

# Nitroreduction of 4-Nitropyrene Is Primarily Responsible for DNA Adduct Formation in the Mammary Gland of Female CD Rats

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Received October 16, 1998

We determined whether DNA adducts derived from 4-nitropyrene (4-NP) are formed via nitroreduction or ring oxidation. DNA adduct markers derived from both pathways were prepared and, consequently, were compared with those obtained *in vivo* in rats treated with 4-NP. Following *in vitro* reaction of 9,10-epoxy-9,10-dihydro-4-nitropyrene (4-NP-9,10-epoxide), an intermediate metabolite derived from ring oxidation of 4-NP, with calf thymus DNA (average level of binding in two determinations was 8.5 nmol/mg of DNA), DNA was enzymatically hydrolyzed to deoxyribonucleosides and the DNA hydrolysates were analyzed by HPLC. Electrospray mass and <sup>1</sup>H NMR spectra of the major products indicated that these adducts are deoxyguanosine (dG) derivatives that resulted from *N*<sup>2</sup>-dG substitution at the 9- or 10-position of the pyrene nucleus. However, these adducts were not detected *in vivo* in the rat mammary gland and liver following the administration of 4-NP. Nitroreduction of 4-NP catalyzed by xanthine oxidase in the presence of DNA resulted in three major putative DNA adducts (level of binding of 12.0 ± 1.1 nmol/mg of DNA, *n* = 4) designated as peak 1 (46%), peak 2 (25%), and peak 3 (17%). Although peak 1 was further resolved into peaks 1a and 1b, both were unstable and gradually decomposed to peak 2, and the latter was unequivocally identified as pyrene-4,5-dione. On the basis of electrospray mass spectral analysis, peak 3 was tentatively identified as a deoxyinosine-derived 4-aminopyrene adduct. None of the adducts derived from nitroreduction of 4-NP catalyzed by xanthine oxidase coeluted with the synthetic standard *N*-(deoxyguanosin-8-yl)-4-aminopyrene prepared by reacting dG with *N*-acetoxy-4-aminopyrene. Nevertheless, HPLC analysis of the hydrolysates of liver and mammary DNA obtained from rats treated with [<sup>3</sup>H]-4-NP yielded four radioactive peaks, all of which coeluted with the markers derived from the nitroreduction pathway. These results indicate that nitroreduction is primarily responsible for DNA adduct formation in the liver and, especially, in the mammary gland which is the organ susceptible to carcinogenesis by this environmental agent.

## Introduction

Nitropolycyclic aromatic hydrocarbons (NO<sub>2</sub>-PAH)<sup>1</sup> are formed in combustion processes and are, therefore, widely distributed in the environment (1, 2). Several NO<sub>2</sub>-PAH are potent mutagens in bacterial and mammalian systems and are carcinogens in rodents (3, 4). However, the extent of their contribution to the pathogenesis of some human cancers has not been clearly defined (5, 6). One

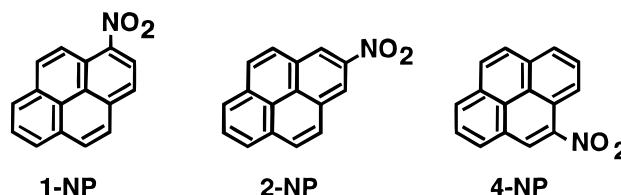


Figure 1. Structures of 1-, 2-, and 4-NP.

of the mononitropyrene (mono-NP) isomers (Figure 1), 1-nitropyrene (1-NP), is the most abundant NO<sub>2</sub>-PAH found in various environmental matrices, including certain food items (7–10). The presence of 1-NP in lung specimens from Japanese and Chinese lung cancer patients has been reported as a consequence of inhaling soot from the combustion of coal and heavy oils used for cooking and indoor heating (11). All three mono-NP isomers are tumorigenic in mouse lung and liver (12, 13). 1-NP induces tumors in the lungs and trachea of hamsters (14). 1-NP and 4-NP have been shown to be tumorigenic in rat mammary glands (15–18); 4-NP was

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<sup>1</sup> Abbreviations: NO<sub>2</sub>-PAH, nitropolycyclic aromatic hydrocarbons; mono-NP, mononitropyrenes; 1-NP, 1-nitropyrene; 2-NP, 2-nitropyrene; 4-NP, 4-nitropyrene; 4-NP-9,10-epoxide, 9,10-epoxy-9,10-dihydro-4-nitropyrene; *N*-acetoxy-4-AP, *N*-acetoxy-4-aminopyrene; dG-C8-4-AP, *N*-(deoxyguanosin-8-yl)-4-aminopyrene; dG, deoxyguanosine; dI, deoxyinosine; poly(dA)-poly(dT), polydeoxyadenylic-polythymidylic acid; poly-(dG)-poly(dC), polydeoxyguanylic-polydeoxycytidylic acid; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethyl sulfoxide.

the strongest tumorigen of the three isomers.

As is the case with other NO<sub>2</sub>-PAH, metabolic activation of mono-NP may involve a nitroreduction and/or a ring oxidation pathway (19–21). The major DNA adduct derived from nitroreduction of 1-NP catalyzed by xanthine oxidase in vitro was identified as *N*-(deoxyguanosin-8-yl)-1-aminopyrene (22). This adduct was detected in *Salmonella typhimurium* as well as in rats and mice treated with 1-NP (22–26). In addition, Herreno-Saenz et al. (27) identified in vitro and in vivo in the rat mammary gland two minor adducts derived from nitroreduction of 1-NP, 6-(deoxyguanosin-*N*<sup>2</sup>-yl)-1-aminopyrene and 8-(deoxyguanosin-*N*<sup>2</sup>-yl)-1-aminopyrene, where the 6- or 8-position of 1-aminopyrene was the site of substitution by the *N*<sup>2</sup>-deoxyguanosine (dG). Although nitroreduction is considered the principal activation pathway of 1-NP, leading to DNA adducts, other reports also suggest the formation of putative DNA adducts derived from ring-oxidized metabolites and/or resulting from a combination of ring oxidation and nitroreduction (24, 28, 29).

In the presence of xanthine oxidase which catalyzes the nitroreduction of 2-NP, both dG and deoxyadenosine adducts [*N*-(deoxyguanosin-8-yl)-2-aminopyrene and *N*-(deoxyadenosin-8-yl)-2-aminopyrene] were formed following its incubation with calf thymus DNA; however, unequivocal identification of these adducts in vivo was not possible, due to low levels of binding (30, 31). On the contrary, nothing is known about the nature of DNA adducts derived from 4-NP despite its remarkable carcinogenic activity when compared to the other two mono-NP isomers. Ring oxidation of 4-NP, catalyzed by rat liver microsomes or 9000*g* rat liver supernatant, resulted in the formation of primarily two metabolites (32). One of these was identified as 4-nitropyrene-9,10-dione; however, in the presence of 3,3,3-trichloropropylene 1,2-oxide, an inhibitor of epoxide hydrolase, 9,10-epoxy-9,10-dihydro-4-nitropyrene (4-NP-9,10-epoxide) was observed. Following the oral administration of 4-NP to female CD rats, we identified metabolites derived from both nitroreduction and ring oxidation (32). In a previous investigation, based on total radioactivity, we found that the degree of binding of 4-NP to rat mammary DNA was at least 3-fold higher than that of 1-NP and 2-NP; we proposed that this may account for the higher tumorigenic activity of 4-NP (33). However, we were not able to delineate whether these DNA adducts are derived from nitroreduction and/or ring oxidation pathways. Thus, in the present study, DNA adduct markers derived from nitroreduction of 4-NP, catalyzed by xanthine oxidase, and from nitroreduction followed by acetylation of 4-NP [*N*-acetoxy-4-aminopyrene (*N*-acetoxy-4-AP)] (34) as well as those derived from an electrophilic intermediate metabolite that resulted from the ring oxidation of 4-NP (4-NP-9,10-epoxide) were prepared. On the basis of cochromatography with DNA adduct markers obtained in vitro, our results indicate that nitroreduction of 4-NP is primarily responsible for DNA adduct formation in the liver and, especially, in the mammary gland, which is the organ susceptible to carcinogenesis by this environmental agent.

## Experimental Procedures

**Caution:** 4-NP is carcinogenic in rodents and should be handled with care.

**Chemicals and Enzymes.** [<sup>3</sup>H]-4-NP (specific activity of 2.1 Ci/mmol) was purchased from Chemsyn Science Laboratories, Inc. (Lenexa, KS), and purified by HPLC prior to use (>98% radiochemically pure). The following enzymes and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): protease K (EC 3.4.21.64; type XI from *Tritirachium album*), RNase A (EC 3.1.27.5; type III-A from bovine pancreas), RNase T1 (EC 3.1.27.3; grade V from *Aspergillus oryzae*), DNase I (EC 3.1.21.1; DN-EP from bovine pancreas), nuclease P1 (EC 3.1.30.1; from *Penicillium citrinum*), phosphodiesterase I (EC 3.1.4.1; type II from *Crotalus adamanteus* venom), alkaline phosphatase (EC 3.1.3.1; type III from *Escherichia coli*), xanthine oxidase (EC 1.1.3.21; grade I from buttermilk), catalase (EC 1.11.1.6; from bovine liver), hypoxanthine, polydeoxyadenylic-polythymidylic acid [poly(dA)·poly(dT)], polydeoxyguanylic-polydeoxycytidylic acid [poly(dG)·poly(dC)], and DNA (type I from calf thymus).

**Syntheses.** 4-NP, 4-NP-9,10-epoxide, and [<sup>3</sup>H]-4-NP-9,10-epoxide were synthesized as described previously (32). The adduct standard *N*-(deoxyguanosin-8-yl)-4-aminopyrene (dG-C8-4-AP) was prepared by reacting dG with *N*-acetoxy-4-AP as reported earlier (34).

**Instrumentation.** HPLC was performed with a Waters Associates (Milford, MA) high-performance liquid chromatograph equipped with a model 510 solvent delivery system, a model U6K septumless injector, a model 440 UV-VIS detector operated at 254 nm, and a model 680 Waters automated gradient controller. HPLC analyses of DNA hydrolysates were carried out with the following systems: system 1, a linear gradient from 20 to 80% methanol in water over the course of 45 min, followed by a linear gradient from 80% methanol in water to 100% methanol over the course of 10 min at a flow rate of 1.5 mL/min using a 10 μm Vydac C<sub>18</sub> reverse phase analytical column (0.46 cm × 25 cm) (Separations Group, Hesperia, CA); and system 2, an isocratic elution at 45% methanol in water for 25 min followed by a linear gradient from 45 to 65% methanol in water over the course of 10 min, which was then held at 65% methanol in water for 30 min at a flow rate of 1 mL/min using a 5 μm Ultrasphere Altex C<sub>18</sub> analytical column (0.46 cm × 25 cm) (Beckman Instruments, St. Louis, MO). Radioactivity was monitored every 6 s with a Flo-one β-flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL). In the case of HPLC analyses of the DNA adducts formed in vivo, a Retriever III fraction collector (ISCO, Inc., Lincoln, NE) was used to collect 1 mL fractions for the radiochromatography. Radioactivity was counted on a Tri-Carb 1900 CA liquid scintillation analyzer (Packard Instruments Co., Meriden, CT).

Mass spectra were obtained on a Hewlett-Packard model HP5988A dual-source mass spectrometer (Hewlett-Packard Co., Palo Alto, CA). LC/MS analyses of the DNA adducts using electrospray ionization with positive and negative ion detection were performed on a Finnigan TSQ-700 instrument (Finnigan Corp., San Jose, CA). The electrospray ionization spray voltage was 7.0 kV, the tube lens voltage 45.8 V, and the voltage of the heated (275 °C) capillary 20 V. The HPLC apparatus used was a Waters Associates System model 600-MS with a 484-MS tunable absorbance detector operated at 254 nm. DNA hydrolysates were separated using the conditions described above. <sup>1</sup>H NMR measurements were carried out at 360 MHz on a Bruker AM-360 WB spectrometer (USA Bruker Instruments, Inc., Billerica, MA) in dimethyl sulfoxide (DMSO-*d*<sub>6</sub>) or DMSO-*d*<sub>6</sub>/D<sub>2</sub>O. UV spectra were prepared with a Beckman model 25 spectrophotometer (Beckman Instruments).

**Modification of Calf Thymus DNA with 4-NP-9,10-epoxide.** [<sup>3</sup>H]-4-NP-9,10-epoxide mixed with the corresponding unlabeled compound was incubated with calf thymus DNA as described in a previous publication (29). Calf thymus DNA dissolved in 50 mM citrate buffer (pH 5.8) at a concentration of 2 mg/mL was purged with N<sub>2</sub> for 10 min. An aliquot of [<sup>3</sup>H]-4-NP-9,10-epoxide (specific activity 0.34 mCi/mmol) in DMSO was added to a DNA solution to give a final concentration of

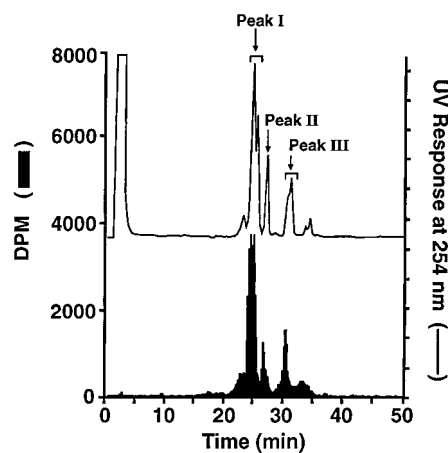
0.2 mM. After incubation for 18 h at 37 °C, followed by three extractions with a mixture of chloroform/isoamyl alcohol (24:1), DNA was precipitated by adding 1 volume of ice-cold absolute ethanol and 1/10 volume of 5 M NaCl. DNA was washed with 70% ethanol, followed by absolute ethanol, dried, and dissolved in 10 mM Tris-HCl buffer (pH 7.4). DNA was quantified by measuring UV absorbance at 260 nm.

On the basis of the amount of radioactivity associated with DNA, the level of the epoxide bound to DNA was determined, and then DNA was hydrolyzed to deoxyribonucleosides by the sequential addition of DNase I, nuclease P1, phosphodiesterase I, and alkaline phosphatase (33, 35). Following purification by loading onto a Sep-Pak C<sub>18</sub> cartridge (Waters Associates, Milford, MA), the DNA hydrolysates were analyzed by HPLC using system 1. Reaction of [<sup>3</sup>H]-4-NP-9,10-epoxide with calf thymus DNA was repeated twice, and upon HPLC analysis major peaks of DNA hydrolysates were collected for spectral analysis.

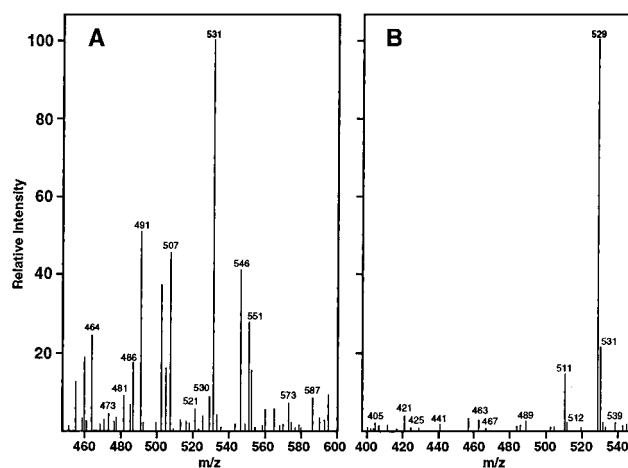
**Modification of Calf Thymus DNA or Polynucleotides with 4-NP in the Reaction Catalyzed by Xanthine Oxidase.** [<sup>3</sup>H]-4-NP was incubated with calf thymus DNA in the presence of xanthine oxidase under anaerobic conditions according to procedures described in the literature and employed in previous investigations (22, 30, 33). Briefly, calf thymus DNA was dissolved in 50 mM citrate buffer (pH 5.8) containing hypoxanthine (0.5 mg/mL) at a concentration of 2 mg/mL. Following the addition of catalase (10 μg/mL), the solution was purged with N<sub>2</sub>. Xanthine oxidase (0.1 unit/mL) and [<sup>3</sup>H]-4-NP in DMSO (3.2%, v/v) mixed with unlabeled 4-NP to give a specific activity of 0.58 mCi/mmol (final concentration of 0.8 mM) was added to the incubation mixture under positive pressure of N<sub>2</sub>. After incubation for 3–4 h at 37 °C, additional aliquots of hypoxanthine and xanthine oxidase were added, and then the mixture was purged with N<sub>2</sub>. After incubation for a further 18 h at 37 °C, the reaction mixture was extracted three times with a mixture of chloroform/isoamyl alcohol (24:1). The DNA was recovered and hydrolyzed after determining levels of binding as described above. Following Sep-Pak purification, the DNA hydrolysates were analyzed by HPLC using system 1 and system 2. Similar incubations, followed by HPLC analysis of DNA hydrolysates, were repeated at least four times.

Incubations of [<sup>3</sup>H]-4-NP with polynucleotides, poly(dA)·poly(dT), or poly(dG)·poly(dC) in the presence of xanthine oxidase were carried out following the procedure reported previously (27) and similar to that described above. Approximately 250 μg of poly(dA)·poly(dT) or poly(dG)·poly(dC) was incubated with 1.5 mg of [<sup>3</sup>H]-4-NP (specific activity of 12.2 mCi/mmol) in the presence of xanthine oxidase. Analysis of deoxyribonucleosides was similarly conducted as described above.

**DNA Binding in Vivo and Analysis of DNA Adducts.** Six female CD rats [CrI:CD(SD)BR], 7 weeks of age, were purchased from Charles River Breeding Laboratories, Inc. (Kingston, NY). They were given a single ip injection of [<sup>3</sup>H]-4-NP at a dose of 24 mg/kg of body weight (1.5 mCi/rat; specific activity of 77 mCi/mmol) in 300 μL of DMSO. Livers and mammary glands were obtained 24 h after the injection of [<sup>3</sup>H]-4-NP. Organs were pooled, and DNA was isolated according to a procedure described previously (33). Briefly, livers were homogenized in 0.01 M Tris-HCl buffer (pH 7.4) containing 1% sodium dodecyl sulfate and 1 mM EDTA. Mammary fat pads were frozen in liquid nitrogen, pulverized, and then homogenized. The homogenate was incubated with protease K for 30 min at 37 °C; it was extracted consecutively with phenol, a 1:1 mixture of phenol and chloroform/isoamyl alcohol (24:1), and then with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 1 volume of ice-cold absolute ethanol and 1/10 volume of 5 M NaCl. DNA was purified by treatment with RNase A and RNase T1 for 30 min at 37 °C; it was then extracted three times with a mixture of chloroform/isoamyl alcohol (24:1) and precipitated with ethanol and NaCl. DNA, dissolved in 10 mM Tris-HCl buffer (pH 7.4), was quantified by measuring the absorbance at 260 nm. The ratio of the absorbance at 260 nm to that at 280 nm was greater than 1.8. After the level of 4-NP bound to DNA was determined on



**Figure 2.** HPLC chromatograms of enzymatic hydrolysates of calf thymus DNA modified with [<sup>3</sup>H]-4-NP-9,10-epoxide (system 1).

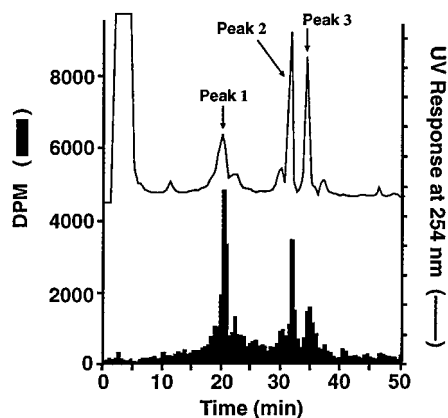


**Figure 3.** (A) Positive and (B) negative ion electrospray mass spectra of peak I of Figure 2.

the basis of total radioactivity, DNA was enzymatically hydrolyzed as described above, and following Sep-Pak purification, the DNA hydrolysates were analyzed by HPLC using system 2.

## Results

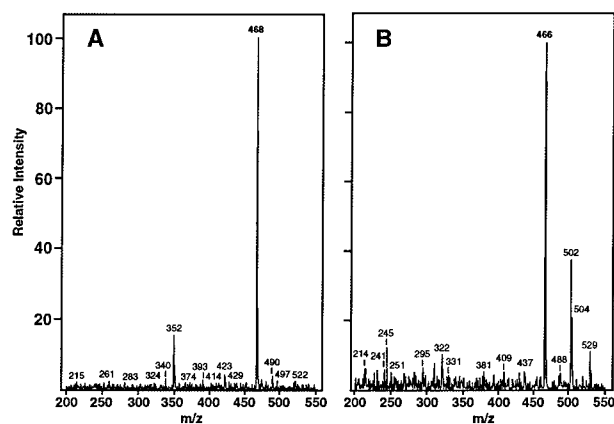
**DNA Adducts Derived from 4-NP-9,10-epoxide.** Incubation of [<sup>3</sup>H]-4-NP-9,10-epoxide, a metabolite derived from ring oxidation of 4-NP and a potential electrophilic intermediate, with calf thymus DNA yielded a level of 8.5 nmol of the epoxide bound per milligram of DNA (an average of two determinations). HPLC analysis of the DNA hydrolysates, using system 1, yielded four radioactive peaks; two major peaks eluted after 24 and 25 min, and two minor products eluted after 27 and 31 min (Figure 2). The first two peaks (peak I) were collected as a mixture and characterized by spectral analysis. Positive (Figure 3A) and negative ion (Figure 3B) electrospray mass spectra of peak I are shown. The signals at *m/z* 531 and 529 were in the positive and negative mode, respectively, indicating that this peak contains dG-derived adducts with a molecular weight of *m/z* 530. The <sup>1</sup>H NMR spectrum of peak I in DMSO-*d*<sub>6</sub> showed that the chemical shifts of the aromatic protons in the pyrene moiety of the adducts are moved slightly downfield as compared to those reported previously for *cis*- and *trans*-9,10-dihydro-9,10-dihydroxy-4-nitropyrene (32). Upon comparison of the chemical shifts of the dihydrodiols, the



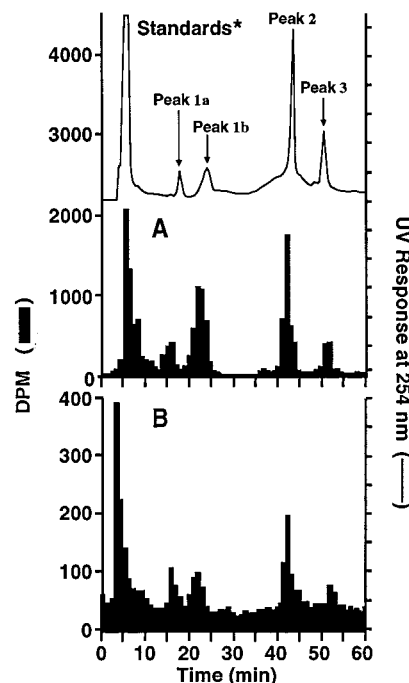
**Figure 4.** HPLC chromatograms of enzymatic hydrolysates of calf thymus DNA modified with  $[^3\text{H}]$ -4-NP in the presence of xanthine oxidase to catalyze the nitroreduction pathway (system 1).

adducts gave additional signals at 7.98, 6.87, 6.20, 4.86–5.11, 4.26, and 3.79 ppm, representing C8-H,  $N^2$ -H, 1'-H, OH's, 3'-H, and 4'-H, respectively. The signals at 6.87 and 4.86–5.11 ppm disappeared upon adding  $\text{D}_2\text{O}$ , as expected. Attachment at the C8-position of the dG base is clearly ruled out by the presence of the signal at 7.98 ppm as a singlet which is characteristic of C8-H. Peak I contains two adducts (adduct a and adduct b) derived from dG as indicated by the presence of a set of two different chemical shifts. The downfield shift could be associated with the carbon ( $\text{C}_9$  or  $\text{C}_{10}$ ) attached to the  $N^2$  of dG (29). Due to the fact that none of these adducts were detected in the mammary glands or in the liver of rats following 4-NP administration (see below), further studies were not pursued to assign the site of substitution ( $\text{C}_9$  or  $\text{C}_{10}$ ) and stereochemistry. However, on the basis of the analysis described above, the chemical assignments of the adducts are described as follows:  $^1\text{H}$  NMR (360 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.89 (s, 1H,  $\text{H}_{5a}$  or  $\text{H}_{5b}$ ), 8.88 (s, 1H,  $\text{H}_{5b}$  or  $\text{H}_{5a}$ ), 8.33 (t, 2H,  $\text{H}_{3a}$  and  $\text{H}_{3b}$ ,  $J = 7.51$  Hz), 8.26 (t, 2H,  $\text{H}_{7a}$  and  $\text{H}_{7b}$ ,  $J = 7.58$  Hz), 7.99 (m, 2H,  $\text{H}_{2a}$  and  $\text{H}_{2b}$ ), 7.98 (s, 2H, C8- $\text{H}_a$  and C8- $\text{H}_b$ ), 7.80–7.91 (m, 6H,  $\text{H}_{1a}$ ,  $\text{H}_{6a}$ ,  $\text{H}_{8a}$ ,  $\text{H}_{1b}$ ,  $\text{H}_{6b}$ , and  $\text{H}_{8b}$ ), 6.87 (m, 2H,  $N^2$ - $\text{H}_a$  and  $N^2$ - $\text{H}_b$ ), 6.20 (m, 2H, 1'- $\text{H}_a$  and 1'- $\text{H}_b$ ), 5.63 (m, 2H,  $\text{H}_{9a}$  and  $\text{H}_{10b}$ ), 5.23 (m, 2H,  $\text{H}_{9b}$  and  $\text{H}_{10a}$ ), 4.86–5.11 (m, OH's), 4.26 (m, 2H, 3'- $\text{H}_a$  and 3'- $\text{H}_b$ ), 3.79 (m, 2H, 4'- $\text{H}_a$  and 4'- $\text{H}_b$ ).

**DNA Adducts Derived from Nitroreduction of 4-NP.** Incubations of  $[^3\text{H}]$ -4-NP with calf thymus DNA in the presence of xanthine oxidase resulted in the formation of DNA adducts at levels of  $12.0 \pm 1.1$  nmol/mg of DNA ( $n = 4$ ). Figure 4 shows primarily three radioactive peaks from HPLC analysis (using system 1) of the DNA hydrolysates. Peaks 1–3 eluting at 20, 32, and 35 min, respectively, accounted for 46, 25, and 17% of the total radioactivity associated with DNA, respectively. Further HPLC analysis (system 2) resolved peak 1 into peak 1a eluting at 16 min and peak 1b at 22 min under the conditions employed (data shown in Figure 6 as UV markers). Both peak 1a and peak 1b were stable in double-stranded DNA as indicated by the following observations. When DNA samples modified by  $[^3\text{H}]$ -4-NP were extracted with ethyl acetate after being stored at 4 °C for 3 months, no appreciable amount of radioactivity was found in the ethyl acetate extracts. Total radioactivity associated with DNA remained the same, and HPLC analyses of DNA hydrolysates yielded similar traces



**Figure 5.** Positive (A) and negative (B) ion electrospray mass spectra of peak 3 of Figure 4.



**Figure 6.** HPLC radiochromatograms of enzymatic hydrolysates of DNA obtained from the liver (A) and mammary tissues (B) of female CD rats following ip injection of  $[^3\text{H}]$ -4-NP (system 2). Standards are UV markers derived from nitroreduction of 4-NP catalyzed by xanthine oxidase.

before and after 3 months of storage at 4 °C. However, at the deoxyribonucleoside level, peaks 1a and 1b were decomposed to yield a peak eluting at 32 min, as was seen when peak 1 was re-injected into the HPLC system following enzymatic hydrolysis of DNA at 37 °C (data not shown). Consequently, we attempted another approach by performing the enzymatic digestion of DNA at 10 °C, a condition used previously to isolate unstable N-7-substituted dG adducts in DNA (36); however, the results were comparable to those from the experiment conducted at 37 °C. Therefore, attempts to obtain material sufficient for spectral analysis of peak 1 were unsuccessful.

On the basis of comparison of its UV, MS, and  $^1\text{H}$  NMR spectral analysis, as well as chromatographic characteristics, with those of a synthetic standard, peak 2 was unequivocally identified as pyrene-4,5-dione (37). Chromatographic characteristics of the degradation product of peak 1 described above suggested that it is likely to be peak 2 (pyrene-4,5-dione). Spectral data of the degradation product of peak 1 (system 1) eluting at 32 min

confirmed this to be pyrene-4,5-dione and that the presence of peak 2 in DNA hydrolysates resulted from decomposition of peak 1.

Peak 3 appears to be stable both in double-stranded DNA and at the deoxyribonucleoside level. Although the amount of peak 3 collected was inadequate for  $^1\text{H}$  NMR analysis, it was sufficient for electrospray mass spectral analysis. As shown in Figure 5, the molecular weight of this adduct is 467, suggesting that it is likely a deoxyinosine (dI)-derived adduct. The incubation of [ $^3\text{H}$ ]-4-NP with poly(dA)·poly(dT) in the presence of xanthine oxidase yielded peak 3; however, similar incubation with poly(dG)·poly(dC) did not produce peak 3, further suggesting that peak 3 is an adduct derived from dI (data not shown).

The standard dG-C8-4-AP derived from nitroreduction of 4-NP was synthesized to determine its possible presence among those DNA adducts formed via nitroreduction of 4-NP catalyzed by xanthine oxidase. The adduct standard dG-C8-4-AP was stable at room temperature. Under the chromatographic conditions employed (system 2), the synthetic standard eluted after 44 min, which was different from elution obtained upon incubating 4-NP with DNA in the presence of xanthine oxidase (peak 1a, 16 min; peak 1b, 22 min; peak 2, 42 min; and peak 3, 52 min; as shown in Figure 6 as the UV markers).

**In Vivo Study.** Rats were sacrificed 24 h after treatment with [ $^3\text{H}$ ]-4-NP, and DNA was isolated from the liver and the mammary gland. HPLC analyses of the liver (Figure 6A) as well as mammary DNA hydrolysates (Figure 6B) using system 2 yielded four radioactive peaks, all of which coeluted with UV standards derived from the nitroreduction of 4-NP catalyzed by xanthine oxidase, including peak 1a (16 min), peak 1b (22 min), peak 2 (42 min), and peak 3 (52 min). None coeluted with the major DNA adducts derived from 4-NP-9,10-epoxide, a ring-oxidized metabolite of 4-NP, or with the adduct standard dG-C8-4-AP which eluted after 44 min.

## Discussion

In this report, we describe for the first time that nitroreduction of 4-NP is primarily responsible for the formation of DNA adducts in the mammary gland of rats *in vivo*. However, the possibility of the presence of dG-C8-4-AP *in vivo* was ruled out. This conclusion is based on a comparison of chromatographic characteristics among adducts obtained *in vitro* via nitroreduction or ring oxidation (4-NP-9,10-epoxide) and those detected *in vivo* following 4-NP treatment.

The two major DNA adducts resulting from the incubation of 4-NP-9,10-epoxide with DNA *in vitro* were derived from the addition of a  $N^2$ -exocyclic amino group of dG; however, no adducts were detected in rats treated with 4-NP. Although there is a great deal of information about the structures of DNA adducts derived from bay region dihydrodiol epoxides, relatively little is known about those derived from K-region epoxides, perhaps due to the relatively weak biological activities of such epoxides compared with those of bay-region diol epoxides. In our previous study, we reported on the identification of DNA adducts derived from 4,5-epoxy-4,5-dihydro-1-nitropyrene *in vitro* (29). Of the three major adducts that were identified, two stemmed from *trans* and one from *cis* addition of the  $N^2$ -exocyclic amino group of dG to the benzylic carbon ( $\text{C}_5$ ) of the 4,5-epoxide ring. The exocyclic

amino groups of DNA bases were also shown to be involved in reactions with K-region epoxides derived from 7,12-dimethylbenz[*a*]anthracene (DMBA) and 10-azabenz[*a*]pyrene (38, 39).  $N^2$ -Substitution of guanosine has been observed in the reaction of a K-region epoxide of DMBA with polyguanylic acid *in vitro*; however, the *cis* or *trans* configuration was not determined (38). Although *trans* addition is favored, *cis* opening of other arene oxides has also been observed (39–41). The fact that none of the adducts derived from 4-NP-9,10-epoxide were detected *in vivo* in the rat liver, or in the mammary gland, did not provide further stimulus to us toward pursuing assignments of the stereochemistry of these adducts.

Putative DNA adducts derived from nitroreduction of 4-NP, catalyzed by xanthine oxidase *in vitro*, were obtained. Clearly, these adducts coeluted with those obtained *in vivo* after treatment with 4-NP. While incubations of DNA with 1- or 2-NP in the presence of xanthine oxidase were shown to produce C8-substituted (deoxyguanosyl)aminopyrene derivatives (22, 30), none of the adducts resulting from similar incubations with 4-NP coeluted with the adduct standard dG-C8-4-AP. Peak 1 consisting of peaks 1a and 1b was stable in the double-stranded DNA, but they were susceptible to decomposition at the deoxyribonucleoside level to yield peak 2. Due to its fragile nature and gradual decomposition to peak 2, it was not possible to obtain any structural information about peak 1. On the other hand, peak 2, as the decomposition product of peak 1, was unequivocally identified as pyrene-4,5-dione.

On the basis of its electrospray mass spectrum and results obtained following incubations of 4-NP with polynucleotides, peak 3 was tentatively identified as a dI adduct. The formation of a modified dI adduct derived from *N*-hydroxy-6-aminochrysene [*N*-(deoxyinosin-8-yl)-6-aminochrysene] *in vitro* has been previously reported along with some evidence to indicate that spontaneous oxidation of *N*-(deoxyadenosin-8-yl)-6-aminochrysene is responsible for the formation of a dI adduct (35). Oxidation of the adduct on the DNA strand, or after isolation of the adduct, did not take place. The same dI adduct was detected in target tissues (lung and liver) of mice treated with 6-nitrochrysene (42). Relevant to the present study, Malia and Basu (43) demonstrated oxidative deamination of cytosine during nitroreduction of 1-NP catalyzed by xanthine oxidase. Thus, it is conceivable that deamination of adenine to inosine also can occur during enzymatic reduction *in vivo* via a similar mechanism. In the present study, although peak 3 is not fully characterized, its presence *in vivo* in the mammary gland is biologically relevant since modified inosine derivatives would be expected to mispair with cytosine bases in the DNA (44, 45).

In summary, adducts derived from a nitroreductive pathway coeluted with those detected in the liver as well as with those in the target organ (mammary gland) of CD rats treated with 4-NP. These findings clearly document the importance of the nitroreduction pathway *in vivo* in the activation of 4-NP.

**Acknowledgment.** We thank the staff of the Research Animal Facility for handling animals and Mrs. Patricia Sellazzo for preparing and Mrs. Ilse Hoffmann for editing the manuscript. This work was supported by NCI Grant CA 35519.

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TX9802318