Formation of 8-Methylguanine as a Result of DNA Alkylation by Methyl Radicals Generated during Horseradish Peroxidase-catalyzed Oxidation of Methylhydrazine*

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Methylhydrazine oxidation promoted by horseradish peroxidase-H2O2 or ferricyanide led to the formation of high yields of methyl radicals and to the formation of 7-methylguanine and 8-methylguanine upon interaction with calf thymus DNA. Methyl radicals were identified by spin-trapping experiments with α-(4-pyridyl-1-oxide)-N-tert-butyl nitrode and tert-nitrosobutane. The methylated guanine products were identified in the neutral hydrolysates of treated DNA by high pressure liquid chromatography (HPLC) analysis and spiking with authentic samples. The structure of 8-methylguanine, a product not previously reported in in vitro DNA oxidation experiments, was confirmed by HPLC chromatography, UV absorbance, and mass spectrometry. The formation of 8-methylguanine suggests a possible role for carbon-centered radicals as DNA alkylating agents.

Covalent binding of chemical carcinogens to cellular macromolecules, especially DNA, is thought to be a critical step in the initiation of the carcinogenic process. Most chemicals require metabolic activation to electrophilic intermediates before reacting with cellular nucleophiles. In the case of hydrazine derivatives, their tumorigenic properties are usually ascribed to bidentation to cationic alkylating metabolites (1-4). Carbon-centered radical alkylating species are less considered in the literature (5, 6), despite several reports demonstrating metabolic activation of hydrazine derivatives to alkyl and aryl radical species, both in vitro (reviewed in Refs. 6 and 7) and in vivo (5, 8, 9). DNA alkylation by carbon-centered radicals is possible on chemical grounds (10). A few in vitro studies have indicated that chemically generated methyl radicals in acidic medium (11, 12) or photochemically generated α-hydroxycarbon radicals (13) attack guanine and adenine bases by substituting at position 8 of purine rings. DNA alkylation at C-8 has not been demonstrated in vivo (10), probably because of lack of appropriate standards and/or loss of the DNA adducts by depurination (14). For example, a recent study has reported detection of an unknown deoxyguanosine adduct in DNA purified from rats treated with 2-nitropropane (15). It was suggested that the adduct could result from attack of DNA by 2-nitropropane-derived alkyl radicals, but no structural evidence has been presented to support this hypothesis (15).

The little attention carbon-centered radicals have received as DNA alkylating species led us to investigate DNA adducts present in neutral hydrolysates of DNA treated with methylhydrazine and horseradish peroxidase-H2O2 or ferricyanide. Previous studies from our laboratories have presented evidence that hydrazine derivatives, namely 2-phenylethyldrazine (16) and 1,2-dimethylhydrazine (17), can alkylate DNA through oxidation to carbon-centered radicals, but the structures of the DNA adducts formed were not determined (16, 17). We now report the formation of 8-methylguanine as a result of DNA attack by methyl radicals generated during chemical and enzymatic oxidation of methylhydrazine.

EXPERIMENTAL PROCEDURES

Materials—Guanine, 7-methylguanine, horseradish peroxidase type VI, POBN,† and t-NB were from Sigma. Calf thymus DNA was from Pharmacia LKB Biotechnology Inc. Methylhydrazine, free base, was from Aldrich. 8-Methylguanine was synthesized and purified as previously described (11). The purified compound was shown to be 95% pure by HPLC and was characterized by its spectroscopic properties: UV (H2O), λmax (nm) 248, 278; NMR (MeSO-D6), 6.49 ppm (s, 2H, 2-NH2 exchanged in D2O), 2.23 ppm (s, 3H, CH3); and mass spectrum, M+1,166 (Fig. 4B), obtained by direct chemical ionization on a Kratos MS 90-1A instrument.

DNA Alkylation—The standard incubation mixture (3.0 ml final volume) contained calf thymus DNA (1 mg/ml), methylhydrazine (20 mM), horseradish peroxidase (75 μM), H2O2 (2 mM), and diethyleneetriaminepentaaetic acid (1 mM) in phosphate buffer (0.1 M), pH 7.8. In the chemical systems, DNA (1 mg/ml) was incubated with methylhydrazine (5 mM) and ferricyanide (12.5 mM) (16). After a 30-min incubation at 37 °C, the reaction was terminated by cooling to 0 °C in an ice bath, and DNA was desalted by dialysis against water at 2 °C for 24 h. Desalted DNA was precipitated by the addition of 6 ml of cold ethanol (−20 °C); the pellet was washed twice with cold ethanol/H2O (80% v/v) and dried under vacuum. From 30 to 50% of the initial DNA was recovered by this procedure.

DNA Hydrolysis and Chromatography—Dried DNA was resuspended in phosphate buffer (10 mM), pH 7.0, to a final concentration of 1–2 mg/ml as ascertained by UV absorption (A260 = 1 for 50 μg/ml DNA). DNA was then processed by neutral thermal hydrolysis followed by mild acid hydrolysis as described before (18). 7-Methylguanine and 8-methylguanine, present in the neutral hydrolysates, and guanine, present in both neutral and mild acid hydrolysates, were analyzed by HPLC accordingly to the separation method of Herron and Shank (19). A Waters composite (model 6000 pump and 6600 pump and diode array detector) high pressure liquid chromatograph attached to a Gilson Holochrom UV detector and to a Shimadzu RF 535 fluorometer was used throughout the study. Peaks were recorded and quantitated with two Hewlett-Packard 3380 integrators. Chromatographic separation was carried out using a Partisil 10 SCX cation exchange

* The abbreviations used are: POBN, α-(4-pyridyl-1-oxide)-N-tert-butyl nitrode; t-NB, tert-nitrosobutane; HPLC, high pressure liquid chromatography.
column (2.5 cm × 4.5 mm, inner diameter), and elution of the compounds was followed by UV at 254 nm and by fluorescence using 285 nm and 365 nm as excitation and emission wavelength, respectively. Neutral hydrolysates (30–100 μl corresponding to 30–100 μg of DNA) or acid hydrolysates (5-20 μl) were eluted with ammonium formate, pH 3.0, at 1.5 ml/min.

ESR Experiments—The reaction mixtures (500 μl, final volume) and incubation conditions were the same as those described for DNA alkylation except that DNA was substituted by POBN (100 mM) or t-NB (57 mM); stock solutions of t-NB were 10 times more concentrated and prepared in HPLC-grade acetonitrile. Aliquots of the incubation mixtures were transferred to small flat cells (100 μl, final volume) and analyzed with a Bruker ER-200 ESR spectrometer. The concentration of radical adducts was estimated as previously described (20).

Characterization of 8-Methylguanine Isolated from Treated DNA—A larger scale incubation of the enzymatic system (15 ml, final volume) was performed and treated as described above. The DNA neutral hydrolysate (500-μl injections) was submitted to HPLC, and the fractions corresponding to the 16.4-min retention time (Fig. 2) were collected, pooled together, and evaporated under nitrogen. This fraction was further analyzed by HPLC (Waters 600E) on a YMC 5-m ODS-AQ reverse-phase analytical column (YMC, Overland Park, KS) eluted with water at 1.5 ml/min. The peak was detected by absorbance at 254 nm using a Waters 990 photodiode array detector, and the spectrum was recorded (Fig. 3). Chemical ionization mass spectra were obtained from the pooled 16.4-min fractions isolated from DNA as described above.

RESULTS

Oxidation of methylhydrazine to carbon-centered radicals promoted by isolated hepatocytes or by rat liver microsomes has been reported before (21). Formation of high yields of methyl radicals during oxidation of methylhydrazine mediated by ferricyanide or horseradish peroxidase was confirmed here by spin-trapping experiments (Fig. 1, Table I). The ESR parameters of the observed POBN-methyl adduct (αH = 2.8 G, αN = 17.3 G) (Fig. 1A) were in agreement with previously reported values (6). Unequivocal identification of the methyl radical was possible by the characteristic 12-line ESR spectrum of the t-nitrosobutane-methyl radical adduct (αH = 17.3; αN = 14.5 (3H)) (Fig. 1C). The composite ESR spectrum displayed in Fig. 1C was ascribed to three main species, i.e. t-NB-methyl radical adduct, t-NB-H radical adduct, and di-tert-butyl nitroxide (22), as labeled and specified in the figure legend. Qualitatively, the results of the spin-trapping experiments were the same for both oxidizing systems, horseradish peroxidase-H2O2 or ferricyanide, but the latter system generated a higher yield of methyl radicals (Fig. 1, Table I). Calf thymus DNA alkylation during oxidation of methylhydrazine promoted by horseradish peroxidase-H2O2 or ferricyanide was ascertained by HPLC analysis of neutral hydrolysates of treated DNA (Fig. 2, Table I). A representative HPLC profile is displayed in Fig. 2B; the peaks were first tentatively identified as guanine, 8-methylguanine, 7-methylguanine, and adenine (23) by comparison of the retention times with appropriate standards (Fig. 2A) and by spiking with authentic samples. Under these conditions 1-methylguanine eluted at 19.6 min and 3-methylguanine at 21.3 min. This identification is not, however, definitive. To prove unequivocally the structure of the 16.4-min retention time compound appeared in the neutral hydrolysates of DNA treated with methylhydrazine and horseradish peroxidase-H2O2 or ferricyanide (Fig. 2, Table I), a larger scale incubation of the enzymatic system was performed as described under "Experimental Procedures." The 16.4-min fractions isolated from neutral DNA hydrolysates (Fig. 2B) were pooled and further analyzed by reverse phase HPLC with water and mass spectrometry. The pooled fraction contained one main compound as ascertained by HPLC with photodiode array detection. Both the retention time, 23.8 min, and the UV spectrum of the main peak were identical to those obtained for the synthetic 8-methylguanine (Fig. 3). The hyperconjugative effect of the 8-methyl group slightly shifted the maximum of guanine at 276 nm to 279 nm (Fig. 3). Based on the retention times and UV spectra (Fig. 3), 8-methylguanine (23.8 min) could be distinguished from 1-methylguanine (44.8 min) and 3-methylguanine (35.9 min). An authentic sample of guanine methylated at the 2-amino group was not available, but its UV spectrum should be identical to that of parent guanine.

Further proof that the adduct isolated from treated DNA was 8-methylguanine was obtained by comparison of the chemical ionization mass spectrum of the pooled fractions (Fig. 4A) with that of the synthetic compound (Fig. 4B). If allowance is made for the fact that the biologically obtained compound is not completely pure, the mass spectra were identical, with molecular ion at m/z 166 (M + 1) and high

![Fig. 1. ESR spectra of spin-trap radical adducts obtained after 30 min of incubation of methylhydrazine in phosphate buffer (0.1 M), pH 7.0, containing diethylenetriaminepentaaetic acid (1 mM), A, methylhydrazine (20 mM), horseradish peroxidase (75 μM), H2O2 (2.0 mM), and POBN (100 mM); B, methylhydrazine (5 mM), ferricyanide (12.5 mM), and POBN (100 mM); C, methylhydrazine (20 mM), horseradish peroxidase (75 μM), H2O2 (2 mM), and t-NB (57 mM). The composite ESR spectrum of C is labeled to show its main components: t-NB-methyl radical adduct (X), t-NB-hydrogen adduct (O), and di-tert-butyl nitroxide (●). Instrumental conditions: microwave power, 20 milliwatts; modulation amplitude, 1 G; time constant, 0.5 s; rate 0.2 G/s; gain, 6.3 × 104 for A and B and 8 × 105 for C.

### Table I

<table>
<thead>
<tr>
<th>System</th>
<th>Yield</th>
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<tbody>
<tr>
<td>POBN-methyl radical adduct</td>
<td>7-MeGua/Gua *</td>
</tr>
<tr>
<td>HRP + H2O2 + POBN</td>
<td>44.4</td>
</tr>
<tr>
<td>HRP + H2O2 + DNA</td>
<td></td>
</tr>
<tr>
<td>Ferricyanide + POBN</td>
<td>6.0</td>
</tr>
<tr>
<td>Ferricyanide + DNA</td>
<td>14.4</td>
</tr>
<tr>
<td>Ferricyanide + DNA + POBN</td>
<td>14.0</td>
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</tbody>
</table>

* Yields expressed in relation to total guanine (Gua), present in neutral and mild acid DNA hydrolysates, and normalized to 100 μg of DNA; average of two independent determinations, the values of which varied about 20%. MeGua, methylguanine.

1 HRP, horseradish peroxidase.
DNA Alkylation by Methylhydrazine-derived Methyl Radicals

**DISCUSSION**

DNA alkylation by methylhydrazine-derived metabolites has been demonstrated before both in vitro (4) and in vivo (26). The identified DNA adducts were 7-methylguanine and 6-methylguanine (4) implying a cationic metabolite as the alkylating species. The results reported here, however, unequivocally establish formation of another adduct, 3-methylguanine, during DNA treatment with methylhydrazine plus an oxidizing system, either horseradish peroxidase-H$_2$O$_2$ or

![Image](image_url)

**FIG. 2.** HPLC profile obtained by fluorescence detection of: A, a standard mixture of guanine (288 ng), 8-methylguanine (73 ng), 7-methylguanine (107 ng), and adenine (288 ng); B, neutral hydrolysates of DNA treated with methylhydrazine, horseradish peroxidase, and H$_2$O$_2$; the HPLC injection corresponds to 30 µg of initial non-hydrolyzed DNA. Experimental conditions are as described under “Experimental Procedures.”

mass fragmentation peaks at m/z 123 and m/z 85, the latter peak being a clear indication that the fragmentation leaves a methylated imidazole moiety rather than a methylated pyrimidine moiety (Fig. 4).

The yield of methylated guanine obtained in the enzymatic and chemical systems was estimated by two independent determinations (Table I). The chemical system generated a higher yield of methyl radicals but led to a lower yield of both 8-methylguanine and 7-methylguanine (Table I). This is probably due to other free radical reactions, such as hydrogen abstraction (10), leading to DNA cleavage (24, 25). In agreement, DNA treated with methylhydrazine-ferricyanide is more prone to depurination, estimated by the higher yields of guanine and adenine present in the neutral hydrolysates, than DNA treated with the enzymatic system.

**FIG. 3.** Photodiode array UV spectra of guanine, 1-methylguanine (MeGua), 8-methylguanine (synthetic), 6-methylguanine (sample), and 3-methylguanine obtained during reverse phase HPLC in water. 8-Methylguanine (sample) was isolated from DNA treated with methylhydrazine and horseradish peroxidase-H$_2$O$_2$, corresponding to the 16.4-min peak in Fig. 2B.

**FIG. 4.** Upper mass region of the chemical ionization spectra of 8-methylguanine. Spectra were obtained from the pooled fractions isolated from neutral hydrolysates of DNA treated with methylhydrazine and horseradish peroxidase-H$_2$O$_2$ (corresponding to the 16.4-min peak in Fig. 2B (A)) and synthetic 8-methylguanine (B).
DNA Alkylation by Methylhydrazine-derived Methyl Radicals

Both 7-methylguanine and 8-methylguanine (Figs. 2, 3) can be used as an analytical tool for investigating DNA alkylation and should occur in the enzymatic oxidation described here. In addition, spin-trapping experiments demonstrated that oxidation of methylhydrazine promoted by horseradish peroxidase-\text{H}_2\text{O}_2 or ferricyanide generates high yields of methyl radicals (Fig. 1, Table I). This is the first demonstration of formation of Smethylguanine in an enzymatic system, and the same mechanism should occur in the enzymatic oxidation described here. Indeed, spin-trapping experiments demonstrated that oxidation of methylhydrazine promoted by horseradish peroxidase-\text{H}_2\text{O}_2 or ferricyanide generates high yields of methyl radicals (Fig. 1, Table I). In addition, the spin-trap POBN was shown to inhibit formation of 8-methylguanine from DNA treated with methylhydrazine-ferricyanide (Table I).

Neutral hydrolysates of DNA treated with methylhydrazine and horseradish peroxidase-\text{H}_2\text{O}_2 or ferricyanide contained both 7-methylguanine and 8-methylguanine (Fig. 2, Fig. 3, Table I). This suggests formation of methylidyazene (27), an intermediate that, through one- or two-electron oxidation, can generate the ultimate alkylation species, the methyl radical, or the methylidencyanion ion, respectively (Scheme I). These species can account for both products characterized in DNA, namely the methylidencyanion ion for 7-methylguanine (28) and the methyl radical for 8-methylguanine (see above).

DNA depurination upon alkylation at N-7 guanine is well known (18, 19, 23), whereas instability of the adduct at C-8 has been described for 8-(benzo[a]pyren-6-yl) deoxyguanosine (14). However, there are DNA C-8 adducts described as stable to heating (29); in addition, high yields of free radicals can facilitate DNA depurination (25). Consequently, further studies will be required to verify the stability of 8-methylguanine DNA adducts under various conditions.

Scheme I summarizes the results obtained up to this point. Further understanding of DNA alkylation by methylhydrazine metabolites will require complete analysis of DNA alkylation products, a task not yet performed for any nucleic acid reaction (28). In spite of this, our demonstration that enzymatically generated methyl radicals alkylate DNA with formation of 8-methylguanine has wide implications for the study of chemical carcinogenesis. On the one hand, formation of 8-alkylated deoxyguanosine may cause DNA misreading during replication as shown before for 8-OH-deoxyguanosine (30). On the other hand, detection of 8-alkylated guanine can be used as an analytical tool for investigating DNA alkylation by carbon-centered radicals both in vitro and in vivo.

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