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Oxidation of Methylhydrazines to Mutagenic Methylating Derivatives and Inducers of the Adaptive Response of *Escherichia coli* to Alkylation Damage

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ABSTRACT

The methylhydrazines, monomethylhydrazine, 1,1-dimethylhydrazine, and 1,2-dimethylhydrazine, are known carcinogens but only weak mutagens in the Ames test. Chemical oxidation of these compounds by potassium ferricyanide greatly enhanced their mutagenicity to an *Escherichia coli ada* mutant and converted them into inducers of the adaptive response of *E. coli* to alkylation damage. Enzymatic oxidation of monomethylhydrazine by horseradish peroxidase-H_2O_2 also yielded products which induced the adaptive response. Thus, methylhydrazines can be oxidized to active DNA-methylating derivatives which generate methylphosphotriesters (the inducing signal of the adaptive response), O^6-methylguanine and/or O^4-methylthymine (the miscoding bases repaired by the Ada protein) in DNA. These observations support the suggestion that metabolic oxidation of methylhydrazines in mammalian systems may be required to generate the mutagenic/carcinogenic derivatives.

INTRODUCTION

Carcinogenic hydrazine derivatives occur naturally in edible mushrooms and tobacco and are used as pharmaceutical drugs, agricultural herbicides, high energy fuels, and chemical intermediates in industry (1–3). Methylhydrazines, including MMH,[1] 1,1-DMH, and 1,2-DMH are known to be carcinogenic in rodents. 1,2-DMH is the most potent of these three carcinogens and induces a high incidence of colon and rectal tumors (1, 2). Although methylhydrazines are well-known carcinogens, they are only weak mutagens of *Salmonella typhimurium* in the Ames test. Preincubation with rat liver homogenates does not affect the mutagenic potency of 1,2-DMH or MMH and only slightly increases that of 1,1-DMH (4–6). The lack of correlation between the carcinogenic and mutagenic potencies of methylhydrazines may reflect the difficulty of reproducing *in vitro* the metabolic steps required for their activation *in vivo*.

DNA alkylation has been observed following treatment of rats with 1,2-DMH or MMH, and methylated bases including N^7-methylguanine and the mutagenic mispairing derivative O^6-methylguanine have been detected (7–9). However, the mechanism of conversion of methylhydrazines to active alkylating species remains unclarified. Methyl-free radicals or methyldiazonium ions formed during methylhydrazine oxidation may be the reactive intermediates which alkylate cellular constituents (reviewed in Refs. 3 and 10). The production of ethane by rats treated with 1,2-DMH suggested the release of methyl radicals *in vivo* (11). Such free radicals were also released *in vitro* from MMH, 1,1-DMH, 1,2-DMH, and methylformylhydrazine on activation by isolated hepatocytes or liver microsomes (12, 13) and on oxidation of 1,2-DMH or MMH by ferricyanide, oxyhemoglobin, or horseradish peroxidase-H_2O_2 (14–17). Oxidation of 1,2-DMH in the latter conditions resulted in nicking and alkylation of DNA (17), and in the case of MMH the methylated bases N^7-methylguanine and C^8-methylguanine were detected (15). This was the first finding of C^8-methylguanine in DNA, and the mutagenicity of this methylated base remains uninvestigated.

The adaptive response of *Escherichia coli* to alkylation damage is specifically induced by methylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-methyl-*N*-nitrosourea. Induction of the response results in the increased expression of four genes, namely, *ada, alkA, alkB*, or *aidB*. The Ada protein, O^6-methylguanine-DNA methyltransferase, demethylates the mispairing bases O^6-methylguanine and O^4-methylthymine in DNA and, consequently, protects against the mutagenicity of alkylating agents (reviewed in Refs. 18 and 19). The Ada protein also regulates the response. It transfers the methyl group from an *S*-stereoisomer of a methylphosphotriester in DNA to one of its own cysteine residues and in so doing is converted into a strong transcriptional activator of the inducible genes. Thus, the generation of methylphosphotriesters in DNA is the inducing signal for the adaptive response (20). Direct protein methylation has also been demonstrated as a mechanism of Ada activation *in vitro* (21). However, the target size *in vivo* of 2 to 4 Ada molecules/cell (22, 23) is very small compared with the number of phosphate linkages in DNA. Activation of this basal level of cellular Ada protein by low levels of alkylating agents will therefore occur most efficiently by the rapid repair of phosphotriesters in DNA. In a recent review (24), we described preliminary experiments indicating that the chemical oxidation products of one methylhydrazine (MMH) induce the response, which implies that these products methylate the phosphate linkages in DNA. The ability to alkylate oxygens suggested that mutagenic O^6-methylguanine may also be generated.

In this paper, we show that the *in vitro* oxidation products of three carcinogenic methylhydrazines, MMH, 1,1-DMH, and 1,2-DMH, are able to induce the adaptive response of *E. coli* and are mutagenic to *E. coli ada* mutants defective in the repair of O^6-methylguanine in DNA.

MATERIALS AND METHODS

Chemicals. MMH, 1,1-DMH, 1,2-DMH dihydrochloride were purchased from the Aldrich Chemical Company. Horseradish peroxidase type VI was from Sigma, peroxidase-conjugated rabbit anti-mouse immunoglobulins was from DAKO Ltd., and the ECL Western blotting detection system was from Amersham International.

Bacterial Strains. *E. coli* B strains, F26 (*his thy sulA*) and BS23 (a Δ*ada-alkB* mutant of F26) (25, 26), and *E. coli* K12 strains, AB1157 (F⁻ *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*) and BS24 (as AB1157 but *ada-5 nal^r rif^r* (27), were laboratory stocks. *E. coli* B GWR109 (as BS23 but *ogt-1*::Kan^r) (28) was obtained from L. Samson, HK117 (*rha lac str polA12 alkB*::Tn3) (29) was obtained from H. Kataoka, and *E. coli* K12 BH20 (AB1157 but *fpg-1*::Kan^r) (30) was obtained from S. Boiteux.

BS87 (as AB1157 but *alkB*::Tn3) was constructed by transduction of AB1157 with a thermoinduced P1 cml clr 100 lysate of HK117 (*alkB*::Tn3). The transduction method was as described previously (27). Ampicillin-resistant transductants were selected and purified on L agar plates containing 40 μg/ml ampicillin at 42°C. The enhanced sensitivity

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[1] Abbreviations: MMH, monomethylhydrazine; 1,1-DMH, 1,1 dimethylhydrazine; 1,2-DMH, 1,2 dimethylhydrazine; $K_3Fe(CN)_6$, potassium ferricyanide; ECL, enhanced chemiluminescence.

of *alkB*::Tn3 (Amp^r) transductants compared with the parent strain AB1157 was demonstrated by streaking 10 μl of cultures (A_{600} 0.4) across a 0–0.1% gradient of methylmethane sulfonate in a final volume of 100 ml L agar in a 10-cm^2 Petri dish and incubating at 37°C.

Treatment of Cultures with Methylhydrazines. Liquid cultures were grown to A_{600} 0.3 in M9 minimal medium (31), 0.2% casamino acids, and, when required, 20 μg/ml thymine. Potassium phosphate (0.1 M), pH 7.5, and diethylenetriamine pentaacetic acid (10 mM) were added, and 1-ml culture aliquots were exposed to methylhydrazines at various final concentrations.

Immunoassay of Induction of the Ada Protein. Culture aliquots of 1 ml were exposed to doses of methylhydrazines that resulted in little or no cell killing for 40 min at 37°C. Where indicated, these treatments were in the presence of 12.5 mM $K_3Fe(CN)_6$ or 2 mM H_2O_2 and varying amounts of horseradish peroxidase. The cells were harvested and lysed in 20 μl detergent buffer (22). Cellular proteins in 7 μl lysate were resolved by 15% polyacrylamide-sodium dodecyl sulfate gel electrophoresis and transferred by electrophoresis to a nitrocellulose filter. The filter was blocked with 5% dried milk for 1 h and exposed to two purified anti-Ada monoclonal antibodies as described previously (22) for 2 h at 20°C . The two antibodies recognize the two major domains of the Ada protein. The filter was washed with 1% Nonidet P-40 in phosphate-buffered saline and incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins diluted 1:7000 in 1% Nonidet P-40 and 1% fetal calf serum in phosphate-buffered saline. After washing in 1% Nonidet P-40, the filter was treated with the ECL solutions as directed by the manufacturers and exposed to Amersham Hyperfilm-ECL for 30 s to several minutes at 20°C.

Mutagenesis. Culture aliquots were exposed to methylhydrazines in the absence or presence of 12.5 mM $K_3Fe(CN)_6$ for 30 min at 37°C. The cells were immediately diluted in M9 salts and 10 mM $MgSO_4$ and plated in M9 minimal soft agar (0.8%) on M9 minimal plates containing the appropriate supplements to select for mutants and survivors. F26-derived strains were monitored for His$^+$ revertants on plates lacking histidine, and AB1157 derivatives were monitored for Arg$^+$ revertants on plates containing a limiting amount of arginine (0.75 μg/ml) (27).

RESULTS

The Ada protein of *E. coli* is induced as part of the adaptive response to alkylation damage. We previously described a sensitive immunoassay in which two anti-Ada monoclonal antibodies were used to monitor induction of this protein (22). The assay was modified, and a chemiluminescence system was used to detect the secondary antibody. The ability of the three methylhydrazines, MMH, 1,1-DMH, and 1,2-DMH, to induce the Ada protein was examined using this assay. Concentrations of up to 1 mM MMH (24), 1,1-DMH, or 1,2-DMH failed to induce the Ada protein of *E. coli* B strain F26 (Fig. 1). However, in the presence of $K_3Fe(CN)_6$, low concentrations of all three methylhydrazines were activated as inducers (Fig. 1), suggesting that they were all converted by oxidation to active methylating derivatives. The lowest doses used which resulted in Ada induction in the presence of ferricyanide were 30 μM MMH, 30 μM 1,1-DMH, and 100 μM 1,2-DMH. Compared with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-methyl-*N*-nitrosourea which induce the Ada protein at nanomolar concentrations (22, 32), the oxidized methylhydrazines were weak inducers.

MMH induced the *E. coli* Ada protein after oxidation by horseradish peroxidase-H_2O_2 (Fig. 2). The induction exhibited a dose dependency on the enzyme concentration (Fig. 2, *lanes 1–5*) and occurred only when all three components, MMH, horseradish peroxidase, and H_2O_2, were added to the cells (Fig. 2, *lanes 5–9*). Thus, oxidation of this methylhydrazine to an active DNA-methylating species also occurred by an enzyme-catalyzed process. The horseradish peroxidase was a particulate

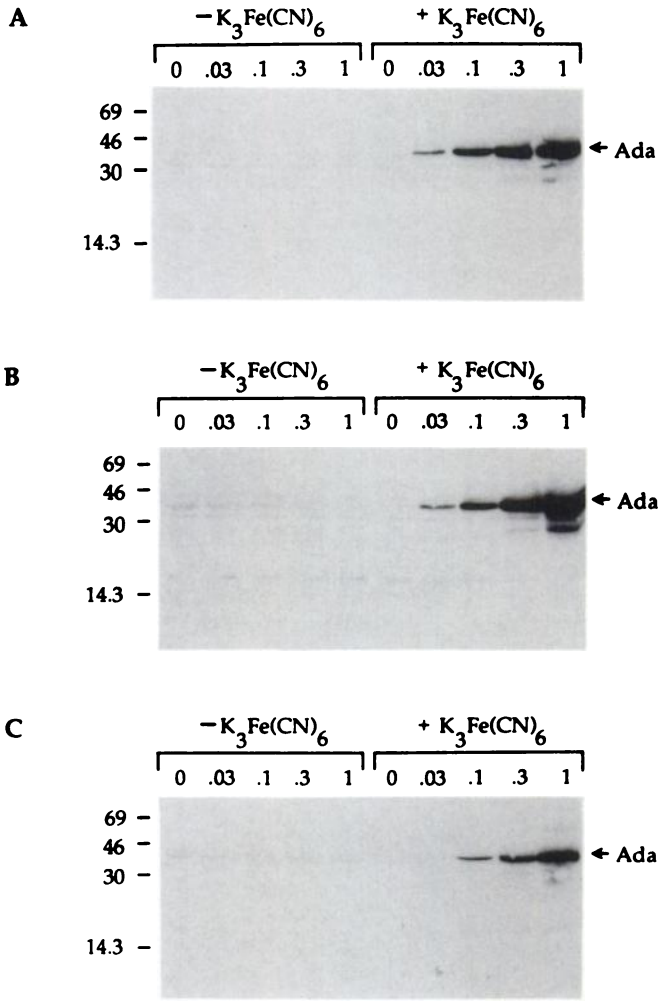

Fig. 1. Induction of the *E. coli* Ada protein by methylhydrazines oxidized by $K_3Fe(CN)_6$. *E. coli* F26 was exposed to various doses of MMH, 1,1DMH, or 1,2-DMH in the absence or presence of 12.5 mM $K_3Fe(CN)_6$. The 39-kDa Ada protein induced in 0.3 ml of culture was monitored by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using two anti-Ada monoclonal antibodies and a chemiluminescence system to detect the peroxidase-conjugated secondary antibody. The exposure time of the autoradiograph was 5 min. The methylhydrazine treatment doses (mM) of MMH (*A*), 1,1-DMH (*B*), or 1,2-DMH (*C*) are indicated above each lane.

suspension and was pelleted with the cells after MMH treatment. The chemiluminescence Western blotting detection system monitored peroxidase activity and detected both the peroxidase-conjugated secondary antibody bound to the induced 39-kDa Ada protein and also the free 44-kDa horseradish peroxidase (33) added to oxidize MMH (Fig. 2, *lanes 2–5* and *9*).

The three carcinogenic methylhydrazines are poor mutagens of *S. typhimurium* in the Ames test with or without added liver microsomes (4–6). *S. typhimurium* has only a weak adaptive response which does not confer any detectable cellular resistance to mutation induction by such agents (34, 35). *S. typhimurium* is therefore a very sensitive bacterium for monitoring the mutagenicity of active methylating agents. Furthermore, 2 mM MMH, 1,1-DMH, or 1,2-DMH were not mutagenic to *E. coli* B strain BS23 (*Δada-alkB*) which completely lacks an adaptive response (Fig. 3, Table 1). However, on oxidation by $K_3Fe(CN)_6$, the mutagenicity of all three agents was dramatically increased >500-fold (Fig. 3, Table 1). This mutagenicity was detected using the *ada* mutant, BS23 (*E. coli* B F26 but *Δada-alkB*) (Fig. 3, Table 1), and also, in the case of MMH, a

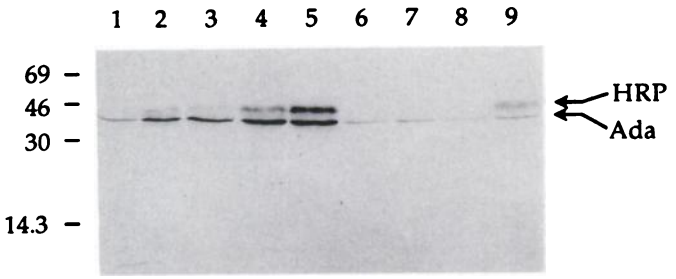

Fig. 2. Induction of the *E. coli* Ada protein by MMH oxidized by horseradish peroxidase and H_2O_2. *E. coli* F26 was exposed to 1 mM MMH, 2 mM H_2O_2, and various amounts of horseradish peroxidase (HRP) (42 kDa) (*lanes 1–5*). *Lane 1*, no horseradish peroxidase; *lane 2*, 25 μM; *lane 3*, 50 μM; *lane 4*, 125 μM; *lane 5*, 250 μM. Control cultures (*lanes 6–9*) were exposed to no addition (*lane 6*), MMH only (*lane 7*), MMH and H_2O_2 (*lane 8*), and MMH and 250 μM horseradish peroxidase (*lane 9*). The induced 39-kDa Ada protein was monitored by Western blotting as described in Fig. 1. The exposure time of the autoradiograph was 1 min.

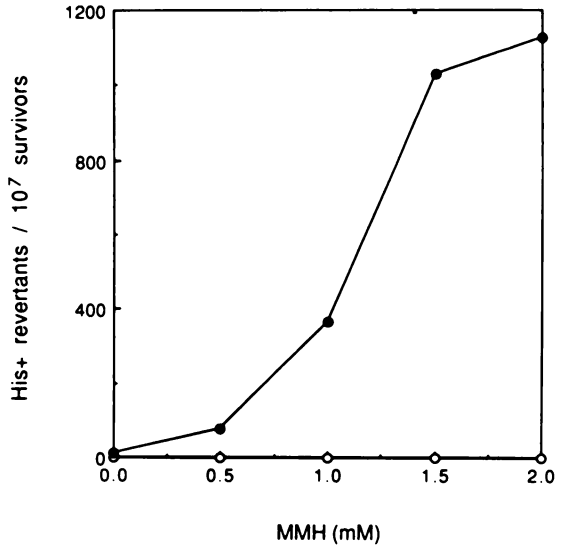

Fig. 3. Mutagenicity of MMH oxidized by $K_3Fe(CN)_6$. *E. coli* BS23 (*thy his Δada-alkB*) in supplemented minimal medium was exposed to various doses of MMH with or without the addition of 12.5 mM $K_3Fe(CN)_6$ for 30 min at 37°C. Cultures were immediately diluted and plated to estimate cell survival and the number of His⁺ revertants. ○, without $K_3Fe(CN)_6$; ●, with $K_3Fe(CN)_6$.

Table 1 *Mutagenicity of 1,1-DMH and 1,2-DMH oxidized by $K_3Fe(CN)_6$*

DMH	$K_3Fe(CN)_6$	His⁺ revertants/10⁷ survivors[a]	
		BS23 (*Δada*)	F26 (*ada⁺*)
–	–	0.3	0.8
–	+	1.0	<0.3
+ 1,1 DMH	–	0.4	0.3
+ 1,1 DMH	+	218.0	12.0
+ 1,2 DMH	–	<0.3	0.3
+ 1,2 DMH	+	141.0	6.3

[a] Strains F26 and BS23 were exposed to 2 mM 1,1-DMH or 1,2-DMH with or without 12.5 mM $K_3Fe(CN)_6$ for 30 min at 37°C and immediately plated to measure the cell survival and number of His⁺ revertants.

second *ada* mutant BS24 (*E. coli* K12 AB1157 but *ada-5*) (Fig. 4*B*). The parental strains (F26 and AB1157, respectively) were approximately 20-fold less mutagenized by the oxidized methylhydrazines than the *ada* mutants (Fig. 4, *A* and *B*, Table 1). Thus, the mutagenic adduct produced by the oxidized methylhydrazines was apparently repaired by the Ada O^6-methylguanine-DNA methyltransferase activity or possibly the product of one of the four genes regulated by Ada (18). *E. coli* has two O^6-

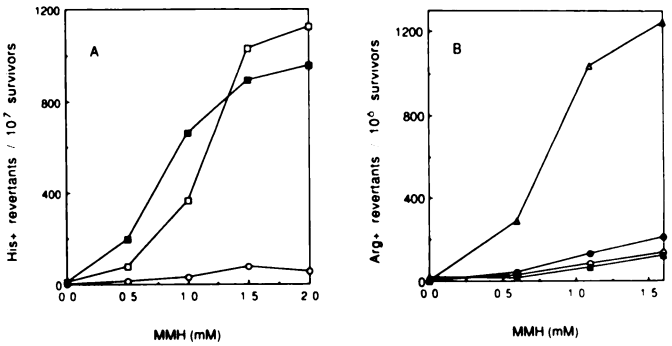

Fig. 4. Sensitivity of *E. coli* mutants defective in DNA repair to the mutagenicity of MMH. *E. coli* cultures in supplemented minimal medium were exposed to various doses of MMH in the presence of 12.5 mM $K_3Fe(CN)_6$ for 30 min at 37°C. Cultures were diluted and plated to estimate cell survival and the number of His⁺ (*A*) or Arg⁺ (*B*) revertants. A: ○, F26; □, BS23 (*Δada-alkB*); ■, GWR109 (*ogt Δada-alkB*). B: ○, AB1157; △, BS24 (*ada-5*); ●, BS87 (*alkB*::Tn3); ▲, BS20 (*fpg*::*kan*ʳ).

methylguanine-DNA methyltransferase activities, the inducible Ada protein and also the constitutively synthesized Ogt protein. GWR109 (F26 but *ogt Δada-alkB*) lacks both these activities and was approximately 2-fold more sensitive than the *ada* mutant BS23 to mutation induction by the lower MMH doses of 0.5 and 1 mM (Fig. 4*A*). The 30 molecules of Ogt protein/cell (36) are consumed in the repair reaction and, therefore, conveyed measurable protection only at low levels of mutagenic damage. These observations imply that the Ada and Ogt O^6-methylguanine-DNA methyltransferase activities are important in defending cells against the mutagenic products of oxidized methylhydrazines.

C^8-Methylguanine of unknown mutagenicity was found in DNA treated with oxidized MMH (15). The *fpg* or *mutM* gene product is a DNA glycosylase which excises mutagenic C^8-hydroxypurines and also imidazole ring-opened purines from DNA damaged by ionizing radiation or photosensitization (37–39). Thus, the *fpg* enzyme excises a range of imidazole ring-modified purines, and the possibility that it might excise C^8-methylguanine from DNA was considered. The *fpg* mutant BH20, however, was not sensitive to the mutagenicity of oxidized MMH (Fig. 4*B*). Thus, C^8-methylguanine is either not excised by the *fpg* gene product or it is not mutagenic. The inducible AlkB protein has an unknown function in DNA repair (40). An *alkB* mutant BS87 was also insensitive to the mutagenicity of oxidized MMH (Fig. 4*B*). Thus, the AlkB protein also was not involved in the repair of mutagenic adducts produced by oxidized MMH.

DISCUSSION

The methylhydrazines, MMH, 1,1-DMH, and 1,2-DMH, are nonmutagens or weak mutagens in the Ames test (4–6). The mechanism of activation of these compounds to mutagenic derivatives therefore required identification. In this paper, we have shown that chemical oxidation of these three methylhydrazines greatly enhanced their mutagenicity to *E. coli ada* mutants. Conditions have therefore been identified in which mutagenicity of methylhydrazines can be readily detected.

The oxidized methylhydrazines induced the Ada protein of *E. coli*. Thus, methylhydrazine oxidation must generate an active derivative which methylates DNA to give rise to the inducing signal of the adaptive response, methylphosphotriesters (20). The ability to alkylate DNA-oxygens suggests that

the mutagenic bases O^6-methylguanine and O^4-methylthymine may also be generated. The Ada O^6-methylguanine-DNA methyltransferase repairs both of these mutagenic bases. Methylation of O^6-guanine and O^4-thymine would therefore explain the sensitivity of *ada* mutants to mutation induction by oxidized methylhydrazines.

O^6-Methylguanine and N^7-methylguanine were found in the DNA of rats treated with 1,2-DMH or MMH (7–9). Oxidation of the methylhydrazines *in vivo* may account for their DNA-methylating ability and their effectiveness as mutagens and carcinogens. In support of this suggestion, enzymatic oxidation of MMH by horseradish peroxidase-H_2O_2 yielded products which induced the adaptive response indicating the alkylation of DNA-oxygens.

1,2-DMH is a more potent carcinogen than 1,1-DMH and MMH (2), but on oxidation 1,2-DMH was least mutagenic at the dose tested (Table 1, Fig. 3) and the least effective in inducing the adaptive response by approximately 3-fold (Fig. 1). The lack of correlation of the relative carcinogenic and mutagenic potencies may be related to different susceptibilities of the methylhydrazines to *in vivo* mechanisms of oxidation or, alternatively, to further means of activation of 1,2-DMH to carcinogenic derivatives.

Aerial oxidation has also been found to increase the mutagenicity of 1,1-DMH in the absence of liver microsomes (41). The presence of nitrosamines in this oxidized 1,1-DMH is unlikely to account for its mutagenicity since nitrosamines require metabolic activation by liver microsomes or hydroxylation to release the mutagenic methyldiazonium ion (42, 43).

The conditions used in these experiments presented in this paper for methylhydrazine oxidation were similar to those of Augusto *et al.* (15) who detected the release of methyl radicals and the production of N^7-methylguanine and C^8-methylguanine in DNA. Methyl radicals released by homolysis of *tert*-butyl peracetate-methylated free guanosine and adenosine at the C^8 position but no *N*- or *O*-methylated products were detected (44). The ultimate alkylating derivatives released on oxidation of methylhydrazines may therefore be nucleophilic methyl radicals which methylate at C^8-guanine and possibly electrophilic methyldiazonium ions which alkylate at N^7-guanine and O^6-guanine (3, 45).

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