Mutagenicity of Cyclic Nitrosamines in *Escherichia coli* following Activation with Rat Liver Microsomes

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SUMMARY

Ten cyclic nitrosamines were tested for mutagenicity in *Escherichia coli* after incubation in vitro with 9000 × g microsomal supernatants prepared from rat liver, and the results were compared with carcinogenicity data from the same species. None of the compounds was mutagenic in the absence of microsomes. Seven carcinogenic compounds, nitrosopyrrolidine, nitrosopiperidine, nitrosohexamethyleneimine, nitrosoheptamethyleneimine, nitrosomorpholine, dinitrosopiperazine, and dinitrosohomopipera- zine, were mutagenic after microsomal activation. One compound, nitrosoheptamethyleneimine, was toxic to the bacteria. Two noncarcinogens, 1-nitrosopiperezine and 1-methyl-4-nitrosopiperazine, and 1 strong carcinogen, 2,6-dimethyl-4-nitrosopiperazine, were not mutagenic with or without microsomal incubation. The liver microsome preparation activated equally well those compounds that are liver carcinogens in Sprague-Dawley rats, and compounds for which the liver is not a target organ.

INTRODUCTION

That many carcinogens can be activated to mutagens by liver microsomal enzyme preparations is now well established (1, 2, 6, 7, 15, 16). This finding has stimulated a great deal of work and speculation concerning the relationship between carcinogenesis and mutagenesis. Much effort has gone into the development of mutagenicity assays for carcinogens, which have great promise for monitoring carcinogens in the environment or in tissues and organs of experimental animals.

The usefulness of mutagenicity assays would be greatly enhanced if it could be established that a quantitative correlation exists between mutagenicity and carcinogenicity of a group of similar compounds. Such data are difficult to assemble because of the diversity of systems used for mutagenicity and carcinogenicity testing. We present here the results of mutagenicity testing of a group of 10 cyclic nitrosamines for which quantitative carcinogenicity data in the rat have been obtained. *Escherichia coli* was used for the mutagenicity assay because of its suitability for studies of the genetics of mutagenesis.

MATERIALS AND METHODS

The 10 cyclic nitrosamines used were prepared and purified according to well-established methods described previously in connection with their chronic toxicity tests (4, 8, 10, 13). The 2 mononitrosopiperazines were used as the hydrochlorides, since they are strong bases and might upset the buffering of the incubation system.

Crude microsome fractions were made according to standard procedures (1) from the livers of Sprague-Dawley rats fed 1% phenobarbital in their drinking water for 3 days prior to sacrifice by cervical dislocation. The livers were washed with sterile 0.15 M KCl by injection into the hepatic portal vein and were excised under aseptic conditions. The livers were homogenized in 3 volumes of cold KCl solution, with a Potter-Elvehjem homogenizer, followed by centrifugation in the cold at 9000 × g for 10 min. Supernatants of homogenates were mixed with an equal volume of 0.01 M phosphate buffer, pH 7.4, and were frozen in 5- or 10-ml portions at −80°. One ml of supernatant is derived from 166 mg of liver.

The bacteria, *E. coli* WU 3610 (tyr−, leu−), obtained from Dr. D. L. George (originally from Dr. E. M. Witkin), contain nonsense mutations at both sites (17). Stationary overnight cultures were diluted 20-fold into Difco nutrient broth and grown for 1 hr, to early log phase (1 to 2 × 10⁶ bacteria/ml). Twenty ml of bacterial suspension were pelleted, and the pellet was resuspended in 10 to 20 ml of a solution of cofactors (1 ml contains 7.5 mg NADP, 10 mg glucose 6-phosphate, 10 mg nicotinamide, and 6.5 mg MgCl₂ in 0.01 M sodium-potassium phosphate buffer, pH 7.4). The suspension containing bacteria and cofactors was warmed to 37°, and glucose-6-phosphate dehydrogenase, 1 μl/ml, was added. The solution was kept warm and was dispensed rapidly into tubes containing the nitroso compounds to be tested, in volumes appropriate to make 2 to 4 ml of 50-μM solution.

When the nitroso compound was dissolved, 0.5-ml aliquots were dispensed into 4 tubes, and volumes of freshly thawed microsomal suspension were added, ranging from 0.1 to 1.0 ml (see Table 1). Phosphate buffer was added to each of the solutions containing less than 1.5 ml so as to make the volumes equal. The volume in each tube was 1.5 ml and the concentration of each test compound was 16.7

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Mutagenicity of cyclic nitrosamines in E. coli with and without rat liver microsomes

Nitrosamines were incubated for 1 hr with aliquots of a 9000 x g rat liver supernatant plus bacteria. The suspensions were plated on selective agar and incubated 2 days. No. of mutants/dish following incubation with different amounts of microsomal supernatant

<table>
<thead>
<tr>
<th>Compound</th>
<th>0 ml</th>
<th>0.2 ml</th>
<th>0.5 ml</th>
<th>1.0 ml</th>
<th>Carcinogenicity in rats</th>
<th>Site</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>NM</td>
<td>4</td>
<td>13</td>
<td>59</td>
<td>130</td>
<td>++++</td>
<td>Liver</td>
<td>3, 11</td>
</tr>
<tr>
<td>NPy</td>
<td>4</td>
<td>40</td>
<td>92</td>
<td>240</td>
<td>+</td>
<td>Liver</td>
<td>3</td>
</tr>
<tr>
<td>NPH</td>
<td>5</td>
<td>25</td>
<td>82</td>
<td>250</td>
<td>+++</td>
<td>Nasal cavity, esophagus</td>
<td>3, 12</td>
</tr>
<tr>
<td>NHex</td>
<td>7</td>
<td>33</td>
<td>150</td>
<td>270</td>
<td>++++</td>
<td>Liver, esophagus</td>
<td>8</td>
</tr>
<tr>
<td>NHept</td>
<td>3</td>
<td>28</td>
<td>46</td>
<td>79</td>
<td>++++</td>
<td>Lung, esophagus</td>
<td>13</td>
</tr>
<tr>
<td>MNP</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>0</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>MeNp</td>
<td>3</td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>DNP</td>
<td>5</td>
<td>8</td>
<td>23</td>
<td>43</td>
<td>+++</td>
<td>Esophagus, liver, nasal cavity</td>
<td>3, 10</td>
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<tr>
<td>DNHP</td>
<td>4</td>
<td>18</td>
<td>47</td>
<td>82</td>
<td>++++</td>
<td>Nasal cavity, esophagus</td>
<td>10</td>
</tr>
<tr>
<td>Me2DNP</td>
<td>5</td>
<td>4</td>
<td>9</td>
<td>6</td>
<td>++++</td>
<td>Nasal cavity, esophagus</td>
<td>10</td>
</tr>
<tr>
<td>DMN</td>
<td>5</td>
<td>3</td>
<td>8</td>
<td>3</td>
<td>++++</td>
<td>Liver</td>
<td>3, 18</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>9</td>
<td>8</td>
<td>3</td>
<td>++++</td>
<td></td>
<td></td>
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</tbody>
</table>

* Numbers are the average of 3 or more experiments, except NHep, which is the average of 2.

Mutants are tyr -> tyr*. 

NP, nitrosopipendine; NHex, nitrosoheptamethyleneimine; MNP, 1-nitrosopiperazine; DNP, 1,4-dinitrosopiperazine; DNPH, 1,4-dinitrosohomopiperazine.

* Compound was toxic at 0.0167 M even in the absence of microsomes. Concentration was reduced 10-fold.

mm. The tubes were incubated with shaking at 37° for 1 hr.

Aliquots of 0.2 ml of each solution (containing 2 x 10⁷ bacteria) were plated on minimum medium E (19) supplemented with 2.5% nutrient broth + 0.4% glucose + 20 μg L-leucine per ml. Plates were incubated for 2 days at 37° and then counted. Under these conditions, bacteria divide several times, allowing expression of mutations and the appearance of a background lawn of bacteria (5). The leucine present supplements 1 deficiency, and the mutants that appear as large colonies are phenotypic revertants from tyr* to tyr*, most likely due to a suppressor (5, 17). In some cases, the samples were diluted and plated for measurement of survival.

RESULTS AND DISCUSSION

In kinetic studies of mutation induction, Gletton et al. (6) showed that the enzymatic activation of DMN* was proportional to the concentration of compound and to the concentration of microsomal protein. Both dose-response curves saturated at the same level of induced mutations. In our experiments, we have kept the nitrosamine concentration constant in order to compare the microsome-dependent activation of the compounds.

The results of the mutagenicity tests of the 10 cyclic nitrosamines are given in Table 1 as number of mutants per plate. Survival as determined by dilution and plating was 70% or more, except with NHep. NHep was toxic or growth inhibitory, with or without microsomes, at the 16.7 mM concentration used. Few or no survivors were seen among the bacteria plated. At a 10-fold lower concentration, however, survival was 70%. None of the compounds showed any mutagenic activity without activation by microsomal enzymes. With rat liver microsomes, 7 of the compounds were mutagenic and 3 had no detectable mutagenic activity.

The almost linear increase in the number of mutants (Table 1) with increasing volume of microsomal suspensions is evidence of a dose response to the mutagen formed. These kinetics indicate that the amount of activating enzyme is the limiting factor in the reaction. In animals, as well, the effective dose of carcinogen might be independent of the amount administered, above the level that saturates the activating enzymes. The relatively low effectiveness of large single doses of nitrosamines in inducing tumors might be explained on this basis.

Most of the cyclic nitrosamines were more active in this system than DMN, although the latter is readily converted into an alkylating moiety (14), while most of the cyclic compounds examined gave rise to little or no detectable alkylation of nucleic acids in rat liver (9). It seems possible, then, that the mutagenic product made by the microsomes is not an alkylating moiety, but some other type of structure which is common to both cyclic and aliphatic nitrosamines.

From our data it appears that liver microsomes convert the various nitro compounds into mutagens to different extents, or that the products vary in their mutagenic effectiveness. The piperazines as a group were consistently the least effective among the cyclic nitrosamines tested. NM, which was included in every experiment as a standard, was intermediate in activity, and NPy, nitrosopiperidine, and nitrosohexamethyleneimine were similar and the most active.

There seems to be no quantitative relationship between
mutagenicity in these tests and carcinogenicity in rats, whether to liver or to other organs. DMN and NM are about equally strong liver carcinogens (affecting different cell types), but show quite different mutagenicities. NPy is a much weaker liver carcinogen (by an order of magnitude or more) than DMN, but is a more effective mutagen. NHeP and 1,4-dinitrosohomopiperazine are not liver carcinogens in rats but are effectively activated to a mutagenic form by rat liver microsomes.

Among the nitrosopiperazines there is a positive correlation between mutagenicity and carcinogenicity, with 1 exception, that being the negative response of the strongly carcinogenic Me₂DNP in the mutagenicity test. One possible explanation is that Me₂DNP is not metabolized to a mutagenic form by liver microsomes, but would be mutagenic if incubated with microsomes from an organ in which it induces tumors. However, Bartsch et al. (2) found that microsomes from a target organ other than the liver did not activate diethylnitrosamine to a bacterial mutagen. The 2 compounds negative for carcinogenicity in this series, 1-nitrosopiperazine and MeNP, are also negative for mutagenicity. In the host-mediated assay, by contrast, one of these compounds, MeNP, gave positive results (20).

In our experiments a reasonable quantitative correlation between the mutagenicity and carcinogenicity of a group of cyclic nitrosamines is not demonstrated. Even among compounds carcinogenic for rat liver, a correlatable mutagenic response to these compounds after activation by microsomes from this organ is not seen. Equally perplexing is the absence of liver carcinogenicity of compounds that are activated by liver microsomes to the same extent as liver carcinogens. An explanation of these results would seem to be important in understanding the relationship between carcinogenesis and mutagenesis.

The lack of a quantitative relationship raises the possibility that a carcinogenic compound might not be detected in a mutagenicity test, as was the case with Me₂DNP in our system. It is possible that some other mutagenicity system might be better at predicting the carcinogenicity of a group of similar compounds. However, the complexities of biological systems make it quite probable that a quantitative correlation between mutagenicity and carcinogenicity is not achievable. Further exploration into these differences is needed as the basis for development of better assay systems for potentially carcinogenic compounds.

REFERENCES

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