Detection of Exocyclic Guanine Adducts in Hydrolysates of Hepatic DNA of Rats Treated with N-Nitrosopyrrolidine and in Calf Thymus DNA Reacted with α-Acetoxy-N-nitrosopyrrolidine

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ABSTRACT

This report describes the isolation and characterization of DNA adducts formed in vitro from α-acetoxy-N-nitrosopyrrolidine and in rats treated with the hepatocarcinogen N-nitrosopyrrolidine. Esterase-catalyzed hydrolysis of α-acetoxy-N-nitrosopyrrolidine in the presence of calf thymus DNA, followed by neutral thermal hydrolysis of the DNA, resulted in formation of three previously unknown Adducts 1-3. They were isolated and characterized by their UV, mass, and proton magnetic resonance spectra as the exocyclic 7,8-guanine adducts 2-amino-7,8,9-tetrahydro-9-hydroxypyrido[2,1-f]purine-4(3H)-one (Adduct 1), and cis- and trans-2-amino-7,8-dihydro-8-hydroxy-6-methyl-3H-pyrrrolo[2,1-f]purine-4(6H)-one (Adducts 2 and 3). Adduct 1 was formed by addition of 4-oxobutyryl diazohydroxide, or a related carbonium ion, to the 7 and 8 positions of guanine. Adducts 2 and 3 resulted from Michael addition of 2-butoxyl diazohydroxide, or a related carbonium ion, to the 7 and 8 positions of guanine. Esterase-catalyzed hydrolysis of α-acetoxy-N-nitrosopyrrolidine in the presence of DNA also produced the exocyclic 1,N2-propanodeoxyguanosine Adducts 4a and 4b which we have previously described. Neutral thermal hydrolysates of hepatic DNA isolated from rats treated with N-nitrosopyrrolidine contained a fluorescent adduct, as previously reported (E. J. Hunt and R. C. Shank, Biochem. Biophys. Res. Commun., 104: 1343, 1982). This fluorescent adduct was shown to be identical to Adduct 1. Adducts 2, 3, 4a, and 4b were not detected in hepatic DNA hydrolysates from these animals. The results of this study provide the first example of a structurally characterized DNA adduct formed in vivo from a cyclic nitrosamine and support the α-hydroxylation hypothesis of cyclic nitrosamine metabolic activation.

INTRODUCTION

In this paper we describe the isolation and structural characterization of DNA adducts formed in vivo from the cyclic nitrosamine NPYR1 and in vitro from α-acetoxy-NPYR (see Fig. 1 for structures). NPYR is important because it occurs in the environment, is carcinogenic in laboratory animals, and is the structural parent of other environmentally prevalent cyclic nitrosamines such as N'-nitrosonornicotine and N-nitrosopropylene. It has been detected in bacon and other cured meats, mainstream and sidestream tobacco smoke, and smokeless tobacco products (1-3). It induces hepatocarcinoma in rats and respiratory tract tumors in mice and hamsters (4).

DNA modification by dialkylnitrosamines such as N-nitrosodimethylamine and N-nitrosodiethylamine is a consequence of metabolic hydroxylation of the carbon adjacent to the nitrosamino nitrogen. This process is called α-hydroxylation and produces an alkyl diazohydroxide intermediate which alkylates nucleophilic sites in DNA with formation of products such as 7-alkylguanine or Oα-alkylguanine (4). In contrast to the dialkylnitrosamines, little is known about the DNA-binding properties of cyclic nitrosamines (5). The diazohydroxides formed upon metabolic α-hydroxylation of cyclic nitrosamines have an aldehyde or ketone group in their structure. The chemistry of these diazohydroxides containing an additional reactive group is different from that of the simple diazohydroxides formed from dialkyl nitrosamines. There are no reports in the literature of the complete structural characterization of DNA adducts formed in vivo from cyclic nitrosamines.

Previous studies on NPYR have demonstrated that it is metabolized by α-hydroxylation in vitro in micromosmal preparations and in vivo in the rat (5-8). The intermediates formed by α-hydroxylation of NPYR can also be produced by esterase-catalyzed hydrolysis of α-acetoxy-NPYR. When this hydrolysis is carried out in the presence of dGuacyclc 1,N2-propanodeoxyguanosine adducts (Fig. 1, Adducts 4a and 4b) are formed, but their presence in the DNA of rats treated with NPYR has not yet been confirmed (9). Hunt and Shank (10), as well as Kruger (11), have reported the chromatographic isolation of putative nucleic acid adducts from rat liver following treatment with NPYR, but their structures were not determined. Therefore, we have further characterized the adducts formed upon reaction with DNA of α-acetoxy-NPYR, and have used these as markers for the identification of NPYR adducts formed in vivo in rat liver.

MATERIALS AND METHODS

Apparatus

HPLC was carried out with a Waters Associates System (Millipore, Waters Division, Milford, MA) equipped with a Model 990 photodiode array detector or a Perkin-Elmer Model 650-10S fluorescence detector (Perkin-Elmer Corp., Norwalk, CT) or a Flo-one/Beta radioactive flow apparatus (Radiomatic Instruments, Tampa, FL). The following solvent elution systems were used.

System 1. A 25-cm x 4.6-mm Partisil-10 SCX strong cation exchange column (Whatman, Clifton, NJ) eluted isocratically with 0.04 M ammonium formate, pH 2.0, at a flow rate of 1 ml/min, with detection by fluorescence (excitation at 290 nm and emission at 380 nm). Retention times were as follows: Adduct 1, 9.0 min; Adduct 2, 7.3 min; Adduct 3, 7.5 min; Adduct 4, 11.1 min.

System 2. Two 25-cm x 4.6-mm Partisil-10 SCX strong cation exchange columns in series eluted isocratically with 0.04 M ammonium phosphate, pH 2.0, at a flow rate of 1.5 ml/min with detection by fluorescence as in System 1. Retention times were as follows: Adduct 1, 12.5 min; Adduct 2, 9.9 min; Adduct 3, 10.4 min; Adduct 4, 14.6 min.

System 3. Two 30-cm x 3.9-mm C18 μBondapak reverse-phase columns (Waters) in series eluted isocratically with 15% methanol in H2O for 5 min, and then with a gradient from 15 to 25% methanol in H2O in 30 min at 1 ml/min, using Curve 6. Detection was by UV absorbance at 254 and 285 nm. Retention times were as follows: Adduct 1, 23.0 min; Adduct 2, 22.5 min; Adduct 3, 28.4 min; Adduct 4, 35.0 min; Adduct 4a, 45.1 min; Adduct 4b, 48.2 min.

System 4. Two 30-cm x 3.9-mm C18 μBondapak reverse-phase col-
Fig. 1. Metabolite and DNA adduct formation from NPYR and \( \alpha \)-acetoxyn-NPYR. Adduct 1 has been detected in vivo in hepatic DNA of NPYR-treated rats (see text). Adducts 1, 2, 3, 4a, 4b, and 4 have been detected in DNA reacted with \( \alpha \)-acetoxyn-NPYR. Adducts 1–3 were isolated as racemic mixtures. Adduct 4, formed by hydrolysis of a mixture of 4a and 4b, is a mixture of enantiomers. Only one enantiomer is shown. Adduct 1 is released from DNA at 37°C or 100°C, the release of Adducts 2 and 3 from DNA at 37°C has not been investigated.

Columns in series eluted with Solvent A for 6 min, then a gradient of 0–100% Solvent B in Solvent A in 60 min, at 1.5 ml/min. Solvent A was 0.1 M phosphate buffer, pH 5.7, and Solvent B was 20% methanol in H2O. Retention times were as follows: Adduct 4, 42.0 min; Adduct 4a, 60.0 min; Adduct 4b, 64.4 min.

Fluorescence spectra were determined on the Perkin-Elmer Model 650-10S instrument. UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer coupled to an IBM XT personal computer. MS were run on a Hewlett Packard Model 5988A instrument, in the electron impact mode, with NH4Cl added to the samples. NMR spectra were recorded on a Bruker Instruments Superconducting Fourier NMR Spectrometer AM 360 WB.

Chemicals

NPYR and crotonaldehyde (2-butenal) were obtained from Aldrich Chemical Co., Milwaukee, WI. Their purities and structures were confirmed by gas chromatography and NMR. \( \alpha \)-Acetoxyn-NPYR was synthesized (12). [3,4-\( ^3 \)H]NPYR (0.94 Ci/mmol; purity, 98%) was obtained from Chemsyn Science Laboratories, Lenexa, KS. Adducts 4, 4a, and 4b were prepared as described (9). DNA, dGua, polyGua, and enzymes were obtained from Sigma Chemical Co., St. Louis, MO.

Reactions of \( \alpha \)-Acetoxyn-NPYR with DNA

Analytical Scale. \( \alpha \)-Acetoxyn-NPYR (148 mg, 0.94 mmol) and porcine liver esterase (100 units) were incubated with calf thymus DNA (52 mg) at 37°C in 10 ml of 0.1 M phosphate buffer, pH 7.0. After 24 h, the incubation mixture was extracted with two 20-ml portions of CHCl3. The DNA was precipitated by addition of 0.1 volume of saturated sodium acetate solution and 2 volumes of cold ethanol, followed by cooling in an ice bath for 30 min. The DNA was dissolved in 10 ml of H2O and reprecipitated by 2 volumes of cold ethanol. The isolated DNA was dissolved in 10 mM sodium cacodylate buffer, pH 7.0 (200 \( \mu \)l/mg DNA), and heated at 100°C for 60 min to release Adducts 1–3. The solution was cooled in an ice bath and 1 N HCl (20 \( \mu \)l/mg DNA) was added to precipitate the DNA. The DNA was collected by centrifugation at 20,000 \( \times \) g for 10 min. The supernatant was analyzed for Adducts 1–3 by HPLC Systems 1 or 2. The collected DNA was hydrolyzed in 0.1 N HCl (200 \( \mu \)l/mg DNA) at 75°C for 45 min. The hydrolysate was analyzed for Adduct 4 b by using System 1. Control incubations were carried out without \( \alpha \)-acetoxyn-NPYR.

Preparative Scale. Calf thymus DNA (1 g) was dissolved in 100 ml of 0.1 M phosphate buffer, pH 7.0, heated at 100°C for 25 min, and then immediately cooled in an ice bath. The temperature of the resulting solution was adjusted to 37°C. \( \alpha \)-Acetoxyn-NPYR (500 mg, 3.2 mmol) and 600 units of esterase were added. The mixture was incubated at 37°C for 7 h, and was then added to 50 ml of H2O. The diluted solution was extracted with 100 ml of CHCl3 by shaking for 15 min and then centrifuged at 10,000 \( \times \) g for 15 min. The aqueous layer was removed, mixed with 150 ml of cold ethanol, and allowed to stand for 30 min in an ice bath. It was then centrifuged at 16,000 \( \times \) g for 20 min to pellet the DNA. The DNA was isolated and dissolved in 100 ml of 10 mM sodium cacodylate, pH 7.0, and the solution was heated at 100°C for 60 min. It was then cooled in an ice bath, and 10 ml of 1 N HCl were added to precipitate the DNA. The supernatant was removed by centrifugation, neutralized with 1 N NaOH, and concentrated to 10 ml by rotary evaporation. Aliquots of the concentrated hydrolysate were injected on HPLC System 3 and the appropriate peaks were collected, combined, concentrated, and then repurified by using System 3. An aliquot of the final purified adduct was analyzed by HPLC System 1.

Reaction of \( \alpha \)-Acetoxyn-NPYR with polyGua

A mixture of \( \alpha \)-acetoxyn-NPYR (70 mg, 0.44 mmol), polyGua (25 mg), and esterase (80 units) in 8 ml of phosphate buffer, pH 7.0, was incubated at 37°C for 24 h. The mixture was extracted twice with 100 ml of CHCl3. The aqueous layer was mixed with 2 volumes of cold ethanol and allowed to cool in an ice bath for 30 min. It was then centrifuged at 8000 \( \times \) g for 15 min to collect the polyGua. It was
dissolved in 2 ml of 10 mm sodium cacodylate buffer, pH 7.0, and the solution was heated at 100°C for 60 min. The resulting hydrolysate was concentrated to dryness and the residue was extracted with three 10-ml portions of methanol:ethanol(1:1). The extracts were combined, concentrated to dryness, dissolved in 0.5 ml of H2O, and analyzed for Adducts 1–3 by HPLC Systems 1 and 3.

Reaction of Crotonaldehyde with dGua

A mixture of crotonaldehyde (70 mg, 1 mmol) and dGua (53 mg, 0.2 mmol) in 20 ml of phosphate buffer, pH 7.0, was incubated at 37°C for 48 h. The solution was extracted twice with 20 ml of CHCl3 and concentrated to dryness by rotary evaporation. The residue was extracted with three 10-ml portions of methanol:ethanol(1:1), the extracts were combined, evaporated to dryness, and dissolved in 2 ml of 10 mm sodium cacodylate, pH 7.0. The mixture was heated at 100°C for 60 min and analyzed for Adducts 1–3 by HPLC Systems 1 and 3. Adducts 2 and 3 were prepared for spectral characterization by repeated injections in System 3.

DNA adducts of NPYR in Vivo

In Rats Treated with Unlabeled NPYR. Groups of 2 male F344 rats (230 g) were treated by gavage with 175 or 350 mg/kg of body weight of NPYR in 0.5 ml of saline. Control rats received saline only. After 16 h, the rats were sacrificed by decapitation. The hepatic DNA was isolated by the modified Marmur method as previously described (13, 14), except that the isolated DNA was incubated with an additional portion of RNase A and reisolated. This procedure was repeated to ensure that there was no significant RNA contamination. The purity of the DNA was established by the absorbance 260–280 ratio (1.8) and by assay for RNA which showed absence of uridine (14). DNA was then hydrolyzed under neutral thermal conditions as described above. The hydrolysates were analyzed and quantified by HPLC Systems 1 and 2. Adduct 1 was further purified for spectral analysis by HPLC Systems 1 and 3.

A portion of this DNA was treated with NaBH4, as follows. NaBH4 (0.14 g) was dissolved in 0.5 ml of H2O and 2.5 µl of this solution was added to a solution of 3.65 mg DNA in 1.0 ml of 0.1 M phosphate buffer, pH 7.0. The mixture was incubated at room temperature for 30 min. The DNA was isolated, hydrolyzed at 100°C for 60 min, and analyzed by HPLC System 2. The level of Adduct 1 was compared to that in DNA that had not been treated with NaBH4.

In Rats Treated with [3,4-3H]NPYR. Each of 2 male F344 rats (285 g) was given [3,4-3H]NPYR (3.1 mg/kg of body weight; 8 mCi) in 0.5 ml saline by gavage. The rats were sacrificed by decapitation 16 h later and DNA was isolated from liver. It was subjected to neutral thermal hydrolysis as described above and analyzed by HPLC System 1 with detection by a radioactive flow detector. Two male F344 rats (250 g) each received [3,4-3H]NPYR (6.7 mg/kg of body weight; 15 mCi) in 0.5 ml saline by gavage. The animals were sacrificed by decapitation and the liver DNA was isolated. It was enzymatically hydrolyzed as follows (15). A mixture of 1 mg DNA and 100 µg DNase was incubated for 10 min at 37°C in 1 ml of 5 mm Tris-HCl-5 mm MgCl2, pH 7.0. Then, 100 µg phosphodiesterase (0.13 unit/mg) and 40 units of alkaline phosphatase were added and the mixture was incubated at 37°C for 60 min. The mixture was then cooled to 0°C in an ice bath, and filtered through a Centri-free filter (Amicon Division, W. R. Grace and Co., Danvers, MA). The filtrate was concentrated and analyzed for Adducts 4a and 4b by HPLC System 4 with detection by a radioactive flow detector. Fractions corresponding in retention time to standard Adducts 4a and 4b were collected, concentrated to dryness, hydrolyzed in 0.1 N HCl at 90°C for 45 min, and analyzed for Adduct 4 by HPLC System 4.

RESULTS

We carried out the esterase-catalyzed hydrolysis of α-acetoxy-NPYR in the presence of calf thymus DNA. After the hydrolysis was complete, the DNA was isolated and heated at 100°C, pH 7. The supernatant was analyzed by HPLC with a strong cation exchange column and fluorescence detection (System 1). This gave the chromatogram illustrated in Fig. 2A. Peak 1 appeared to have the same retention time as an adduct observed by Hunt and Shank in liver DNA of rats treated with NPYR (10). To confirm this, we treated rats with NPYR, isolated hepatic DNA, and subjected it to neutral thermal hydrolysis and HPLC analysis. As illustrated in Fig. 2B, the peaks formed in vitro from α-acetoxy-NPYR and in vivo from NPYR eluted at the same retention times. This was confirmed by coinjection of the in vitro and in vivo peaks. They also coeluted under reverse-phase HPLC conditions (System 3). Their fluorescence spectra, illustrated in Fig. 3 were identical, as were their UV spectra (not shown). These data established that the DNA adduct formed in vitro from α-acetoxy-NPYR was identical to that formed in vivo from NPYR. The estimated concentrations of the adduct formed in vivo were 320 and 390 pmol/mg DNA following NPYR doses of 175 and 350 mg/kg of body weight, respectively.

When the neutral thermal hydrolysates of the DNA that had been treated with α-acetoxy-NPYR were analyzed by HPLC System 2, three peaks were observed which were not present in control DNA (Fig. 4). A major peak, retention time 12.5 min, was identical to Peak 1 of Fig. 2. Two minor peaks, Peaks 2 and 3, eluted at 9.9 and 10.4 min; these two peaks coeluted with adenine under the conditions used for Fig. 2. In order to obtain more information about Peaks 1–3, we reacted crotonaldehyde (2-butenal, Compound 5 of Fig. 1) with dGua, since in previous studies we had found that crotonaldehyde and α-acetoxy-NPYR formed some common DNA adducts such as Adducts 4a and 4b of Fig. 1. Since crotonaldehyde is more readily available than α-acetoxy-NPYR, this appeared to provide a relatively simple way of obtaining some of the adducts in amounts sufficient for further structural characterization. Analysis of the neutral thermal hydrolysates of the dGua that had been reacted with crotonaldehyde gave Peaks 2 and 3 as well as Adducts 4a and 4b (see Fig. 5). Peak 1 was not detected.

We then investigated optimum reaction conditions for obtaining Peaks 1–3 in quantities sufficient for spectral chara-
Fig. 3. Fluorescence spectra of Peak 1 of Fig. 2, A and B. A and B, excitation (emission at 380 nm) and emission (excitation at 290 nm) spectra of Peak 1 formed by reaction of o-acetoxy-NPYR and DNA in the presence of esterase. C and D, excitation and emission spectra of Peak 1 isolated from hepatic DNA of NPYR-treated rats.

Fig. 4. Chromatogram obtained upon HPLC analysis of neutral thermal hydrolysates of calf thymus DNA that had been allowed to react with crotonaldehyde. HPLC analysis was carried out with System 2, as described in "Materials and Methods." Peaks 1–3 were identified as Adducts 1–3 of Fig. 1 (see text). Gua, guanine; Ade, adenine.

Fig. 5. Chromatogram obtained upon HPLC analysis of neutral thermal hydrolysates of dGua that had been reacted with crotonaldehyde. HPLC analysis was carried out with System 3. Peaks 2 and 3 were identified as Adducts 2 and 3. The structures of Adducts 2, 3, 4a, and 4b are shown in Fig. 1 (the absolute configurations of Adducts 4a and 4b have not been assigned). Gua, guanine; dG, dGua.

were obtained in the reactions with single-stranded DNA, although Peak 1 was detected in the neutral thermal hydrolysates of all these reactions, indicating that it was a product of reaction with dGua in DNA. Peaks 2 and 3 were most readily obtained by reacting crotonaldehyde with dGua, followed by neutral thermal hydrolysis.

The UV spectra of Peaks 1–3 under neutral, acidic, and basic conditions are illustrated in Fig. 6, A–C. These spectra were similar to that of 7-methylguanine (Fig. 6D) and other 7-alkylguanines (16). The MS of Peaks 1–3 are illustrated in Fig. 7. All had molecular ions at m/e 221, corresponding to the addition of a 4-oxobutyl residue to guanine.

The proton NMR spectra of Peaks 1–3 are presented in Figs. 8–10 and Table 1, together with their structures and the assignments of each proton. In the spectrum of Peak 1 (Fig. 8), the peaks at 10.62, 6.10, and 5.68 ppm disappeared upon addition of D2O, confirming their assignments as exchangeable protons. The multiplet at 4.64 ppm, assigned to H9, became a doublet of doublets, as expected. The peak at 6.5 ppm, presumed to be due to an impurity, also disappeared upon D2O addition. A homonuclear 2-dimensional correlated spectrum of Peak 1 confirmed the assignments of the protons in the new ring. The 2-dimensional spectrum showed the coupling of H6a(4.03 ppm) with H7a (2.1 ppm), and of H9(4.64 ppm) with H8a,b (1.8–2.0 ppm) and OH (5.68 ppm).

In the spectrum of Peak 2 (Fig. 9), decoupling experiments were used to confirm the assignments. Irradiation of the peak at 4.34 ppm, assigned to H6, caused the methyl doublet at 1.57 ppm to collapse to a singlet, and the multiplets at 1.87 ppm and 3.00 ppm to simplify. Irradiation at 1.87 ppm (H7a) caused the multiplets at 4.34 ppm (H6) and 3.00 ppm (H7b) to simplify, and the doublet of doublets at 4.92 ppm to collapse to a doublet. Irradiation at 4.92 ppm (H8) converted the peaks at 1.87 ppm to...
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Fig. 6. UV spectra at pH 1, 7, and 12 of A (Peak 1), B (Peak 2), C (Peak 3) of Fig. 4, and D (7-methylguanine). Peaks 1–3 were identified as Adducts 1–3 of Fig. 1.

(H2a) and 3.0 ppm (H3b) to doublets of doublets.

In the spectrum of Peak 3 (Fig. 10), irradiation at 4.56 ppm (H4) caused the methyl doublet at 1.50 ppm to collapse to a singlet, and the multiplet at 2.4–2.5 ppm to simplify. Irradiation at 5.02 ppm (H5) converted the OH doublet at 5.77 ppm to a singlet, and simplified the 2.4–2.5 ppm multiplet. Irradiation at 5.77 ppm (OH) converted the 5.02 ppm multiplet to a doublet of doubles. These experiments confirmed the assignments of the protons as summarized in Fig. 10 and Table 1.

The chemical shift data summarized in Table 1 are consistent with the assigned structures of Peaks 1–3 as Adducts 1–3.

All spectral data support the structures of Adduct 1 as (±)-2-amino-6, 7, 8, 9-tetrahydro-9-hydroxypyrrolo[2,1-f]purine-4(3H)-one and Adducts 2 and 3 as (±)-cis- and trans-2-amino-7,8-dihydro-8-hydroxy-6-methyl-3H-pyrrolo[2,1-f]purine-4(6H)-one. The UV spectra indicate that all three adducts are substituted at position 7 of guanine. The NMR spectra are those of substituted guanines lacking a C8 proton. No aldehyde protons were observed in the NMR spectra. Since, according to the MS, only one 4-oxobutyl residue has been added in each adduct, these spectral data require the formation of a new ring between positions 7 and 8 of guanine. We considered the possibility that the orientation of the new exocyclic ring in Adducts 1–3 could be in the opposite sense to that shown in Table 1; e.g., the hydroxy group could be attached to the carbon bound to position 7. Chemical shift data are inconsistent with such structures.

The carbinol proton of Adducts 4a and 4b, which is bound to a carbon bearing a hydroxyl group and to N1 of guanine, resonates at 6.2 ppm, which is about 1–1.5 ppm further downfield than the carbinol protons of Adducts 1–3. This supports the orientation of the ring shown in Table 1. In addition, treatment of these adducts with base, under conditions which result in ring opening of Adducts 4a and 4b, caused no change, indicating that the carbinol carbon is not attached to a ring nitrogen.

NOE in the NMR spectrum of Adduct 3 supports its assignment as the trans-isomer. Using NOE difference spectroscopy (17), a strong NOE was observed between the methyl group and H6 of Adduct 3, as expected for the configuration shown.

Fig. 7. MS of A (Adduct 1), B (Adduct 2), and C (Adduct 3). Adduct 1 was isolated from the reaction of α-acetoxy-NPYR with single-stranded DNA in the presence of esterase, and Adducts 2 and 3 were isolated from the reaction of crotonaldehyde with dGua.

Fig. 8. Proton NMR spectrum of Peak 1 of Fig. 4, identified as Adduct 1. DMSO, dimethyl sulfoxide.
Adduct 3 (4.56 ppm) compared to H6 of Adduct 2 (4.34 ppm) in DMSO, dimethyl sulfoxide.

In addition the downfield shifts of the methyl group of Adduct 2 (1.57 ppm) compared to Adduct 3 (1.50 ppm) and of H6 of Adduct 3 (4.56 ppm) compared to H6 of Adduct 2 (4.34 ppm) appear to be consistent with the assigned configurations. These downfield shifts may result from transannular deshielding by the hydroxyl group attached to carbon 8.

Adducts 1–3 were released upon neutral thermal hydrolysis of DNA which had been reacted with α-acetoxy-NPYR. Following neutral thermal hydrolysis, the DNA was recovered and hydrolyzed with acid. This gave Adduct 4 of Fig. 1, identified by cochromatography with a standard and by treatment with NaBH4 and base, leading to reductive ring opening (9). The estimated amounts of Adducts 1–4 formed in the reactions of DNA with a-acetoxy-NPYR with DNA were (nmol/mg DNA): Adduct 1, 3.1; Adduct 2, 0.024; Adduct 3, 0.06; Adduct 4, 0.59.

To obtain further information on the formation of these adducts in vivo, rats were treated with [3,4-3H]NPYR. Hepatic DNA was isolated and subjected to neutral thermal hydrolysis. Analysis by HPLC gave a chromatogram in which a radioactive peak (approximately 13,000 dpm) coeluted with Adduct 1, in agreement with the results described above. It corresponded to 0.7 pmol/mg DNA. Adducts 2 and 3 were not detected in this sample. In a second experiment, rats were treated with [3,4-3H]NPYR and hepatic DNA was isolated, hydrolyzed enzymatically, and analyzed by HPLC for Adducts 4a and 4b of Fig. 1. A peak coeluted with one of the Adducts 4a and 4b markers, but acid hydrolysis of this material did not yield Adduct 4. The results of these experiments demonstrate that the levels of Adduct 1 formed in hepatic DNA of rats treated with [3,4-3H]-NPYR are greater than those of Adducts 2–4, which were not detected (detection limit, approximately 0.1 pmol/mg DNA).

Since Adduct 1 was quantitatively the most important of the four adducts detected in vivo, we carried out further experiments to investigate its structure and stability in DNA. We wanted to determine whether Adduct 1 was present in its cyclic form in DNA, or whether it may have had an open chain free aldehyde structure in DNA (see Compound 6 of Fig. 11) and cyclized during the 100°C hydrolysis procedure. Solutions of hepatic DNA from rats treated with NPYR were incubated with NaBH4 at pH 7.0, conditions which are known to reduce aldehyde groups in adducts related structurally to Compound 6 (18). Neutral thermal hydrolysis of this DNA gave Adduct 1 in the same quantity as obtained without NaBH4 treatment, indicating that the cyclic form of Adduct 1 is present in DNA.

The stability of Adduct 1 at 37°C was investigated by incubation of DNA that had been treated with α-acetoxy-NPYR. The release of Adduct 1 followed first-order kinetics, $t_1/2 = 13.6$ h.

**DISCUSSION**

Adduct 1 is the first example of a structurally characterized DNA adduct formed in vivo from a cyclic nitrosamine. Previous studies have shown that NPYR forms nucleic acid adducts in vivo but their structures were not determined (10, 11). Limited data are available on the DNA adducts formed in vivo from other cyclic nitrosamines. N'-Nitrosonornicotine apparently causes 4-(3-pyridyl)-4-oxobutylation of rat liver DNA but the structure of the adduct is unknown (14). Tentative evidence for 7-(2-hydroxyethyl)guanine in the liver DNA of rats treated with N-nitrosomorpholine has been presented, and 1,6-hexanediol has been identified in hydrolysates of liver RNA isolated from rats treated with N-nitrososoxamethyleneimine (19, 20). The results of this study with NPYR, a prototypical cyclic nitrosamine, should therefore provide insights on the mechanisms of DNA adduct formation by other cyclic nitrosamines, and ultimately on their mechanisms of carcinogenesis.

Structure-activity studies, metabolism experiments, and mutagenicity data have all indicated that α-hydroxylation is a major pathway of metabolic activation of NPYR (5, 21, 22). The formation of Adduct 1 from NPYR in vivo supports this hypothesis. The structure of this adduct is consistent with the generation of unstable intermediates 1 and 2 during the metabolism of NPYR. The reaction of position 7 of guanine with dialdehyde 2, or a related carbonium ion, agrees with the results obtained with other alkyl diazohydroxides. The ring closure to form the cyclic adduct is consistent with the electrophilicity of the aldehyde group and the nucleophilicity of C8 of a 7-substituted dGua. Fig. 11 presents a mechanism for formation of Adduct 1 in DNA. The present results thus demonstrate that α-hydroxylation of NPYR leads to DNA modification in vivo, although the significance of Adduct 1 in carcinogenesis is unknown at present.
Assess the formation of Adducts 4a and 4b in DNA of NPYR-treated animals.

The formation of exocyclic DNA adducts has been reported in numerous in vitro studies in which bifunctional electrophiles were reacted with DNA (27). However, evidence for the presence of such adducts in vivo in mammalian systems is limited. Vinyl chloride-DNA interactions have been studied fairly extensively in vivo. Although the presence of the cyclic adducts 1, N⁵-ethenodeoxycytidine in hepatic DNA of vinyl chloride-treated animals has been reported (28), this observation was not confirmed in a subsequent study, possibly due to differences in protocols (29). In a preliminary report, the presence of a cyclic N²,3-guanine adduct in hepatic DNA of acetylaminostilbene-treated rats has been indicated (30). Adduct 1 in hepatic DNA of NPYR-treated rats would thus appear to be one of the few examples of an exocyclic DNA adduct formed in vivo in a mammalian system.

Adduct 1 accounted for only part of the radioactivity present in hepatic DNA of rats treated with [3,4-³H]NPYR. The characterization of other NPYR-DNA adducts is in progress. A complete profile of the NPYR-DNA adducts formed in vivo will allow a more rational assessment of the potential biological significance of Adduct 1 versus other NPYR-DNA adducts.

However, the release of Adduct 1 from DNA in vivo would result in the generation of apurinic sites which are postulated to induce miscoding (31). This may play a role in NPYR carcinogenesis.

Note Added in Proof
Adducts 4a, b have recently been detected in hepatic DNA of NPYR-treated rats, approximately 0.043 pmol/mg DNA.

REFERENCES


Detection of Exocyclic Guanine Adducts in Hydrolysates of Hepatic DNA of Rats Treated with N-Nitrosopyrrolidine and in Calf Thymus DNA Reacted with α-Acetoxy-N-nitrosopyrrolidine

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