Articles

N-Nitrosobenzylmethylamine Is Activated to a DNA Benzylating Agent in Rats

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The ability of rat tissues to activate the esophageal carcinogen, *N*-nitrosobenzylmethylamine (NBzMA), to a DNA benzylating intermediate was investigated. [3-^{3}H]NBzMA was prepared and given to male F344 rats. Tissues were harvested 4 h after treatment, and DNA was isolated. HPLC analysis with radiochemical detection of chemical and enzymatic hydrolysates of DNA from liver and lung revealed the formation of benzyl adducts. Benzyl alcohol, N^2-benzylguanine, 3-benzyladenine, N^6-benzyladenine, and 7-benzylguanine were the major radioactive components in the hydrolysates. An unknown adduct was also observed. The adduct distribution was similar to that observed in [3-^{3}H]benzylnitrosourea ([3-^{3}H]BzNU)-treated calf thymus DNA. However, enzymatic hydrolysates of [3-^{3}H]BzNU-treated DNA also contained significant levels of O^6-benzyl-2′-deoxyguanosine (O^6-BzdG). This radioactive adduct disappeared upon incubation of the DNA with a crude preparation of the repair protein, O^6-alkylguanine−DNA alkyltransferase isolated from rat liver. These data provide evidence that O^6-BzdG is probably rapidly repaired *in vivo.* No benzylation of esophageal mucosal DNA was detected. The level of DNA benzylation observed in tissues from [3-^{3}H]NBzMA-treated rats was several orders of magnitude lower than the level of DNA methylation in these same tissues. Therefore, these data indicate that DNA benzylation plays a minor role, if any, in the carcinogenic activity of NBzMA.

Introduction

N-Nitrosobenzylmethylamine (NBzMA)[1] is a highly selective esophageal carcinogen in laboratory animals (*1*−*3*). This compound is a possible human esophageal carcinogen (*4*, *5*). Carcinogenicity is believed to be initiated by cytochrome P450-catalyzed hydroxylation adjacent to the nitroso group; this reaction forms metabolites that decompose to DNA reactive compounds. Since NBzMA is an asymmetric nitrosamine, it has two potential activation pathways (Figure 1). Benzyl hydroxylation leads to a methylating agent, whereas methyl hydroxylation generates a benzylating agent (*6*, *7*). The available data support the formation of O^6-methylguanine (O^6-mG) as the important step in tumor initiation by this compound. High levels of methyl adducts are detected in target tissues relative to nontarget tissues (*8*−*10*). Furthermore, O^6-mG derived from NBzMA persists longer in the esophagus relative to nontarget tissues, such as liver, following a single dose (*11*, *12*). In addition, mutations detected in activated H-*ras* and p53 oncogenes (*13*−*15*) are consistent with the formation of O^6-mG, not O^6-benzylguanine (O^6-BzG; *16*). Moreover, the mutational frequency of O^6-mG was 2 times higher than that of O^6-BzG in Rat4 TK$^-$ cells (*16*).

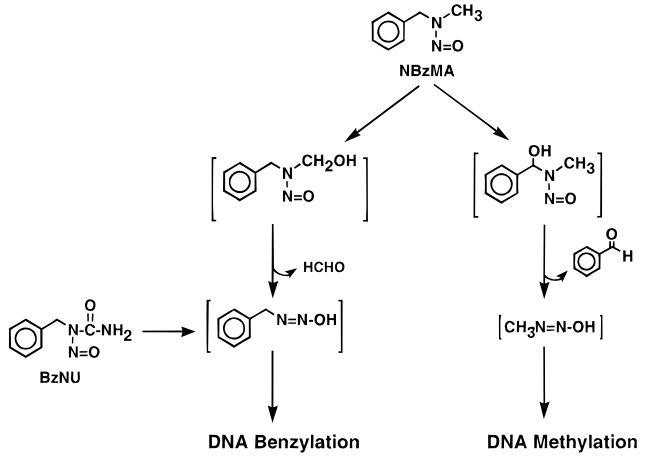

Figure 1. Proposed activation pathways of NBzMA and BzNU.

The role of the DNA benzylation pathway in NBzMA-induced esophageal carcinogenesis has not been extensively investigated. The only reported attempt to measure benzyl adducts was performed in Wistar rats treated with [*methylene*-^{14}C]NBzMA (iv, 0.017 nmol/kg) of low specific activity (11.3 mCi/mmol; *9*). No benzyl adducts were detected. The limits of detection were 0.5−1 benzylated bases in 10^6 bases. However, liver microsomes catalyze both benzylation and methylation pathways as indicated by formaldehyde and benzaldehyde formation (*6*, *17*−*21*). The relative amount of these two metabolites varied with the concentration of NBzMA

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[1] Abbreviations: AGT, O^6-alkylguanine−DNA alkyltransferase; 3-BzA, 3-benzyladenine; 7-BzA, 7-benzyladenine; 3-BzdC, 3-benzyl-2′-deoxycytidine; 7-BzG, 7-benzylguanine; BzNU; *N*-benzyl-*N*-nitrosourea; MNU, *N*-methyl-*N*-nitrosourea; N^2-BzdG, N^2-benzyl-2′-deoxyguanosine; N^2-BzG, N^2-benzylguanine; N^6-BzA, N^6-benzyladenine; N^6-BzdA, N^6-benzyl-2′-deoxyadenosine; 7-mG, 7-methylguanine; NBzMA, *N*-nitrosobenzylmethylamine; O^6-BzdG, O^6-benzyl-2′-deoxyguanosine; O^6-BzG, O^6-benzylguanine; O^6-mG, O^6-methylguanine; SDS, sodium dodecyl sulfate.

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used and the source of microsomes. In esophageal microsomes, there was at least 100 times more benzaldehyde than formaldehyde formed (*17*), indicating that the methylation pathway likely predominates in the target tissue.

DNA benzylation may be involved in the carcinogenic activity of NBzMA. The benzylating agent, *N*-benzyl-*N*-nitrosourea (BzNU), is a locally acting carcinogen in animals (*22*). Rat liver S9 can activate NBzMA to a mutagen in TA100 but not TA1535 *Salmonella* strains (*23*). This strain selectivity parallels that observed for BzNU but not the methylating agent, *N*-methyl-*N*-nitrosourea (MNU; *23*). BzNU as well as the benzylating *N*-(acetoxymethyl)-*N*-benzylnitrosamine are mutagenic in a variety of *in vitro* mutagenicity assays (*23*−*25*). The mutagenic activity of these benzylating species suggests that phenylmethanediazohydroxide is capable of alkylating DNA. Therefore, if benzylation of DNA occurs *in vivo*, it could potentially contribute to the carcinogenic activity of NBzMA.

DNA adducts generated by benzylating agents have not been extensively characterized. Reaction of BzNU with guanosine produced 7-, N^2-, and O^6-benzylguanosine (*26*). This compound also alkylated the N^6- and 1-positions of adenosine (*26*). In addition, 7-benzylguanine (7-BzG) has been identified as a liver DNA adduct isolated from [^{14}C]BzNU-treated Sprague-Dawley rats (*27*).

The possibility that NBzMA is activated to a DNA benzylating agent *in vivo* was re-examined by treating rats with [3-^{3}H]NBzMA of high specific activity and characterizing the adducts present in DNA isolated from target and nontarget tissues. As described below, DNA benzylation does occur in rats treated with NBzMA. However, the levels of DNA benzylation are orders of magnitude lower than those of DNA methylation. The adduct distribution in the DNA from these tissues was compared to that observed in [3-^{3}H]BzNU-treated calf thymus DNA. The major difference was the absence of O^6-benzyl-2′-deoxyguanosine (O^6-BzdG) in the *in vivo* benzylated DNA, a likely result of efficient DNA repair.

Experimental Procedures

Caution: *NBzMA and BzNU are carcinogens in experimental animals.*

Materials. NBzMA was obtained from the NCI Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO). Phenol saturated with 10 mM Tris (pH 8) and 1 mM EDTA, calf thymus DNA, nuclease P_1, phosphodiesterase I (type VII), DNase I (type II), proteinase K, RNase K, and RNase T_1 were purchased from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase was purchased from Boehringer Mannheim (Indianapolis, IN). [3-^{3}H]Benzylamine hydrochloride was purchased from Amersham (Arlington Heights, IL). All other chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). 3-Benzyladenine (3-BzA), 7-benzyladenine (7-BzA), 3-benzyl-2′-deoxycytidine (3-BzdC), N^2-benzyl-2′-deoxyguanosine(N^2-BzdG), N^6-benzyl-2′-deoxyadenosine (N^6-BzdA) standards were a generous gift from Dr. Robert Moschel, NCI-FCRDC, Frederick, MD. N^2-Benzylguanine (N^2-BzG) and N^6-benzyladenine (N^6-BzA) were generated by heating the corresponding 2′-deoxynucleoside in 0.1 N HCl. O^6-BzG and O^6-BzdG were synthesized according to literature procedures (*28*, *29*). 7-BzG was produced by reacting benzyl bromide with 2′-deoxyguanosine in DMF: ^{1}H NMR (DMSO-d_6) δ 10.75 (s, N1-H), 8.1 (s, C8-H), 7.4 (m, aromatic H), 6.1 (br s, 2-NH_2), and 5.4 (s, CH_2). Rat liver O^6-alkylguanine−DNA alkyltransferase (AGT) was prepared as previously described (*30*).

(3-Bromobenzyl)methylamine Hydrochloride. The preparation of (3-bromobenzyl)methylamine hydrochloride was adapted from a published procedure (*31*). Trifluoromethyl sulfonic anhydride (3.2 g, 0.011 mol) in CH_2Cl_2 (11 mL) was added dropwise to 3-bromobenzylamine (4.2 g, 0.025 mol) in 35 mL of CH_2Cl_2 at 0 °C. The reaction was stirred for 1 h at room temperature, washed two times with 1.0 N HCI (20 mL) and three times with water (20 mL), and dried over $MgSO_4$. The resulting oil was reacted with iodomethane (0.7 mL, 0.011 mol) in the presence of potassium carbonate (1.6 g, 0.016 mol) in acetone (25 mL). The acetone was removed under reduced pressure, and the product was added dropwise as an anhydrous diethyl ether solution (11 mL) to a stirred suspension of lithium aluminum hydride (1.4 g, 0.037 mol). The reaction was stirred for 2 days at room temperature. Then, water (4.4 mL) was added dropwise, followed by 15% NaOH (4.4 mL) and, finally, additional water (13 mL). The ether layer was filtered, dried over $MgSO_4$, and concentrated to yield an oil (1.5 g). HCl in ether (0.1 N) was added to the oil, and the resulting white precipitate was collected by filtration and washed with ether (1.5 g). NMR of the product demonstrated that we had obtained (3-bromobenzyl)methylamine that contained approximately 20% benzylmethylamine. This side product resulted presumably from reductive debromination by lithium aluminum hydride. ^{1}H NMR ($CDCl_3$) δ 7.5 (s, 1H, C2), 7.4 (d, 1H, C4), 7.3 (m, 1H, C6), 7.2 (dd, 1H, C5) 3.8 (s, 2H, CH_2N), and 2.5 (s, 3H, NCH_3).

[3-^{3}H]Benzylmethylamine. (3-Bromobenzyl)methylamine hydrochloride was converted to its free base and dissolved in ethanol containing 5% triethylamine. The reductive tritiation was performed in the presence of tritium gas and 5−10% Pd/$CaCO_3$ for 1−2 h at atmospheric pressure and room temperature. The catalyst was removed by filtration. Then 0.1 volume of dilute HCl was added and the solvent was removed under reduced pressure. The product was stored in ethanol at −80 °C (sp act., approximately 8.3 Ci/mmol).

[3-^{3}H]NBzMA. [3-^{3}H]Benzylmethylamine hydrochloride (200 mCi, 6.1 mg, 0.0386 mmol) was dissolved in 30% acetic acid (1 mL). Sodium nitrite (16.4 mg, 0.240 mmol) was added. One and a half hours later, the reaction mixture was extracted with CH_2Cl_2 (3 × 3 mL). The organic phase was washed with water, dried over $MgSO_4$ (anhyd), and concentrated under reduced pressure. The reaction product was purified using a short silica gel column eluted with CH_2Cl_2. The fractions containing product were combined, dried over $MgSO_4$, and stored at −80 °C in CH_2Cl_2. The identity and purity were confirmed by coelution with standards on reverse-phase HPLC (Phenomenex, Torrance, CA, Bondclone C18 column, 3300 × 3.9 mm) linked to radioflow detection (FLO-one/Beta, Radiomatics Instruments, Tampa, FL), using 3% acetic acid (solvent A) and 95% methanol (solvent B). The column was eluted with a linear gradient from 100% solvent A to 50% solvent A/50% solvent B over 50 min. Yield: 93.7 mCi (47%, 98% radiochemically pure, sp act., 5.2 mCi/mmol).

[3-^{3}H]BzNU. [3-^{3}H]Benzylamine hydrochloride (22 mCi, 3.6 mg, 25 μmol) was dissolved in 500 μL of water. Potassium cyanate (4.5 mg, 56 μmol) and 0.1 N H_2SO_4 (125 μL) were added, and the reaction was heated at 110 °C with stirring for 15 min. After the reaction was placed on ice, 5 N H_2SO_4 (1 mL) was added. Then an aqueous solution of sodium nitrite (100 μL, 3.75 mg, 55 μmol) was added slowly over 5 min. The reaction was stirred at 0 °C for 20 min, and then extracted with ether (8 × 1 mL). The ether layer was dried over $MgSO_4$ and concentrated to 4 mL. It was then applied to a Florisil column (1.5 × 13 cm). The product was eluted with ether. The fractions containing product were pooled, concentrated under reduced pressure, and dissolved in ethanol (5 mL). The radioactivity coeluted with cold standard on TLC (20% ethyl acetate in hexane). Yield: 3.3 mCi (15%, sp act., approximately 880 mCi/mmol).

[3-^{3}H]BzNU-Treated DNA. [3-^{3}H]BzNU (3.3 mCi, 0.00375 mmol) was reacted with calf thymus DNA (50 mg) in 50 mM sodium cacodylate (pH 6.0) containing 1 mM EDTA (10 mL) at room temperature overnight. The DNA was precipitated from the solution by the addition of 2 M NaCl (2 mL) and ice cold

ethanol (15 mL). It was redissolved in 100 mM sodium phosphate (pH 7.0, 2.3 mg/mL) and dialyzed against the same buffer overnight at 4 °C. Following the addition of 2 M NaCl (3 mL) and 20 mL of ice cold ethanol, the DNA was isolated, washed with ice cold ethanol several times, dried under an N_2 stream, and stored at −20 °C.

Animal Treatment. Twenty male F344 rats (140−165 g, Charles River) were treated with [3-^{3}H]NBzMA (2.5 mg/kg, 1860 mCi/mol) in saline (sc). NBzMA was soluble in saline at the concentration used (7.3 mM). The animals were sacrificed 4 h after injection. Lungs and liver were removed and frozen at −80 °C for DNA isolation. Esophageal mucosae were stripped from the esophagus using forceps prior to storage at −80 °C. The lungs were pooled in four groups of five, and the esophageal mucosae were grouped into two groups of ten.

DNA Isolation. Several DNA isolation methods were attempted (*32*−*34*). A modification of a published combined Kirby−Marmur method (*32*, *33*) was chosen since it yielded the cleanest DNA and was more successfully adapted to smaller amounts of tissue, such as esophageal mucosae. The tissue was minced and homogenized in 1% sodium dodecyl sulfate (SDS) containing 1 mM EDTA (10 mL/g of tissue). The esophageal mucosae were frozen and crushed or powdered before they were homogenized in the SDS solution. Proteinase K (0.5 mg/g of tissue) was added, and the mixture was incubated at room temperature for 30 min. Then 1 M Tris-HCl (pH 7.4, 0.5 mL/10 mL of solution) was added. The homogenate was extracted for 5 min with 1 volume of phenol (saturated with 10 mM Tris, pH 8, and 1 mM EDTA) containing 0.1% (v/v) cresol (phenol reagent). Since benzyl adducts can be unstable in acid (*35*), buffer-washed phenol was used to ensure neutral pH. Following centrifugation at 14000*g* for 15 min, the aqueous phase was removed and extracted with an equal volume of phenol reagent−chloroform−isoamyl alcohol (25:24:1) for 3 min. This mixture was centrifuged for 15 min. The resulting aqueous phase was further extracted with 1 volume of chloroform−isoamyl alcohol (24:1) for 3 min. This mixture was centrifuged for 15 min. The aqueous phase was removed and made 0.5 M in NaCl. The DNA was precipitated from this solution by the slow addition of ice cold ethanol (1 volume). The esophageal DNA was not precipitated at this point. Lung and liver DNA were harvested, washed with 80% ethanol, and redissolved in 150 mM NaCl, 15 mM sodium citrate (pH 7.0), and 1 mM EDTA (2 mL/g of tissue). This mixture was incubated with RNase A (200 μL/g of a 50 μg of RNase A/mL of 0.3 M NaCl solution) and RNase T_1 (100 units/g of tissue) for 30 min at room temperature. This solution was extracted with an equal volume of chloroform−isoamyl alcohol (24:1) and centrifuged for 15 min. The aqueous solution was extracted as described until there was no discernible interface (2−3 times). DNA was then precipitated from this solution with ice cold ethanol. At this point, the lung and esophageal DNA was washed extensively with 80% ethanol followed by absolute ethanol. The DNA was dried under an N_2 stream and stored desiccated at −20 °C.

The liver DNA was dissolved in distilled water (0.75 mL/g of tissue). An equal volume of phosphate reagent (2.5 M K_2HPO_4 containing 1.6% phosphoric acid) was added. Addition of a similar volume of 2-methoxyethanol removed polysaccharides. The mixture was centrifuged at 20000*g* for 30 min at 4 °C. Addition of an equal volume of 1% cetyltrimethylammonium bromide in water precipitated the DNA from the upper layer. The DNA was washed three times with cold deionized water, followed by two washes with 2% sodium acetate in 70% ethanol. Then it was allowed to stand in 2% sodium acetate in 70% ethanol for at least 15 min at room temperature, to convert the DNA to its sodium salt. It was then washed extensively with absolute ethanol, dried under an N_2 stream, and stored desiccated at −20 °C until analysis.

Samples of this DNA (200 μg) were hydrolyzed with nuclease P_1 (5 units) in 10 mM Tris-HCl (pH 7.0, 200 μL) containing 0.5 M sodium acetate (pH 5.1, 10 μL) for 30 min at 37 °C. Alkaline phosphatase (5 units) and 0.4 M Tris-HCl (pH 7.5, 80 μL) were added, and the hydrolysis was continued for another hour at 37 °C. HPLC analysis of these hydrolysates was achieved on a C18 column (Phenomenex Bondclone) linked to UV detection. They were eluted with isocratic 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH, and 10 mM acetic acid (pH 5.1) containing 5% methanol. A comparison of the guanosine levels to 2′-deoxyguanosine levels demonstrated less than 8% RNA contamination.

DNA Hydrolysis Conditions. DNA (3−5 mg) was dissolved in 10 mM sodium cacodylate (pH 7.0, 1−1.5 mL) and heated to 100 °C for 30 min. This treatment releases positively charged purines such as 7-BzG, 3-BzA, and 7-BzA from the DNA. After cooling on ice, 1/10 volume of 1 N HCl was added to precipitate the DNA. Following centrifugation, the supernatant was removed for HPLC analysis. The pellet was heated in 0.8 N HCl for 5−6 h. The volume of the 0.8 N HCl hydrolysate equaled the final volume of the neutral thermal hydrolysate after addition of 1 N HCl. Some of the DNA was directly hydrolyzed with 0.8 N HCl (0.2−2.7 mg in 0.5−1 mL) for 5−6 h at 80 °C. All samples were neutralized to pH 7 prior to analysis by HPLC.

The following conditions yielded the best results for the enzymatic hydrolysis of the benzylated DNA. DNA (3−5 mg) was incubated with DNase I (72 units/mg of DNA) in 10 mM Tris (pH 7.4)/5 mM $MgCl_2$ for 2 h at 37 °C (total volume: 2−5 mL). Then, phosphodiesterase I (0.1 unit/mg of DNA) was added and the incubation was continued at 37 °C for another 2 h. At that time, alkaline phosphatase (600 units) was added. After 1 h at 37 °C, the hydrolysates were cooled on ice, spiked with unlabeled benzyl DNA standards, and filtered using Centrifuge Micropartition Systems, MW cutoff 30 000 (Amicon, Beverly, MA). The hydrolysates were frozen at −20 °C until analysis. The HPLC analyses were performed using from 1.8 to 4.5 mL of the hydrolysates.

Incubations of [3-^{3}H]BzNU-Treated DNA with AGT. [3-^{3}H]BzNU-treated DNA (1 mg) was incubated with semipurified rat liver AGT (7 pmol) in HEPES (pH 7.8) containing 1 mM EDTA and 1 mM dithiothreitol (total volume: 2.5 mL) for 30 min prior to enzymatic degradation of the DNA as described above. Controls were conducted in the absence of the AGT preparation.

HPLC Conditions. The DNA hydrolysates were analyzed using reverse-phase HPLC linked to radioflow detection (FLO-one/Beta, Radiomatics Instruments, Tampa, FL). The mixtures were separated on a Phenomenex Bondclone C18 column (300 × 3.9 mm) using one of two gradient systems. System 1 used 20 mM sodium phosphate (pH 4.5, solvent A) and 95% methanol (solvent B). After 5 min at 100% A, a linear gradient was run from 100% solvent A to 40% solvent A/60% solvent B over 60 min. System 2 employed 20 mM sodium phosphate (pH 7.0 solvent C) and solvent B. After a 5 min isocratic period at 80% solvent C/20% solvent B, a 60 min linear gradient to 40% solvent C/60% solvent B was used to elute the hydrolysates. When a 2 mL HPLC sample loop was utilized, 2−3 injections of the sample were made during the initial isocratic portion of the gradient. The multiple injections affected only the BzOH peak. When a 5 mL sample loop was used, the separation between 3-BzA and the unknown adduct was poor.

Methylation Levels. The amounts of 7-methylguanine (7-mG) and O^6-mG in lung and liver DNA were quantitated using published methods (*36*). Levels of 7-mG, O^6-mG, and guanine were determined by HPLC analysis with fluorescence detection (LC 240 fluorescence detector; Perkin Elmer, Norwalk, CT). The hydrolysates were separated using two Partisil 10 SCX columns in tandem (Phenomenex, Torrance, CA). Neutral thermal hydrolysates were eluted with 100 mM ammonium phosphate (pH 2.2), and mild acid hydrolysates were eluted with the same buffer plus 10% methanol (flow: 1 mL/min). Quantitation was achieved using standard curves prepared for each analysis. Minimum detection limits were 1.0 pmol of O^6-mG and 4.6 pmol of 7-mG.

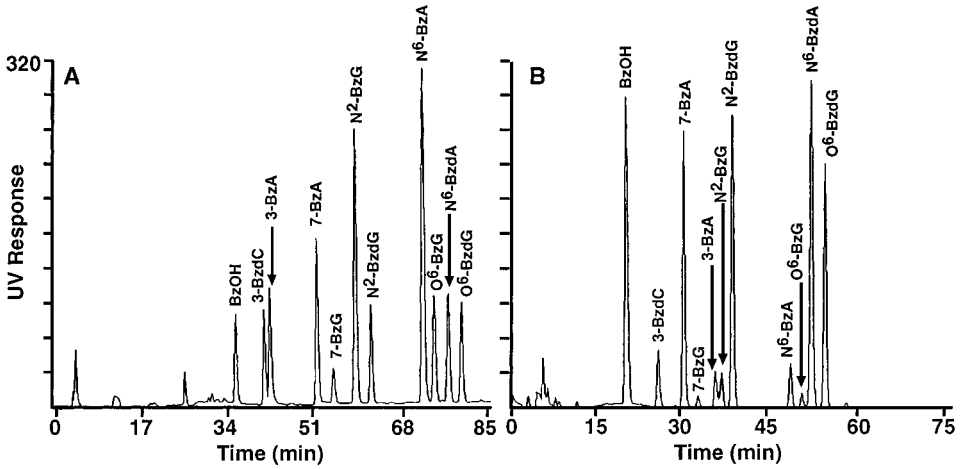

Figure 2. HPLC chromatograms obtained for benzyl DNA adduct standards obtained using a 20 mM sodium phosphate−methanol gradient. A = pH 4.5 and B = pH 7.0. See Experimental Procedures for details.

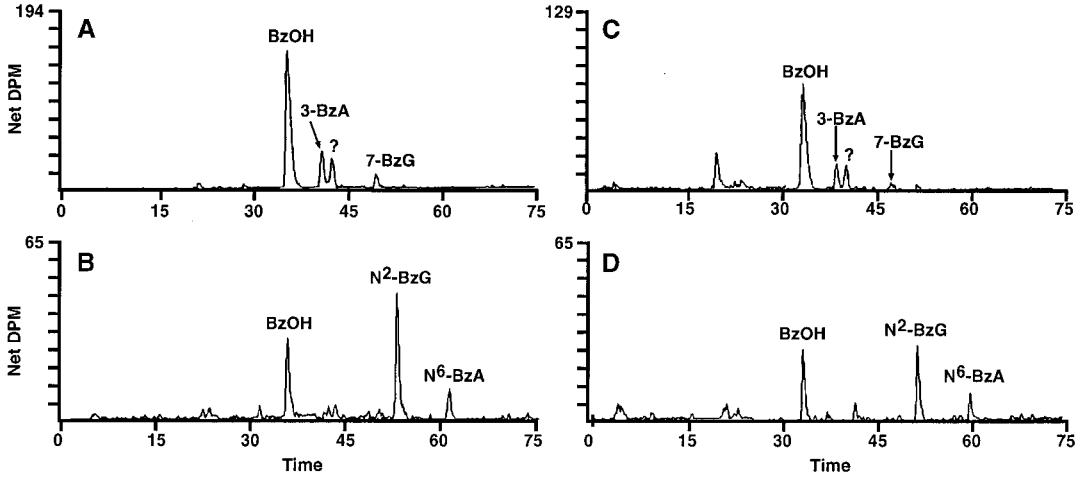

Figure 3. Representative radiograms of neutral thermal (A and C) and subsequent 0.8 N HCl (B and D) hydrolysates of lung (A and B) and liver (C and D) DNA isolated from [3-^{3}H]NBzMA-treated rats. The hydrolysates were eluted from a C18 column using 20 mM sodium phosphate (pH 4.5) with a methanol gradient. See Experimental Procedures for details.

Table 1. Quantitation of BzOH and Benzylated Adducts in DNA Isolated from [3-^{3}H]NBzMA-Treated Rats[a]

		levels of BzOH and adducts (pmol/μmol of guanine)[b]					
tissue	hydrolysis conditions	BzOH	3-BzA	7-BzG	N^2-BzG[b]	N^6-BzA[b]	O^6-BzG[b]
liver	enzyme (n = 3)	0.7 ± 0.2	0.3	nd[c]	0.3 ± 0.03	0.1 ± 0.03	≤0.07 ± 0.00
	neutral thermal (n = 4)	1.0 ± 0.2	0.2 ± 0.1	0.06 ± 0.03	nd	nd	nd
	0.8 N HCl[d] (n = 3)	0.2 ± 0.1	nd	nd	0.2 ± 0.1	0.1 ± 0.03	nd
lung	enzyme (n = 3)	0.3 ± 0.1	0.1	<0.03	0.1 ± 0.03	≤0.03 ± 0.03	≤0.03 ± 0.01
	neutral thermal (n = 3)	0.7 ± 0.2	0.1 ± 0.1	0.03 ± 0.01	nd	nd	nd
	0.8 N HCl (n = 3)	0.1 ± 0.03	nd	nd	0.1 ± 0.02	0.03 (2); nd (1)	nd

[a] Twenty rats were treated with [3-^{3}H]NBzMA (2.5 mg/kg, 1860 mCi/mmol) in saline (sc). The rats were sacrificed at 4 h after treatment. See Experimental Procedures for details. [b] In the enzyme hydrolysates, these adducts were detected as the 2′-deoxyguanosine or 2′-deoxyadenosine derivatives and are expressed as pmol/μmol of 2′-deoxyguanosine. [c] nd = not detected. The detection limits were 150 dpm, approximately 0.03 ± 0.01 pmol/μmol of guanine except where noted. [d] The pellet obtained from the neutral thermal hydroylsate was heated in 0.8 N HCl for 5−6 h. See Experimental Procedures for details.

Results

***In Vivo* [3-^{3}H]NBzMA Experiments.** Standards of benzyl alcohol (BzOH) and the expected benzyl DNA adducts were separated from one another on a C18 column using one of two mobile phase systems (Figure 2). HPLC analysis with radiochemical detection of the neutral thermal hydrolysates of lung and liver DNA from [3-^{3}H]NBzMA-treated rats indicated the presence of four radioactive species. Radioactivity coeluted with BzOH, 3-benzyladenine (3-BzA), and 7-BzG standards (Figure 3A,C). The radioactive peak eluting just after 3-BzA in the pH 4.5 mobile phase did not coelute with any of the standards. The radiograms of the strong acid hydrolysates contained three major radioactive peaks, coeluting with standards for BzOH, N^2-BzG, and N^6-BzA (Figure 3B,D). Adduct levels are displayed in Table 1.

No radioactivity coeluted with O^6-BzG, a previously reported benzyl guanine adduct (*26*). The absence of this adduct in the acid hydrolysate may have resulted from its instability in acidic solutions; it decomposes to BzOH and guanine (*35*). In order to detect this adduct, the DNA was also subjected to enzymatic hydrolysis. Radiograms obtained for these enzyme hydrolysates (Figure 4) contained radioactive peaks that coeluted with BzOH, 3-BzA, N^2-BzdG, and N^6-BzdA. There was radioactivity that migrated in the region of O^6-BzdG; however, it did not

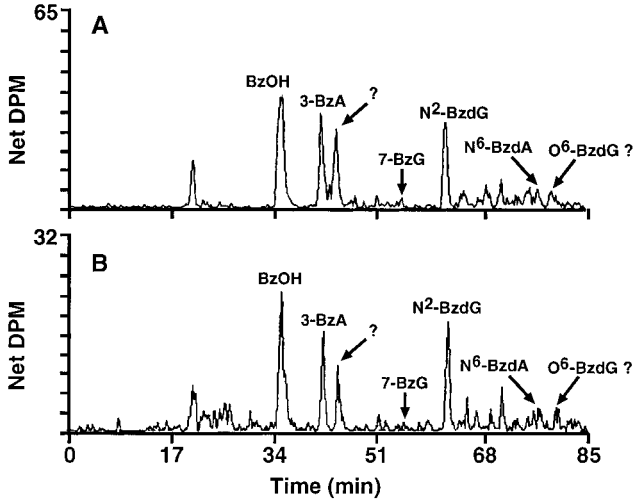

Figure 4. Representative radiograms of enzymatic hydrolysates from lung (A) and liver (B) DNA isolated from [3-^{3}H]-NBzMA-treated rats. The hydrolysates were eluted from a C18 column using 20 mM sodium phosphate (pH 4.5) with a methanol gradient. See Experimental Procedures for details.

coelute with the standard. The enzyme hydrolysates also contained a radioactive peak that had a similar retention time as the unknown peak in the neutral thermal hydrolysates.

A comparison of the adduct levels in enzymatic hydrolysates and chemical hydrolysates demonstrated that these two methods yielded similar results with the following exceptions (Table 1). Levels of BzOH were higher in chemical hydrolysates than in enzymatic hydrolysates. The amount of radioactivity eluting in the region of O^6-BzdG did not account for the difference. There were also measurable levels of 7-BzG in the neutral thermal hydrolysates but not in the enzyme hydrolysates. The levels of 7-mG in liver and lung DNA (120 ± 30 and 250 ± 70 pmol/μmol of guanine, respectively; $n = 3$) were several orders of magnitude higher than the levels of total benzylation in these tissues (4.3 ± 1.2 and 1.9 ± 0.7 pmol of benzylation/μmol of guanine, respectively). O^6-mG was also detected in lung DNA mild acid hydrolysates (46 ± 28 pmol/μmol of guanine; $n = 3$). No detectable O^6-mG was found in liver DNA hydrolysates. Similar results have been reported and ascribed to efficient O^6-mG repair by the liver (*37*).

Isolation of esophageal DNA yielded small amounts (0.4 mg). In order to ensure adduct detection, this DNA was subjected to direct strong acid hydrolysis (0.8 N HCl, 80 °C for 6 h) prior to HPLC analysis. Preliminary studies were conducted with similar quantitities of lung DNA (0.3−0.6 mg). BzOH was the major radioactive species present in the lung DNA hydrolysates; this compound represented one-third of the total binding (0.5 ± 0.1 pmol of BzOH/μmol of guanine and 1.5 ± 0.2 pmol of total benzyl adducts/μmol of guanine). The total amount of [^{3}H] binding to the esophageal DNA was in the same range as the lung DNA (1.8 pmol/μmol of guanine). However, none of this radioactivity coeluted with BzOH or the benzyl adduct standards (data not shown). The limit of detection of BzOH in these samples was 0.08 pmol/μmol of guanine. Therefore, the radioactivity is probably not covalently bound to the DNA. The small amounts of esophageal DNA prevented measurement of DNA methylation. Using the same dose, Siglin *et al.* reported approximately 50 pmol of O^6-mG/μmol of guanine in F344 rat esophageal mucosal DNA 24 h following NBzMA treatment (*37*).

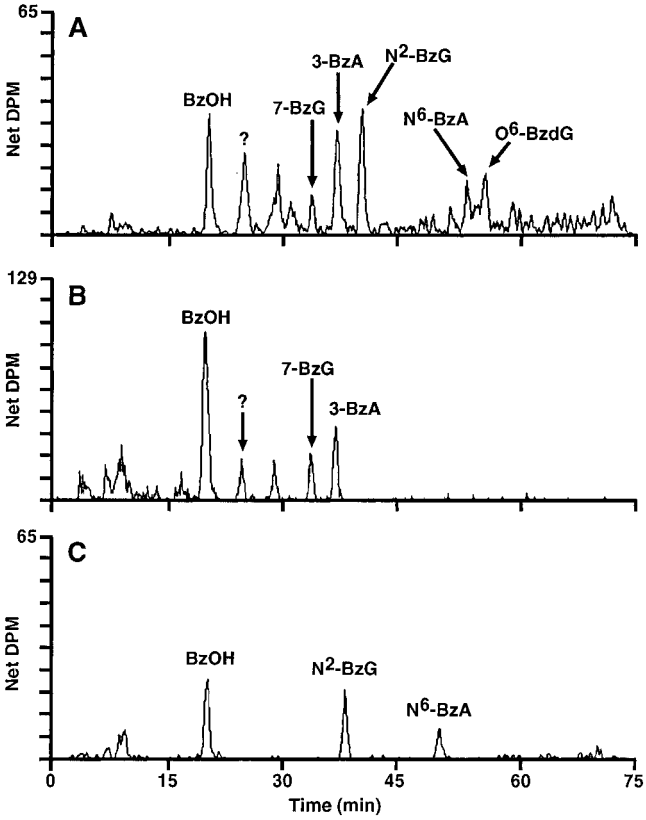

Figure 5. Representative radiograms of enzymatic (A), neutral thermal (B), and subsequent 0.8 N HCl hydrolysates (C) of [3-^{3}H]BzNU-treated calf thymus DNA. The hydrolysates were eluted from a C18 column using 20 mM sodium phosphate (pH 7.0) with a methanol gradient. See Experimental Procedures for details.

***In Vitro* [^{3}H]BzNU Experiments.** In order to determine relative adduct yields in the absence of DNA repair, adduct levels were determined in [^{3}H]BzNU-treated calf thymus DNA. Radiograms of the chemical and enzymatic hydrolysates were obtained using the pH 7 mobile phase and are displayed in Figure 5. The adduct levels are listed in Table 2. These hydrolysates contained radioactive peaks that coeluted with BzOH, 7-BzG, N^2-BzG, 3-BzA, and N^6-BzA. The radioactive peak coeluting with BzOH was extractable by methylene chloride. The unknown that eluted at 45 min using the pH 4.5 HPLC system elutes at 24 min with the pH 7.0 mobile phase. The enzymatic hydrolysates of this DNA also contained a significant peak that coeluted with O^6-BzdG. This radioactive peak is not present in hydrolysates obtained from [^{3}H]BzNU-treated DNA that had been incubated with a crude preparation of rat liver AGT. AGT repairs O^6-benzylguanine (*38*). Interestingly, the unknown that eluted at 24 min also disappeared with this treatment.

Discussion

The data presented in this report represent the first comprehensive study of DNA benzyl adducts formed by benzylating agents both *in vivo* and *in vitro.* Male F344 rats were injected with [3-^{3}H]NBzMA (2.5 mg/kg in saline, sc) and sacrificed 4 h after treatment. This dose of NBzMA produces esophageal tumors in rats when administered in a multiple dose regimen (*14*). The 4 h time point was chosen in order to determine the initial

Table 2. Quantitation of BzOH and Benzylated Adducts in [3-^{3}H]BzNU-Treated DNA

hydrolysis conditions	levels of BzOH and adducts (pmol/μmol of guanine)[a]					
	BzOH	3-BzA	7-BzG	N^2-BzG[a]	N^6-BzA[a]	O^6-BzG[a]
enzyme ($n = 4$)	4.8 ± 0.7	4.4 ± 0.9	1.7 ± 0.7	5.0 ± 0.8	2.7 ± 0.5	2.8 ± 0.2
neutral thermal ($n = 3$)	10.7 ± 1.9	4.5 ± 1.0	2.4 ± 1.0	nd[b]	nd	nd
0.8 N HCl[c] ($n = 3$)	6.8 ± 3.6	nd	nd	3.8 ± 1.4	2.0 ± 0.9	nd

[a] In the enzyme hydrolysates, these adducts were detected as the 2′-deoxyguanosine or 2′-deoxyadenosine derivatives and are expressed as pmol/μmol of 2′-deoxyguanosine. [b] nd = not detected; limits of detection were 150 dpm. [c] The pellet obtained from the neutral thermal hydrolysate was heated in 0.8 N HCl for 5−6 h. The solution was neutralized prior to analysis by HPLC. See Experimental Procedures for details.

Table 3. Relative Ratios of BzOH and Benzyl Adduct Formation[a]

tissue	hydrolysis conditions	BzOH	3-BzA	7-BzG	N^2-BzG[b]	N^6-BzA[b]	O^6-BzG[b]
liver	enzyme	2.3	1.0	<0.1	1.0	0.3	<0.2
	chemical[c]	6.0	1.0	0.3	1.0	0.5	<0.2
lung	enzyme	3.0	1.0	<0.3	1.0	≤0.3	≤0.3
	chemical[c]	8.0	1.0	<0.3	1.0	≤0.3	<0.3
CT DNA	enzyme	1.0	0.9	0.3	1.0	0.5	0.6
	chemical[c]	4.6	1.1	0.6	1.0	0.5	<0.05

[a] Relative to N^2-BzG. [b] In the enzyme hydrolysates, these adducts were detected as the 2′-deoxyguanosine or 2′-deoxyadenosine derivatives. [c] Adduct levels in the neutral thermal and 0.8 N HCl hydrolysates were combined.

adduct distribution in the absence of significant DNA repair. Previous studies indicated that NBzMA is rapidly metabolized (*7*, *8*, *39*). DNA was isolated from the esophageal mucosa since the mucosa is the primary site of NBzMA activation in the esophagus (*12*, *17*).

These results demonstrate that NBzMA is metabolized to a DNA benzylating species *in vivo* as evidenced by the detection of benzyl adducts in lung and liver DNA. The levels of DNA benzylation are several orders of magnitude less than the levels of DNA methylation in the same tissue. The major adducts detected were N^2-BzG, 3-BzA, N^6-BzA, and 7-BzG. BzOH is the primary radioactive species present in the hydrolysates. [3-^{3}H]BzNU reacts with calf thymus DNA to form the same adducts. In this case, O^6-BzdG was clearly detected. An unknown adduct was present in enzymatic and neutral thermal hydrolysates of both [3-^{3}H]BzNU-treated DNA and DNA from [3-^{3}H]NBzMA-treated rat tissues.

The source of BzOH in the hydrolysates is unknown. It could result from decomposition of one or more labile adducts under the hydrolysis conditions. Consistently, BzOH levels were higher in the acid hydrolysates than in the enzyme hydrolysates. Benzyl phosphate adducts likely decompose to BzOH under the acid hydrolysis conditions. Alternatively, positively charged adducts such as 7-benzyl-2′-deoxyguanosine, 1-benzyl-2′-deoxyadenosine, and 3-benzyl-2′-deoxyadenosine might also decompose partially to BzOH under our hydrolysis conditions. O^6-BzdG is another possible source of BzOH in the acid hydrolysates. The possibility that BzOH is noncovalently associated with the DNA cannot be completely excluded.

The relative adduct levels formed *in vivo* and *in vitro* were similar, with some significant differences (Table 3). The hydrolysates of DNA from NBzMA-treated rat tissues contained less 7-BzG relative to other benzyl adducts than in the [3-^{3}H]BzNU-treated DNA hydrolysates. Enzyme hydrolysates of the DNA isolated from NBzMA-treated rat tissues also contained more BzOH than those of the *in vitro* treated DNA. Little, if any, O^6-BzdG was observed in the DNA from NBzMA-treated rats, whereas this adduct was a substantial DNA adduct in the BzNU-treated DNA. A crude preparation of the repair protein AGT was able to remove the benzyl group from the O^6-position of guanine. These results demonstrate that O^6-BzdG is probably rapidly repaired *in vivo*.

These data are consistent with previous studies examining the reaction products between BzNU and nucleosides (*26*, *40*). Alkylation of guanosine by BzNU under aqueous conditions generated 7-, O^6-, and N^2-BzG in a ratio of 0.5:1.2:1. Reactions between adenosine and BzNU generated predominately N^6-BzA with a small amount of 1-BzA (10:1; *26*). The authors did not report the formation of 3-BzA. In the nucleoside reactions, the benzylating agent was more reactive with the exocyclic sites than ring nitrogens (*26*). The relative ratios of 7-, O^6-, and N^2-guanine adducts in [3-^{3}H]BzNU-treated DNA were 0.6:0.6:1. Benzylation of guanine's exocyclic sites predominates as predicted by the previous study. The ratio of 3-BzA to N^6-BzA in [3-^{3}H]BzNU-treated DNA was 1:0.5. Unlike the nucleoside reactions, alkylation of the ring nitrogen of adenosine in DNA predominates. In this current study, 1-BzA was not detected (data not shown). It is unlikely that this adduct underwent a Dimoth rearrangement to 6-BzdA, since studies with 1-benzyladenosine indicate that this is a minor reaction (*41*).

A comparison of the total levels of guanine adducts to adenine adducts suggests that the benzylating agent reacts equally well with both of these bases. These observations demonstrate that the benzylating agent contains chemical properties intermediate between a simple alkylating agent such as methanediazohydroxide and arylalkylating agents such as the epoxides of styrene oxide and benzo[*a*]pyrene (*26*, *42*). According to the rules proposed by Dipple (*42*), the adduct distributions predict that the benzylating intermediate likely reacts with DNA with significant S_N1 character where the positive charge is somewhat delocalized as originally hypothesized (*26*).

DNA benzylation is lower than DNA methylation in all tissues studied. These studies appear to indicate that the tissues activate more NBzMA to a DNA methylating agent than to a DNA benzylating species. However, *in vitro* metabolism studies with F344 rat liver microsomes predict that more of the benzylating species is formed relative to the methylating species in the liver (*21*). Therefore, it is possible that the methylating metabolite is more DNA reactive than the benzylating metabolite. The benzylating agent should have a shorter half-life

than the methylating species and, therefore, is less likely alkylate DNA (*43*−*45*). Consistently, MNU is apparently at least 10-fold more reactive than BzNU with calf thymus DNA under comparable *in vitro* reaction conditions.[2] In addition, *in vivo* DNA benzylation levels may be further reduced by enzyme mediated detoxification of the benzylating agent. Glutathione can partially suppress the mutagenic activity of the benzylation pathway catalyzed by F344 rat liver S9 (*23*).

No detectable levels of benzyl adducts were observed in the esophageal DNA. This is consistent with the low rate of demethylation of NBzMA observed in esophageal microsomes (*17*). However, DNA isolated from cultured esophageal mucosa incubated with [3-^{3}H]NBzMA did release low levels of [^{3}H]BzOH upon strong acid hydrolysis, demonstrating that the esophagus may be capable of activating NBzMA to a benzylating agent to a small extent.[3] BzOH was a minor metabolite of NBzMA under the conditions studied. The esophagus may also have enhanced means of detoxifying the benzylating intermediate. Independent of the reason, the levels of benzylation in the esophagus were lower than those detected in the nontarget tissues, lung and liver. This result differs from published studies indicating that levels of DNA methylation are higher in the esophagus than the lung or liver of F344 rats 6 h following a subcutaneous dose of NBzMA (3.5 mg/kg; *10*). Therefore, these data indicate that DNA benzylation likely plays a minor role, if any, in the carcinogenic activity of NBzMA.

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