N-Methyl-N'-nitro-N-nitrosoguanidine: Reactions of Possible Significance to Biological Activity with Mammalian Cells

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SUMMARY

Mammalian cells exposed to N-methyl-N'-nitro-N-nitrosoguanidine show an immediate inhibition of DNA, RNA, and protein synthesis, as judged by radioactive precursor incorporation. DNA synthesis as determined by thymidine-3H uptake is most sensitive in this respect. The inhibition is not due to impaired uptake or phosphorylation of precursor. Studies at the enzyme level show DNA polymerase to be inhibited by N-methyl-N'-nitro-N-nitrosoguanidine in a dose-dependent manner. Experiments with exogenous thiols indicate that thiol groups of DNA polymerase may be targets for reaction with the guanidino radical resulting from N-methyl-N'-nitro-N-nitrosoguanidine decomposition. Further studies suggest that these and other groups possibly involved in substrate binding may be altered and that enzyme kinetics favor a mixed mode of enzyme inhibition.

INTRODUCTION

Activity of MNNG as a potent carcinogen has been established (10, 36, 40); yet the biological systems in which it is known to react are not confined to mammals and extend to the viruses (37). For example, in bacterial cells (2), synthesis of protein and RNA was inhibited after exposure to MNNG. These effects were possibly related to the inhibition by MNNG of in vitro synthesis of protein (4) and RNA (22), which was attributed in both examples to an alkylation by methyl groups derived from the inhibitor. The alkylation of nucleic acids in vitro by MNNG has been studied (6, 21, 23), and the effect of monofunctional alkylating agents on nucleic acid synthesis in mammalian cells has been examined (13, 41, 42). The present study was prompted by the lack of information about the biochemical effects in mammalian cells exposed to MNNG. A preliminary report of this work has been given (1).

MATERIALS AND METHODS

Chemicals and Media. All chemical reagents were Analar grade (The British Drug Houses, Ltd., Poole, England) or equivalent. Puck's N16 and Evan's NCTC 109 were supplied by Microbiological Associates, Bethesda, Md., and Eagle's minimal essential medium was obtained from Burroughs Wellcome, London, England. MNNG, purchased from Aldrich Chemical Company, Milwaukee, Wis., was dissolved to 0.01 M in pH 6 phosphate buffer at room temperature in the dark. The solution was sterilized by filtration before storage as aliquots in tinfoil-wrapped bottles at —20°.; a fresh sample was thawed for each experiment. Ethanolic MNNG solution (50 mM) was used to study the approximate half-life of MNNG in growth medium, from the rate of decrease in absorption at 399 mμ (21) measured with a Unicam SP800 spectrophotometer. Thymidine-6-3H, uridine-5-3H, and deoxyadenosine-3H (generally labeled) with specific activities, in Ci/mole, of 17.7, 16.0, and 2.4, respectively, and amino acid-14C mixture, specific activity 54 mCi/milliatom of carbon, were all provided by the Radiochemical Centre, Amersham, England. α-TTP-32P, specific activity 740 Ci/mole, was obtained from International Chemical and Nuclear Incorporation, Irvine, Calif.

Tissue Culture and Radioactive Procedures. Hamster fibroblasts (Don cell line) from the Cell Repository of the American Type Culture Collection, Rockvile, Md., were maintained at 37° in humidified 5% CO₂ in air as monolayers in Roux flasks, with medium of the following composition: Puck's N16 (60%, v/v), NCTC 109 (20%, v/v), and fetal calf serum (20%, v/v). The cells were routinely monitored for Mycoplasma infection according to the method of Fogh and Fogh (12) and were in any event replaced after less than 100 generations from stocks kept at —196°. Experiments of cell multiplication or radioactive precursor incorporation were made with replicate cultures in 60-mm plastic Petri dishes (Esco Rubber Co., London, England) each inoculated with 5 X 10⁵ cells and 5 ml growth medium. This number resulted in cultures in early exponential phase after overnight incubation and was used to standardize approximately the population maturity for each experiment. The cells were harvested with 0.25% trypsin in balanced salt solution and then enumerated with a Coulter Counter, Model D. Where necessary, medium was harvested from overnight control cultures to provide "conditioned" medium.

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2 The abbreviation used is: MNNG, N-Methyl-N'-nitro-N-nitroso-guanidine.

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Cells were exposed as monolayers to MNNG by addition of aliquots of stock solution to give the stated initial concentration in the medium, and the dishes were returned to incubate. Cell multiplication was estimated from the total number per dish at different times thereafter. Cytotoxicity was judged by colony formation of cells harvested with trypsin after 1 hr exposure to MNNG, and then plated according to the method of Ham and Puck (15) on lethally irradiated cells of the same line in 60-mm plastic Petri dishes. The colonies were stained with Harris's hematoxylin to aid visibility.

Exposure to thymidine-6-3H (10 μCi/dish, 1 hr), uridine-5-3H (2.5 μCi/dish, 20 min), deoxyadenosine-3H (10 μCi/dish, 1 hr) or amino acids14C (0.5 μCi/dish, 30 min) at the different times indicated was terminated by replacement of medium with ice-cold 5% trichloracetic acid. Cells were removed from the surface with a rubber policeman and collected quantitatively on Millipore filters (0.45 μ) to be washed twice with 10 ml ice-cold 5% trichloracetic acid. Cells harvested in 5% trichloracetic acid after exposure to deoxyadenosine-3H were treated according to the method of Munro and Fleck (31) to determine the radioactivity actually present in DNA. Soluble extracts of cells exposed to thymidine-3H (20 μCi/dish, 1 hr) were prepared in 0.2 N perchloric acid, and the constituents were separated by chromatography (20) for estimation of radioactivity. 3H and 14C radioactivity was assessed by liquid scintillation spectrometry with a Packard Tri-Carb spectrometer.

**Extraction and Assay of DNA Polymerase Activity.** Cells cultivated in bulk (16) in Eagle's minimal essential medium with 10% tryptose phosphate broth (Difco, Detroit, Mich.) and 10% fetal bovine serum were scraped off the glass surface and collected by centrifugation at 600 X g. Crude cytoplasmic extracts were prepared and assayed according to the method of Keir and Shepherd (19), with exclusion of 2-mercaptoethanol from solutions. Protein content of the extracts was estimated by the method of Lowry et al. (24) before storage at 0° for use within the week of preparation. "Primer" in the assay was a thermally denatured sample of calf thymus DNA prepared either in the laboratory (18) or commercially (Sigma Chemical Co., St. Louis, Mo.). Other reagents for addition to the assay system were dissolved where possible in the same buffer as the cell extract. The amount of 32P radioactivity incorporated into acid-insoluble material was assessed with a Nuclear-Chicago gas flow counter. DNA was alkylated in vitro according to the method of McCalla (27).

**RESULTS**

**Effect of MNNG on Cell Division (Chart 1).** In experiments where control cells showed a doubling time of about 12.5 hr, addition of MNNG to 25 μM in the medium immediately inhibited division of cells. Cells exposed in early logarithmic growth showed no resumption of division over a period of 28 hr, although cells exposed in midphase of logarithmic growth showed a slight increase in cell number after 24 hr. Exposure to 5, 10, or 20 μM in early logarithmic growth was also inhibitory but division of cells resumed after approximately 5 hr. Spectroscopic studies gave a value of 90 min for MNNG half-life in the cell growth medium routinely used, and this was in agreement with published data (21, 29). When the medium with 20 μM MNNG was removed after 1 hr and replaced with prewarmed, conditioned medium, the growth inhibition and recovery was not altered.

**Effect of MNNG on Cell Viability.** For determination of viability of cells beyond 2 division cycles after MNNG exposure, survival (colony-forming ability) was assessed with single cells harvested from monolayers exposed for 1 hr to MNNG in early logarithmic phase. A typical dose-survival curve from such experiments is shown in Chart 2. An initial shoulder preceded an apparent exponential decrease in viability, and it is perhaps significant that the shoulder effect increased with cells exposed in the middle of exponential phase (cf., inhibition of cell division above). A concentration of 15 μM MNNG was calculated from the first graph as the value which reduced survivors to 50% of control and is shown for comparison with those of other cytotoxic agents in Table 1.

No permanent transformation of colony morphology was observed in the immediate descendents of the exposed cells.

**Effect of MNNG on Nucleic Acid and Protein Synthesis.** Cells in early logarithmic phase were assessed for ability to
incorporate radioactive precursor into DNA, RNA, or protein immediately after exposure to MNNG. The rate of DNA synthesis, as judged from the amount of thymidine-6-³H incorporated into DNA, was most sensitive to inhibition and showed a dose-dependent relationship. RNA and protein synthesis, as judged by uridine-5-³H and amino acids-¹⁴C incorporation, respectively, were also affected, but the inhibition at equivalent MNNG concentrations was always less than for DNA synthesis (Chart 3).

In another experiment to measure inhibition of DNA synthesis rate, MNNG was added simultaneously to the medium of replicate cultures and the cells examined immediately and after different intervals of incubation for ability to incorporate thymidine-6-³H into DNA. A relationship was again observed between the degree of immediate inhibition and concentration of MNNG in the medium; recovery of incorporation also occurred but to a variable extent and to normal values only in cell populations which would be expected to resume cell division (i.e., those treated with 10 μM MNNG). However, another notable feature was the initial decrease in incorporation of precursor by control populations (Chart 4). This was most probably the result of

![Chart 2. Survival of cells exposed to MNNG. MNNG was added to the medium of cells in logarithmic growth. After 1 hr at 37°, cells were harvested and plated for colony formation on lethally irradiated feeder layers of the same cell line. The colony count per dish (mean of triplicates) after 9 days is shown as the ratio of colonies formed by control cells treated similarly, plotted on a logarithmic scale against initial concentration of MNNG at exposure. Cell count per dish (mean of triplicates) at time of exposure: △, 1 X 10⁶; ○, 1.5 X 10⁶.]

![Chart 3. Inhibition of metabolism in cells exposed to MNNG. The incorporation by cells exposed to thymidine-6-³H (TdR, 10 μCi/dish, 1 hr), uridine-5-³H (2.5 μCi/dish, 20 min), and amino acid-¹⁴C mixture (0.5 μCi/dish, 30 min) into DNA, RNA, and protein, respectively, was assessed in separate experiments in the period immediately following addition of MNNG to the medium. Radioactivity incorporated into DNA and RNA (mean of triplicates ± S.E.) and protein (mean of duplicates) is shown as the percentage of controls. MNNG concentration is shown below the appropriate columns.]

![Table 1](https://example.com/table1.png)

**The reported concentration which reduces survivors to 50% control (ID₅₀) for different alkylating agents acting on mammalian cells**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Exposure time (hr)</th>
<th>ID₅₀ (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfur mustard</td>
<td>0.5</td>
<td>0.3</td>
<td>7</td>
</tr>
<tr>
<td>MNNG</td>
<td>1</td>
<td>15</td>
<td>Present study</td>
</tr>
<tr>
<td>Methyl-methanesulfonate</td>
<td>3</td>
<td>100</td>
<td>13</td>
</tr>
<tr>
<td>N-Methyl-N-nitrosourethan</td>
<td>2</td>
<td>370</td>
<td>34</td>
</tr>
<tr>
<td>N-Dimethylnitrosamine</td>
<td>2</td>
<td>Inensitive</td>
<td>34</td>
</tr>
</tbody>
</table>
Chart 5. Immediate inhibition of precursor incorporation into DNA by MNNG. Cells in logarithmic growth were exposed to different concentrations of MNNG and immediately examined for ability to incorporate thymidine-6-3H (10 μCi/dish, 1 hr) or deoxyadenosine-3H (10 μCi/dish, 1 hr, at 50 μM MNNG only) into DNA. Total incorporated radioactivity per dish (mean of at least 3 ± S.E.) is expressed as the ratio of controls and is plotted on a logarithmic scale against MNNG concentration. •, thymidine-6-3H; ●, deoxyadenosine-3H.

Effect of MNNG on in Vitro DNA Polymerase Reaction. Addition of MNNG directly to the assay system did not cause significant reduction of enzyme activity and, furthermore, DNA alkylated with MNNG also functioned efficiently as “primer” after denaturation (Table 2). However, a distinct inhibition was observed when the enzyme fraction alone was preincubated at 37° with MNNG for increasing periods before addition of the other components for assay of activity. The effect was maximal after 60 min preincubation and was directly related to the concentration of MNNG. For example, activity after incubation with 1 mM MNNG amounted to only 15% of an untreated preincubated sample...
Chart 6. Effect of MNNG on the incorporation of thymidine-6-3H into thymidine deoxyribonucleotides by cells. Acid-soluble extracts were prepared from cells exposed to thymidine-6-3H (20 μCi/dish, 1 hr) during the 2nd hr after MNNG addition to the medium. The constituents were separated by chromatography for individual assessment of incorporated radioactivity. The results are expressed as the percentage of total radioactivity per run in each of the deoxyribonucleotides (mean of 4 runs ± S.E.). The total radioactivity extracted from the different cell populations was (10^4 dpm/dish ± S.E.): control, 2.52 ± 0.75; 20 μM MNNG, 2.18 ± 0.41; 100 μM MNNG, 2.56 ± 0.25. TdR, thymidine.

Table 2
In vitro DNA polymerase activity after different pretreatments of enzyme preparation or “primer” (DNA) components of the reaction is shown as the amount of precursor incorporated and as percentage inhibition (all mean of duplicates)

<table>
<thead>
<tr>
<th>Condition of pretreatment</th>
<th>dTMP-32P incorporated (μmole/mg protein/hr)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.76</td>
<td>0</td>
</tr>
<tr>
<td>MNNG, 1 mM*</td>
<td>0.56</td>
<td>85.1</td>
</tr>
<tr>
<td>Guanidine, 1 mM#</td>
<td>3.08</td>
<td>18.1</td>
</tr>
<tr>
<td>MNNG, 0.05 mM#</td>
<td>3.6</td>
<td>4.3</td>
</tr>
<tr>
<td>MNNG, 2 mM^c</td>
<td>3.88</td>
<td>0</td>
</tr>
<tr>
<td>MNNG, 2 mM plus cysteine, 2 mM^c</td>
<td>3.6</td>
<td>4.3</td>
</tr>
</tbody>
</table>

*Enzyme preparation alone preincubated for 60 min with MNNG or guanidine.
#Enzyme preparation from cells previously exposed for 60 min to MNNG.
^CDNA pretreated with MNNG.

(Chart 7). Preincubation of enzyme fraction with the related compound guanidine at the same molarity only reduced the activity to 82% preincubated control, and there was no significant reduction of enzyme activity extracted from intact cells treated with MNNG (Table 2).

In an effort to characterize the reaction responsible for the inhibition of enzyme, other compounds were included with MNNG during the 60-min preincubation described above to observe any altered effect. Thiol compounds caused the greatest reduction of the inhibition effect. With GSH at twice the concentration of MNNG, enzyme activity increased from 15% to more than 74% of preincubated untreated samples; but with 2-mercaptoethanol, even at 5 times the concentration of MNNG, activity was only 60% (Table 3). No reversal of inhibition was obtained when GSH was added only at the end of the period of preincubation.

Primer DNA and deoxyribonucleoside triphosphates, the substrates of DNA polymerase, were included alone or in combination—at the concentration used for enzyme assay—during the preincubation of cell extracts and inhibitor. With 1 mM MNNG, enzyme activity increased from 15% to around 50% of controls in the samples that contained DNA, but this increase must be viewed in the knowledge that activity of controls treated similarly was enhanced (Table 4). When precursor triphosphates alone were included, activity of controls was also reduced. This latter effect was not explained but did not appear to be due to contaminating phosphatases, as no difference was noted when fresh tri-
phosphates were added along with the other components of the reaction mixture at the end of preincubation.

Effect of MNNG on DNA Polymerase Reaction Kinetics. Studies of enzyme kinetics were made because the above results suggested that DNA polymerase activity was possibly inhibited by reaction or interference with enzyme-substrate binding sites. The results of a typical experiment are shown in Chart 8 as Lineweaver-Burk reciprocal plots. The control reaction is nonlinear, in deviation from simple Michaelis-Menten kinetics, and furthermore shows substrate (DNA) inhibition. The MNNG-inhibited reaction approaches linearity, however, which allows interpretation as outlined in "Discussion."

The results of another experiment to examine the inhibited enzyme rate at each of 2 substrate (DNA) concentrations for different concentrations of MNNG are presented in Chart 9. The graphs are again nonlinear, which is characteristic of "mixed" mode of inhibition. It is not possible to determine enzyme/inhibitor constants for such reactions by graphic procedures alone (8).

DISCUSSION

The inhibition of DNA synthesis was examined closely in the present study, and the observed inhibition of in vitro DNA polymerase reaction (Chart 7) must be considered for a possible relation to the in vivo effect of MNNG.

The biological activity of the nitrosamines is believed to result from the production of diazoalkanes as alkylating intermediates (9, 25, 42), and indeed the activity of MNNG as a methylating agent is well known (6, 21, 23). Yet inhibition of DNA polymerase activity is less when thiol groups are present (Table 3), which is contrary to experiences for DNA reacted with MNNG, where addition of cysteine increased the extent of methylation (6, 27). The presence of cysteine reduced the extent of guanidine-14C binding from MNNG to DNA, although the reaction sites were not characterized (27). Later work (38) disclosed lysine as a target site for guanidination of egg albumin reacted with MNNG, in agreement with known reactivity with amino groups (30). The simple nature of the experiments in the present study do not permit precise distinction of the reactive groups of DNA polymerase enzyme but appear to favor enzyme thiol groups (Table 3). It is also possible that exogenous thiols protect by reduction of MNNG half-life, as spectroscopic studies of MNNG decomposition in the presence of GSH revealed a change to 2nd-order reaction kinetics with a more rapid initial rate.
Quantitative comparison (39) has shown that guanidination occurs more extensively than methylation of both nucleic acids and proteins reacted with MNNG, the more so of basic histone proteins. Profound effects on the function of proteins might be expected to follow guanidination since guanidine itself is a powerful denaturant (14). The irreversibility of DNA polymerase inactivation by MNNG and the effect on DNA polymerase of guanidine itself (Table 2) support such an action. The enzyme kinetic studies (Chart 9) indicate that inhibition is both partially competitive and noncompetitive (8), but any interpretation is further complicated by the multiplicity of DNA polymerase substrates as well as the low degree of enzyme purity. Reaction at enzyme active centers was not established, but a change to linear kinetics (Chart 8) could be interpreted to result from an altered affinity of enzyme for one of its substrates (8). Such a possibility, furthermore, would be in agreement with the enzyme model of Erhan (11), who proposes that 1 region of the enzyme, which binds and orients the DNA strands for replication, is rich in lysine and other basic amino acids. Another nitrosamide, 1,3-bis(2-chloroethyl)-1-nitrosourea, has been reported (43) to inhibit DNA polymerase reaction, and the mode of action proposed, isocyanate production, has some similarity to the present results.

A conformational change in DNA polymerase subsequent to attachment of DNA substrate could lead to protection through inaccessibility of reactive groups. A conformational difference also, between enzyme as it exists in vivo and as it is assayed in vitro, might account for the delay of in vitro inhibition (i.e., the requirement for a period of preincubation). The difference of approximately 20-fold between concentrations which cause equivalent in vitro and in vivo inhibition, although a common observation with many metabolic inhibitors, may be resolved by studies of the final intracellular location of MNNG. The nitrosamides are lipophilic (35), which would favor concentration of MNNG in lipid-rich structures, such as cell membranes, and lead to sensitivity of membrane-associated events. DNA synthesis is reported to be associated with bacterial cell membranes (17) and to be initiated at the nuclear membrane of human cells (5). In addition, MNNG is known to cause mutation at the replication point of the bacterial chromosome (3). However, the relationship of these reports to the results of this study is at present not obvious. The situation is further complicated by knowledge that the conformation and molecular environment of the polynucleotide strands are important factors determining the effect of MNNG (38).

The present work emphasizes the possibility that reactions other than methylation may have a significant influence on the biological consequences of exposure to MNNG. No account has been taken of the observed alteration in the synthesis of RNA and protein, and no allowance has been
made under "Discussion" for an effect of MNNG on intra-
cellular organelles, which has been recorded with other
experimental systems (26, 32). Certainly, the wide range of
chemical groups which could be altered by reaction (either
methylation or guanidination) with decomposition products
of MNNG is sufficient to account for its potent cytotoxicity
(Table 1).

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