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IDENTIFICATION OF N^5-METHYL-N^5-FORMYL-2,5,6-TRIAMINO-4-HYDROXYPYRIMIDINE AS A MAJOR ADDUCT IN RAT LIVER DNA AFTER TREATMENT WITH THE CARCINOGENS, N,N-DIMETHYLNITROSAMINE OR 1,2-DIMETHYLHYDRAZINE

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A major and previously undetected carcinogen-DNA adduct was found in the livers of rats given N,N-dimethylnitrosamine or 1,2-dimethylhydrazine. This adduct, which accounted for 55% of the total methyl residues in DNA at 72 hours after carcinogen treatment, was chromatographically identical to a synthetic purine ring-opened derivative of 7-methylguanine and could be released from the isolated hepatic DNA by a specific *E. coli* glycosylase. The synthetic ring-opened adduct was characterized by mass and NMR spectroscopy as N^5-methyl-N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine and appears to exist in two rotameric forms.

The reaction of chemical carcinogens with cellular DNA is generally considered to be a critical step in the initiation of tumorigenesis (1-3). For the carcinogenic alkylating agents, which include nitrosamines, nitrosamides, and hydrazines, the major DNA alkylation product formed *in vivo* immediately after carcinogen treatment is a 7-alkylguanine (2,3). Although this type of lesion was originally postulated to be associated with a carcinogenic or mutagenic response (4), the rapid loss of the 7-alkylguanine from DNA and the inability to correlate its presence with tissue or species sensitivity to alkylating carcinogens led investigators to consider the role of minor adducts in the neoplastic process. Over the last decade, numerous studies have shown that alkylation of oxygen atoms in DNA bases yields adducts such as O^6-alkylguanines that are persistent in DNA and are closely correlated with carcinogenesis (2,3). With the recent development of a high pressure liquid chromatography (hplc) procedure (5) for the rapid quantitation of all 15 known base alkylation products, we have re-examined the DNA alkylation profile in rat liver after treatment with two extensively studied carcinogens, N,N-dimethylnitrosamine (DMN) and 1,2-di-

ABBREVIATIONS: hplc, high pressure liquid chromatography; DMN, N,N,-dimethylnitrosamine; DMH, 1,2-dimethylhydrazine; 7-Me-G, 7-methylguanine; O^6-Me-G, O^6-methylguanine; 3-Me-G; 3-methylguanine; 3-Me-A, 3-methyladenine; 1-Me-A, 1-methyladenine; 3-Me-dC, 3-methyldeoxycytidine; O^2-Me-C, O^2-methylcytosine; DMSO-d_6, deuterated dimethylsulfoxide.

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methylhydrazine (DMH). We now report the identification *in vivo* of a novel 8,9-purine ring-opened derivative of 7-methylguanine (7-Me-G). The existence of such a product *in vitro* after alkaline treatment of methylated nucleic acids (6-8) or of 7-methylguanosine (9-12) has been previously indicated, but the accumulation of a ring-opened adduct *in vivo* was considered unlikely (13). In addition, a formamidopyrimidine-DNA glycosylase, which has been purified from *E. coli* (14) and is present in rodent liver (13), was recently shown to catalyze the specific removal of a purine ring-opened derivative of 7-Me-G from DNA that had been methylated *in vitro* and then treated with alkali (14).

MATERIALS AND METHODS

2,5,6-Triamino-4-hydroxypyrimidine hemisulfate was purchased from Vega Biochemicals (Tucson, AZ). [^{14}C-Methyl]DMN (9.4 mCi/mmol) and [^{14}C-methyl)DMH (8.4 mCi/mmol) were obtained from New England Nuclear (Boston, MA) and diluted with non-radiolabeled carcinogen purchased from Aldrich Chem. Co.(Milwaukee, WI) to a specific activity of 0.44 and 1.85 mCi/mmol, respectively. Female Sprague-Dawley rats (NCTR Strain 11; 200 g) were treated with [^{14}C]DMN i.p. or [^{14}C]DMH s.c. at a dose of 20 mg/kg body weight as previously described (15,16). After 12 or 72 hours, the animals were sacrificed and liver DNA was isolated by solvent extractions and hydroxyapatite chromatography (17). The DNA was subjected to neutral-thermal, acidic, and/or enzymatic hydrolyses and analyzed for methylated DNA adducts as detailed earlier (5). The purine ring-opened 7-Me-G derivative was quantitatively released by the sequential neutral-thermal (60%) and acidic (40%) hydrolyses. Synthetic ring-opened 7-Me-G was prepared both by alkaline treatment of 7-methylguanosine (14) and by alkaline dialyses and subsequent hydrolysis of methylated DNA (14). Purification of the synthetic and *in vivo* ring-opened products was achieved by hplc on a μ Bondapak C_{18}-Semiprep reversed phase column (*cf.* Fig. 1B). Synthetic derivatives were further purified on a Hamilton PRP-1 preparative column (4% acetonitrile in water; 2 ml/min; retention times for the two components = 13.5 and 16 min, respectively). After removal of the solvent under reduced pressure, samples were analyzed by mass spectrometry (Finnigan 4023 with a thermal desorption probe) and by proton magnetic resonance spectroscopy (Bruker WM500). Incubation of hepatic DNA from [^{14}C]DMN-treated rats with purified *E. coli* formamidopyrimidine-DNA glycosylase (14) and subsequent estimation of ethanol-soluble radioactivity was performed as previously described (8).

RESULTS

In accordance with earlier studies (15,16), administration of [^{14}C]DMN or [^{14}C]DMH to rats resulted in high levels of radioactivity covalently bound to hepatic DNA. Upon hydrolysis of the DNA and analysis by hplc (*cf.* Materials and Methods), we confirmed the presence of 7-Me-G as the major alkylated DNA adduct in the liver at 12 hours after carcinogen treatment. Smaller amounts of the known adducts, O^6-methylguanine (O^6-Me-G), 3-methylguanine (3-Me-G), 3-methyladenine (3-Me-A), and 1-methyladenine (1-Me-A) were also detected (Table 1). However, an appreciable amount of an unknown component was also found in chromatograms of both the neutral-thermal and acidic DNA hydrolysates. This component, designated as X, was relatively polar and eluted at 3.5 min from the hplc cation exchange column (Fig. 1A). By 72 hours after dosing, levels of each of the known adducts had decreased markedly while component X appeared to have increased and now accounted for 55% of the total DNA binding (Table 1).

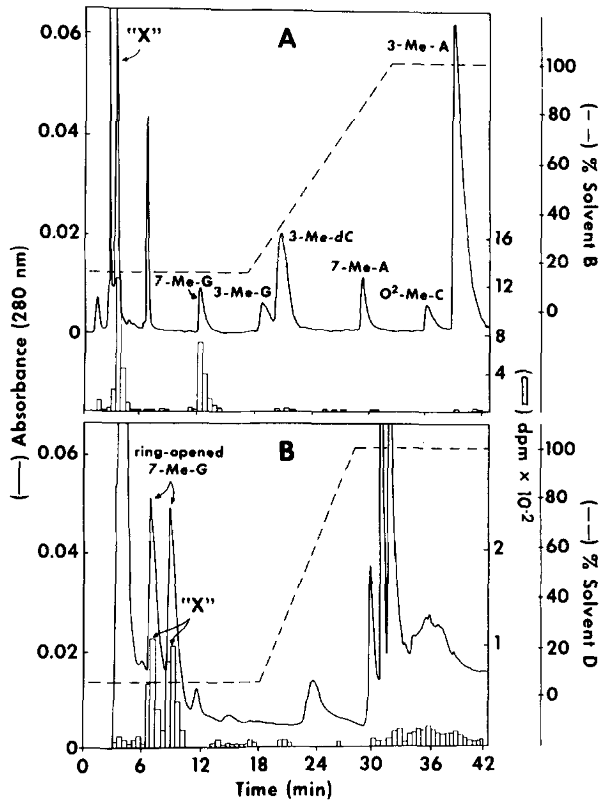


Fig. 1. A. Hplc profile of an alkylated liver DNA hydrolysate at 72 hours after [^{14}C]DMH treatment. Isolated DNA was hydrolyzed under neutral-thermal conditions (100°C, 30 min), and chromatographed on a Partisil-10 SCX/M9 cation exchange column. Solvent A was 6% methanol while Solvent B was 0.20 M ammonium formate-8% methanol. The UV markers arise from addition of synthetic standards to the analytical sample.
B. Rechromatography of component X obtained in Frame A with synthetic ring-opened 7-Me-G on a μBondapak-C_{18} reversed phase column. Solvent C was 0.01 M ammonium phosphate (pH 5.1)-3% methanol while Solvent D was 100% methanol.
Similar chromatograms were obtained on rat liver DNA hydrolysates obtained at 72 hours after [^{14}C] DMN treatment.

Since the increase in component X and the decrease in 7-Me-G were of a similar magnitude (Table 1), the identity of X as a derivative of 7-Me-G was subsequently investigated. A purine ring-opened derivative of 7-Me-G was prepared both by: 1) alkaline treatment of 7-methylguanosine followed by acidic hydrolysis; and 2) alkaline dialysis of methylated calf thymus DNA (14) followed by neutral-thermal _or_ acidic hydrolysis. This synthetic product was found to have the same retention time on the hplc ion exchange column as did component X obtained from rat liver _in vivo_. Reversed phase hplc, which was found to separate the synthetic purine ring-opened 7-Me-G derivative into two interconvertible forms (_vide infra_), further indicated that the _in vivo_ component X and the synthetic products had the same chromatographic properties (Fig. 1B). Addition-

Table 1. DNA Alkylation Products in Rat Liver After DMN or DMH Treatment[a]

Carcinogen	Time of Sacrifice	Methylated Adducts/10^6 Nucleotides: 7-Me-G	X^b	0^6-Me-G	3-Me-G	3-Me-A	1-Me-A
DMN	12 hr	850	105	90	7	24	8
	72 hr	235	310	8	$<1^c$	5	$<1^c$
DMH	12 hr	179	17	26	2	5	3
	72 hr	56	71	1	$<1^c$	1	$<1^c$

[a] As described in Materials and Methods, female rats were given [^{14}C]DMN (i.p.) or [^{14}C]DMH (s.c.) and sacrificed at 12 and 72 hours. Hepatic DNA was isolated and adducts were quantified by hplc. The values above represent an average of duplicate determinations, which were within 15% of each other.

[b] The X designates the previously unknown adduct which is now identified as N^5-methyl-N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine.

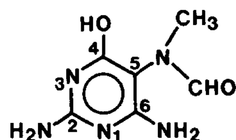
[c] Judged to be the limit of detection. Radioactivity associated with the other 10 known base alkylation products were below detection limits.

al support for the identification of X as a ring-opened 7-Me-G derivative was provided by treatment of the *in vivo* modified DNA with purified *E. coli* formamidopyrimidine-DNA glycosylase, which has been shown to specifically hydrolyze purine ring-opened guanine adducts in DNA (8,14,18). Incubation of hepatic DNA (from DMN-treated rats) or methylated calf thymus DNA (after alkaline treatment) with this glycosylase for 20 minutes released 5-10% of the bound radioactivity.

Although previous reports indicated that the alkaline decomposition product of 7-Me-G was a purine ring-opened derivative (6-12), we sought to obtain additional spectroscopic data and to establish the structure of the synthetic adduct used in this study. For this purpose, the synthetic product was purified by reversed phase hplc (Fig. 1B) and its two individual components were collected. Rechromatography of these fractions on a PRP-1 column (*cf.* *Materials and Methods*) indicated that each component was slowly converted to the other to give a 1:1 mixture after 4-6 hours. Both components were again collected and each was analyzed by mass and NMR spectroscopy. The thermal desorption mass spectra of both samples were identical and showed the expected molecular ion at m/z 183 with major fragments at 155 (-CO) and 140 (-CO,-CH_3). Likewise, 500 MHz proton NMR spectra (acquisition time = 6-8 hours) of both samples in deuterated dimethylsulfoxide (DMSO-d_6) and in D_2O were identical and established the identity of this adduct as N^5-methyl-N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine (Table 2). The doubling of certain resonances, whose relative intensities were solvent-dependent, was consistent with restricted rotation about the C-5-N bond. This suggested that the two peaks observed on reversed phase hplc (Fig. 1B) were rotational isomers. Construction of space-filling molecular models of this ad-

Table 2. 500 MHZ ^{1}H-NMR Spectral Parameters[a] for:

N^5-Methyl-N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine



Chemical Shift in ppm from TMS	Multiplicity (No. of Protons)	Assignment
10.03[b], 9.92[c]	2S (1)[d]	4-OH
8.00[c], 7.73[b]	2S (1)	N^5-CHO
6.25[b], 5.94[c]	2S (2)[d]	6-NH_2
6.19	S (2)[d]	2-NH_2
2.93[c], 2.80[b]	2S (3)	N^5-CH_3

[a] The solvent was DMSO-d6. TMS = tetramethylsilane; S = singlet. The assignments were made by comparison with spectral parameters for authentic 2,5,6-triamino-4-hydroxypyrimidine hemisulfate.

[b,c] The relative intensities of these resonances were 90:10 in DMSO-d_6. Upon addition of an equal volume of D_2O and equilibration overnight, this ratio for the non-exchangeable protons became 65:35.

[d] Exchangeable in D_2O.

duct supported the existence of rotamers due to steric hindrance between the methylformamido group and the exocyclic atoms of the pyrimidine ring, with the transition state being coplanar and the energy minima being orthogonal (20).

DISCUSSION

The biological importance of an 8,9-purine ring-opened guanine derivative was first suggested by Hems who identified N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine as a major product formed after treatment of guanosine with ionizing radiation (21). More recently, a similar ring-opened N-7-alkylated guanine derivative of aflatoxin B_1 was identified in rat liver DNA as a persistent lesion which accumulated after multiple doses of the carcinogen. Consequently, this adduct was proposed to play an important role in the initiation of aflatoxin carcinogenesis (19,22,23). Likewise, we have recently reported that an 8,9-ring-opened C-8-arylamino-guanine derivative is a major DNA adduct of the urinary bladder carcinogen, 2-naphthylamine, and that it persists in the urothelium but not the liver of dogs administered this carcinogen (24,25). Other purine ring-opened derivatives have been detected _in vitro_ after reaction of DNA with phosphoramide mustard (18) or with N-hydroxy-2-aminofluorene (26).

In this study, we have found that two different carcinogenic alkylating agents, DMN and DMH, give rise to a purine ring-opened 7-Me-G adduct _in vivo_.

At 12 hours after carcinogen dosing, this adduct represented only about 7-10% of the total hepatic DNA alkylation products. However, at 72 hours the levels of the ring-opened adduct were increased 3 to 4-fold and now accounted for 55% of the total binding to the DNA (cf. Table 1). This data is comparable to our preliminary finding (27,28) that this purine ring-opened adduct is the only persistent lesion in the bladder DNA of rats given a carcinogenic intraurethral dose of N-methylnitrosourea. Since 7-Me-G in the *in vitro* methylated DNA has been reported to be resistant to purine ring-opening under physiological conditions (6), we suggest that the *in vivo* formation of the ring-opened derivative may be enzymatically mediated. The existence of glycosylases catalyzing the removal of this adduct from DNA has already been described (13,14).

The role of purine ring-opened 7-alkylguanine adducts in carcinogenesis by nitrosamines, hydrazines, and direct-acting alkylating agents has not been previously addressed. However, the presence of N^5-methyl-N^5-formyl-2,5,6-triamino-4-hydroxypyrimidine as a major product in hepatic DNA after DMN and DMH dosing strongly suggests that this adduct should be considered as a potential mutagenic and carcinogenic lesion.

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