-Schuchardt (Darmstadt, West Germany). *N*-Nitrodimethylamine (m.p. 55–56°), *N*-nitrodiethylamine (b.p. 48°), and *N*-nitrosomorpholine (m.p. 52–53°) were synthesized by Dr. A. S. Petrov (Laboratory of Organic Chemistry, N. N. Petrov Research Institute of Oncology, Leningrad, USSR) by oxidation of the respective *N*-nitrosamines with hydrogen peroxide in trifluoroacetic acid (10).

The purity of *N*-nitrodimethylamine, *N*-nitrodiethylamine, and *N*-nitrosomorpholine was ascertained by obtaining a single peak in a gas-liquid chromatograph coupled with a thermal energy analyzer (column, 2 m x 0.25 inch outside diameter, packed with Carbowaax 20M on Chromosorb W HP, 80 to 100 mesh, at 140°, using helium as a carrier gas and the thermal energy analyzer, Model 502 Thermo Electron Corporation). The retention times (min) of the nitramines and the corresponding nitrosamines (first and second figure, given in parentheses, respectively) were *N*-nitro/nitrosodimethylamine (3.5; 1.8); *N*-nitro/nitrosodiethylamine (4.5; 2.3); *N*-nitro/nitrosomorpholine (18.2; 10.8). Using the gas chromatographic method described above, a mixture of 7 nitrosamines and their corresponding nitramines (including dimethyl- and diethylnitramine) could easily be separated (33). The presence of nitrosamines (limit of detection, 20 pg of DMNA or DENA per injection and 50 pg of *N*-nitrosomorpholine per injection) as trace contaminants in the respective nitramine samples assayed can thus be firmly excluded. After thin-layer chromatography on silica gel F₂₅₄ (Merck) using as eluant n-hexane:diethyl ether:methylene chloride (4:3:2, by volume, for *N*-nitrodimethylamine and *N*-nitro...
diethylamine; 5:7:10, by volume, for 1/4-nitromorpholine) and were obtained commercially.

Immediately before use in mutagenicity assays. All other products were suspended in 0.9% NaCl solution (3 to 5 x 10^9 bacteria/ml for use in host-mediated assays). All other products were obtained commercially.

Animals and Pretreatment

Adult female BD-VI rats (100 to 120 g) were bred in the laboratory of the International Agency for Research on Cancer and fed on a Charles River CRF diet. Groups of 4 animals received a single i.p. injection of Aroclor 1254 (500 mg/kg body weight) 5 days before they were killed.

Bacterial Strains

S. typhimurium strains TA1530 and TA100 were provided by Professor B. N. Ames (University of California, Berkeley, Calif.). The presence of R factor was checked by seeding the TA100 and TA1530 strains was checked with methyl methanesulfonate and N-methyl-N'-nitro-N-nitrosoguanidine, respectively (1). The bacteria were grown overnight in nutrient broth at 37° and then centrifuged at 1900 x g for 15 min; 3 to 5 x 10^6 bacteria/assay were used for in vitro mutagenicity tests or were suspended in 0.9% NaCl solution (3 to 5 x 10^6 bacteria/ml for use in host-mediated assays).

Tissue Preparations

The livers of decapitated rats were excised, washed in an equal volume of 0.15 M KCI, minced with sterile scissors in 3 volumes of 0.15 M KCI (3 ml/g wet tissue), and homogenized in a Potter-Elvehjem-type homogenizer. The homogenate of the pooled livers from 4 rats was centrifuged at 9000 x g for 10 min; the resulting supernatant (S-9) was distributed in 2- to 6-ml portions and stored below -70° for 3 to 8 weeks. All procedures were carried out at 0-4° with sterile glassware and under sterile conditions, as described previously (4).

Mutagenicity Assays

Liquid Incubation Assays (Procedure A). These were used in preference to plate incorporation assays, since the former more readily detect the mutagenic action of DMNA and DENA (2).

The medium (made up to a final volume of 460 µl) contained 120 µl of S-9, cofactors [3.2 µmol of MgCl₂, 0.32 µmol of NADP⁺, 2 µmol of glucose-6-phosphate], 8 µmol of Sörensen phosphate buffer (pH 7.4), 80 µl of a bacterial suspension of strain TA1530 or TA100, and various concentrations of the test compound dissolved in 20 µl of DMSO. In some experiments, ascorbic acid was dissolved in 20 µl of 0.068 M Sörensen phosphate buffer (pH 7.4), and disulfiram was dissolved in 20 µl of DMSO and added to the incubation medium before the test compounds; final concentrations of 0.3 to 7.4 µM ascorbic acid and 0.05 to 0.1 µM disulfiram were used. Some control experiments were also carried out in which the test compound and disulfiram was dissolved in buffer instead of in DMSO.

The assay mixture was then flushed with oxygen for 15 sec and further incubated in an atmosphere of oxygen at 37° in stoppered glass tubes (10 ml) with shaking (80 strokes/min). In some experiments, the assay mixture was flushed with nitrogen for 40 sec and further incubated under nitrogen. After 80 min of incubation, enzyme-catalyzed reactions were stopped by adding 540 µl of ice-cold 0.9% NaCl solution. Two 0.5-ml aliquots were each added to 2 ml of histidine-poor soft agar and plated on plates of minimal glucose agar. The number his⁺ revertant colonies was counted after 48 hr of incubation at 37°. Except for N-nitrodiethylamine, none of the compounds showed any gross toxicity to the background lawn of bacteria within the concentrations tested. In control assays run in parallel, the cofactors (NADP⁺, glucose 6-phosphate) and/or the test compound and/or ascorbic acid and disulfiram were omitted. The results reported were calculated from 2 or more independent series of experiments.

Host-mediated Assay (Procedure B). The host-mediated assay, described previously for mice (18, 22, 24), was adapted for rats with the following modifications. Groups of 4 to 6 rats received a single p.o. application of each of the N-nitramines studied (0.2 g/kg body weight) dissolved in 0.5 ml DMSO per p.o. intubation. Control animals received the same volume of solvent only. After 10 min, 1 ml of the bacterial suspension of either TA1530 or TA100 strain in 0.9% NaCl solution (3 to 5 x 10⁸ bacteria) was injected i.p. into each rat. One, 3, and 6 hr after inoculation, the rats were killed by decapitation, and 2 ml of a 0.9% NaCl:5 mM Sörensen phosphate buffer solution (pH 7.4) was injected i.p. into each rat. The bacteria were removed from the peritoneal cavity with a sterile syringe; the suspension was centrifuged at 100 x g for 10 min. The supernatant was centrifuged a second time at 1900 x g for 15 min. The bacterial pellet was resuspended in 1 ml of ice-cold 0.9% NaCl solution. For scoring the number of his⁺ revertants, 0.2-ml aliquots of the 10-fold diluted bacterial suspension were added to 2 ml of histidine-poor soft agar and plated on plates of minimal glucose agar. For the determination of survivors, 6 x 0.2-ml aliquots of the 10⁻⁵ and 10⁻⁶ dilutions were added to 2 ml of histidine-containing soft agar and plated. Colonies were counted after 48 hr of incubation at 37°.

Procedure C. In another series of experiments, 12 rats were treated with a single p.o. dose of N-nitrodiethylamine, 0.2 g/kg body weight, and 10 min later were given injections of cells of TA1530 as described under Procedure B. After 1, 3, and 6 hr, the i.p. liquid, liver, kidneys, and lungs were isolated from the decapitated animals. The organs were washed in an equal volume of 0.15 M KCI, minced with sterile scissors in 3 volumes of 0.15 M KCI (3 mg/g wet tissue), and gently homogenized in a Potter-Elvehjem-type homogenizer. After centrifugation at 200 x g for 10 min, the supernatant fraction of the organ homogenate was centrifuged at 1900 x g for 15 min. The sediments bacteria were then resuspended in 10 ml of 0.9% NaCl solution. Aliquots (0.2 ml) of the 10⁻⁵ dilution were plated on Petri dishes for scoring of his⁺ revertants (4 plates) and 10⁻⁵ and 10⁻⁶ dilutions for the detection of survivors (6 plates).
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TA100, was flushed with nitrogen before incubation for 80 min under nitrogen. The number of revertants in the presence of nitrogen was only 25% of that seen in assays with oxygen.

N-Nitromorpholine, a member of the class of heterocyclic nitramines, was also tested for mutagenicity in the presence of rat liver microsomal enzymes. With concentrations up to 30 mM, the number of revertants in strain TA1530 increased linearly at a rate of 12 revertants per mM concentration of N-nitromorpholine (Chart 2). In similar experiments with strain TA100, N-nitromorpholine produced a lower mutagenic effect. At a 100 mM concentration, only 1.1 revertants per mM concentration were observed. N-Nitrodimethylamine and N-nitromorpholine both showed remarkable strain specificity in vitro, in that N-nitrodimethylamine was not detectable in TA1530 but was mutagenic in TA100, while N-nitromorpholine was about 9 times more active in TA1530 than in TA100. In contrast to the nitramines studied, a nitro aromatic nitramine, N-methyl-N,2,4,6-tetranitroaniline (a compound which also contains C-nitro groups) was found to be a direct-acting mutagen in S. typhimurium (35).

For comparative purposes, we investigated the microsomal-mediated mutagenesis in strains TA1530 and TA100 of the N-nitroso analogs, DMNA, DEMA, and N-nitrosomorpholine (Charts 3 to 5). In the case of DMNA and DEMA, nonlinear, sigmoidal dose-response curves were obtained with both strains; but with the N-nitrosomorpholine, the dose-response curves were linear up to a concentration of 12.5 mM, and mutagenic activities (expressed as the number of revertants per mM concentration) in the 2 strains were similar. Within the concentration range studied, DMNA and DEMA produced a slightly greater number of mutant colonies in strain TA100 than in strain TA1530, while the reverse was true for N-nitrosomorpholine. When the mutagenic effects of the 3 N-nitramines in vitro were compared with those of the respective N-nitroso compounds assayed under similar conditions, the mutagenic activity of the N-nitrosamines was, in general, found to be much greater than those of the respective N-nitramines, i.e., DMNA and N-nitrosomorpholine were about 60 to 70 times more mutagenic than was the respective N-nitramine in the same bacterial strain, TA100.

RESULTS AND DISCUSSION

Using liquid incubation assays and S. typhimurium TA100 as a sensitive indicator organism for the detection of electrophilic metabolites which can react with DNA, N-nitrodimethylamine was shown to be mutagenic in the presence of a liver S-9 fraction from Aroclor-treated rats, cofactors for microsomal monooxygenases, and oxygen. The number of his* revertant colonies increased linearly with concentrations up to 750 mM N-nitrodimethylamine (Chart 1). Analysis of N-nitrodimethylamine by thin-layer chromatography and gas-liquid chromatography excluded DMNA as a possible mutagenic impurity. Previous analytical studies (33) on a mixture of nitramines and the corresponding nitrosamines revealed that the dimethyl and diethyl compounds can be efficiently separated by the gas chromatographic method used (see "Materials and Methods"). Mirvish et al. (21) reported the carcinogenicity of N-nitrodimethylamine, with no detectable level of DMNA contamination. These results, akin to those presented herein, support the notion that no trace contaminants but nitramines themselves act as mutagens-carcinogens.

Omission of the NADPH-generating system from the assays reduced the number of revertants to the spontaneous level seen in strain TA100, indicating that N-nitrodimethylamine is not a direct-acting mutagen nor can it be activated by bacterial enzymes, e.g., nitro reductases. Oxidative metabolism of N-nitrodimethylamine by measuring 14CO2 production in the presence of rat liver slices has been reported (12). The oxygen requirement for the metabolic activation of N-nitrodimethylamine into mutagens was demonstrated in experiments in which the incubation mixture, containing 200 or 500 mM N-nitrodimethylamine, rat liver S-9, cofactors, and S. typhimurium strain as described under Procedure B. Mutation frequencies were expressed as the number of his* revertants per 106 surviving cells. Because the number of surviving bacteria in nitramine-treated rats did not differ from that of control animals, results obtained (Procedures B and C) are also expressed as a ratio of mutation frequencies in the experimental group versus the solvent control-treated rats.

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We also studied the inhibition of the microsome-mediated mutagenesis by disulfiram and ascorbic acid. As shown in Charts 1 and 2, addition of 0.1 mM disulfiram or 7.4 mM ascorbic acid to the liquid incubation medium efficiently inhibited the mutagenesis of both N-nitrosodimethylamine and N-nitromorpholine. With 750 mM N-nitrodimethylamine, mutagenicity was reduced by 97% with disulfiram and by 78 to 87% with ascorbic acid; at concentrations of 200 mM N-nitrodimethylamine and below, mutagenicity was completely suppressed by the 2 agents (Chart 1). With 20 and 30 mM N-nitromorpholine, mutagenesis was reduced by 98 and 90% with disulfiram and by 55 and 51% with ascorbic acid, respectively (Chart 2). Control experiments carried out in the presence of either disulfiram or ascorbic acid and a fortified rat liver microsomal fraction showed that disulfiram had no mutagenic effects; however, a marginal (1.5- to 2-fold) increase in the number of revertants of strain TA100 was seen with ascorbic acid confirming earlier observations (14). Therefore, this number of revertants was subtracted from all values plotted in Chart 1 and those from similar experiments.

Disulfiram (0.1 mM) was also shown to be a powerful inhibitor of the microsome-mediated mutagenesis of each of the 3 N-nitrosamines (Charts 3 to 5); the mutagenicity of 50 mM DMNA or DENA was inhibited by 99% and that of 12.5 mM N-nitrosomorpholine was inhibited by 87%. The inhibitory action of disulfiram was similar in all S. typhimurium strains used. The inhibitory effect of 7.4 mM ascorbic acid was also studied (Charts 3 to 5). The mutagenicity of 50 mM DMNA was inhibited by 29 and 73% in strains TA1530 and TA100, respectively; that of 50 mM DENA was inhibited by 48 and 38%, respectively; and that of 12.5 mM N-nitrosomorpholine was inhibited by 32 and 28%, respectively. A lower concentration of disulfiram, 0.05 mM, still efficiently suppressed the mutagenicity of DMNA, DENA, and N-nitrosomorpholine; however, at 0.3 mM, ascorbic acid had little effect (data not shown). Because Sugimura et al. (31) reported that DMSO inhibits the microsome-mediated formation of mutagens by DMNA, we included control experiments in which the test compounds and disulfiram were dissolved in buffer instead of DMSO. In these studies, we have obtained similar inhibitory effects of mutagenesis using N-nitrodimethylamine, DENA, DMNA, N-nitrosomorpholine, or N-nitromorpholine as substrates, ruling out an interference of the solvent (data not shown).
It has been shown that disulfiram and its cleavage product [formed in the presence of rat liver cytosol (30)] impairs the monooxygenase activity of microsomes in vitro (16); further, the trapping of electrophiles by the sulfhydryl group of the diethyliocarbamate moiety cannot be excluded as another means by which the effective concentration of mutagenic metabolites reaching bacterial DNA is reduced in our in vitro assays. This inhibitory effect of disulfiram on the liver microsome-mediated mutagenicity of several nitrosamines is matched by an inhibitory action of disulfiram on the induction of liver tumors in rats in vivo by some N-nitrosodialkylamines (29). In comparison with disulfiram, much higher concentrations of ascorbic acid were required to inhibit the microsome-mediated mutagenicity of N-nitrodimethylamine, N-nitromorpholine, and their N-nitroso analogs; inhibition of bacterial mutagenesis was less efficient, particularly with higher concentrations of the N-nitroso or N-nitramine compounds. As previously proposed, alkylating agents released from nitrosamines react with ascorbic acid, thus competing for reaction with bacterial DNA (9, 14, 15). A similar mechanism may apply for the nitramines studied.

We extended our mutagenicity studies to N-nitrodiethylamine, a compound which also produces liver tumors in rats, mice, and fish (27). Under experimental conditions identical to those used to demonstrate the mutagenicity of N-nitrodimethylamine or N-nitromorpholine, no appreciable mutagenic effect was found in S. typhimurium TA100. Substitution of strain TA100 by TA1530 revealed only borderline mutagenicity, accompanied by toxicity to the bacteria (data not shown). N-nitrodiethylamine was also not mutagenic when tested at a concentration of 150 to 250 mM in the plate incorporation assay or in the preincubation assay (substrate and fortified S-9 preincubated for 20 min at 37°) (25) (data not shown). The reasons for this lack of mutagenicity of N-nitrodiethylamine in vitro may be related to the inefficiency of the in vitro metabolic activation system used and the volatility of the test compound (b.p. 48°).

Since some compounds such as 1,2-dimethylhydrazine, not detected as mutagens in the Salmonella-microsome test (20–36), were active in the host-mediated assay (24), we have therefore further tested N-nitrodiethylamine in the host-mediated assay. Although mice were used in most host-mediated assays (18, 22, 24), we have chosen rats for a better comparison with the in vitro mutagenicity data of N-nitramines. N-Nitrodimethylamine, N-nitrodiethylamine, and N-nitromorpholine were tested in the host-mediated assay in rats using S. typhimurium TA1530; the indicator organism was inoculated for up to 6 hr into the peritoneal cavity following a single p.o. dose of the nitramine (Procedure B). Each of the 3 nitramines was found to be mutagenic, causing a linear, time-dependent increase in the mutation frequency (number of revertants per 10⁶ surviving cells) when plotted on a semilogarithmic scale (Chart 6). With all of the N-nitramines, induced mutation frequencies were highest after 6 hr of inoculation of the bacteria and the increase above control values in solvent-treated rats was 16-fold for N-nitromorpholine, 11-fold for N-nitrodimethylamine, and 4-fold for N-nitrodiethylamine. The host-mediated assay in rats was also carried out with each of the 3 nitramines using S. typhimurium TA100 strain. Following reisolation of the cells after 1, 3, and 6 hr of inoculation, mutation frequencies as compared to solvent-treated control animals did not increase significantly (maximum, 0.7- to 0.8-fold above the respective solvent control values). This may be attributable to the observed lower survival of TA100 strain carrying a deep rough mutation (1) following inoculation, than of the strain TA1530 (data not included).

In order to study a possible organ-specific metabolic activation reaction of N-nitrodiethylamine, rats were treated with a single p.o. dose (or solvent only) prior to the inoculation of S. typhimurium TA1530 cells into the i.p. cavity; the bacterial cells were then isolated from the peritoneal fluid, liver, kidneys, and lungs (Procedure C). When the ratio of mutation frequencies (number of revertants per 10⁶ survivors in nitramine-treated over the solvent-treated rats) was determined as a function of inoculation time (Table 1), bacteria recovered both from the liver or the peritoneal liquid showed a similar time course. The nitramine-induced mutation frequencies were highest 3 hr after inoculation, being approximately 6-fold increased above solvent-treated control rats. Six hr after inoculation, mutation frequencies declined to 4 times the control value. In

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**Table 1**

<table>
<thead>
<tr>
<th>Bacteria recovered from</th>
<th>Mutation frequency (revertants/10⁶ survivors)</th>
<th>Ratio of mutation frequency (A:B)</th>
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<td>Peritoneal liquid</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>370; 260*</td>
<td>100; 130*</td>
</tr>
<tr>
<td>3</td>
<td>1300; 1160</td>
<td>240; 180</td>
</tr>
<tr>
<td>6</td>
<td>1350; 1390</td>
<td>310; 330</td>
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<td></td>
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</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1500; 1200</td>
<td>460; 400</td>
</tr>
<tr>
<td>3</td>
<td>3730; 3630</td>
<td>800; 520</td>
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<td>6</td>
<td>3310; 3000</td>
<td>840; 860</td>
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<tr>
<td>Kidneys</td>
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<td>270; 180</td>
<td>190; 140</td>
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<tr>
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<tr>
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<td>150; 170</td>
<td>100; 100</td>
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<tr>
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<td>340; 300</td>
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<tr>
<td>6</td>
<td>680; 310</td>
<td>250; 200</td>
</tr>
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</table>

* Each figure corresponds to results obtained in individual experiments.
contrast to liver and peritoneal fluid, bacteria isolated from
valuable secretarial assistance, E. Heseltine for editorial help, and Dr. A. Aitio for
technical advice and J. Michelon for carrying out Chromatographie analyses.

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