DNA Binding by 1-Nitropyrene and Dinitropyrenes in Vitro and in Vivo: Effects of Nitroreductase Induction

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Includes the Commentary by the Institute's Health Review Committee

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DNA Binding by 1-Nitropyrene and Dinitropyrenes in Vitro and in Vivo: Effects of Nitroreductase Induction

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DNA Binding by 1-Nitropyrene and Dinitropyrenes in Vitro and in Vivo: Effects of Nitroreductase Induction

Frederick A. Beland

ABSTRACT

1-Nitropyrene, the predominant nitropolycyclic aromatic hydrocarbon found in diesel exhaust, is both a mutagen and a tumorigen. 1,6-Dinitropyrene is present in diesel exhaust in much smaller quantities than is 1-nitropyrene, but is much more mutagenic and carcinogenic. In an attempt to understand this difference in biological potencies, we have compared the extents to which these two nitropyrenes bind DNA in vivo. We have also determined the effect of 1-nitropyrene pretreatment upon the induction of nitroreductases and the subsequent DNA binding by both 1-nitropyrene and 1,6-dinitropyrene. In subsequent experiments, we have examined the importance of acetylation phenotype in the formation of DNA adducts from dinitropyrene in vivo.

After a single intraperitoneal injection of 1-nitropyrene, covalent DNA binding could not be detected in vivo; however, 1,6-dinitropyrene formed N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene as the major DNA adduct in rat liver, kidney, urinary bladder, and mammary gland, with the highest levels being found in the bladder. The capability of liver microsomes to catalyze the oxidative metabolism of 1-nitropyrene was unchanged after treating rats with a single dose of 8 mg of 1-nitropyrene per kilogram of body weight. Cytochrome P-450, reduced nicotinamide adenine dinucleotide phosphate (NADPH2)-cytochrome P-450 reductase, and cytochrome b5 levels were also unchanged, while slight increases were detected in reduced nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase and epoxide hydrase activities. Liver cytosolic and microsomal nitroreductase activities toward both 1-nitropyrene and 1,6-dinitropyrene were increased twofold, and cytosolic nitroreductase activity toward 1-nitrosopyrene and 1-nitro-6-nitrosopyrene was elevated by about 20 percent. DNA binding of both 1-nitropyrene and 1,6-dinitropyrene in vitro was twofold higher when cytosol from rats pretreated with 1-nitropyrene was used. However, pretreatment of rats with 1-nitropyrene only slightly increased the amount of in vivo DNA binding by 1,6-dinitropyrene except in the kidney, where there was a 60 percent increase.

In the presence of S-acetylcoenzyme A, liver cytosol from slow-acetylator phenotype hamster strains Bio. 1.5 and 82.73 catalyzed the binding of two-to-three times more 1,8-dinitropyrene to DNA than was observed with the fast-acetylator phenotype strain Bio. 87.20. Similarly, when 1,8-dinitropyrene was administered in vivo and the extent of binding was assayed in liver, bladder, and intestinal DNA, there was more binding in strain Bio. 1.5 than in strain Bio. 87.20. A similar relationship was observed in mice; after a single intraperitoneal injection, there was more hepatic DNA binding by 1,6-dinitropyrene in the slow-acetylator phenotype strain A/J than in the fast-acetylator phenotype strain C57BL/6J.

These results suggest that although nitroreduction is involved in DNA adduct formation by 1,6- and 1,8-dinitropyrene, other factors (for example, O-acetylation of N-hydroxyarylamine intermediates) limit the extent of DNA binding in vivo.

INTRODUCTION

Nitro-derivatives of polycyclic aromatic hydrocarbons (nitro-PAHs) have been detected in a variety of environmental samples, including airborne particulates, coal fly ash, and diesel emissions (reviewed in Beland et al. 1985; Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986). These compounds are formed in a wide variety of combustion processes and from atmospheric reactions of PAHs with nitrogen oxides (Beland et al. 1985; Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986), which results in their ubiquitous distribution. Since some of the nitro-PAHs are extremely potent bacterial mutagens (Beland et al. 1985; Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986), many of these compounds have been considered as a potential human health risk.

These results suggest that although nitroreduction is involved in DNA adduct formation by 1,6- and 1,8-dinitropyrene, other factors (for example, O-acetylation of N-hydroxyarylamine intermediates) limit the extent of DNA binding in vivo.

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2 A list of abbreviations appears at the end of this report for your reference.
Likewise, while both positive and negative results have been obtained regarding the tumorigenicity of 1-nitropyrene (El-Bayoumy et al. 1982, 1984; Ohgaki et al. 1982, 1985; Hirose et al. 1984; Nesnow et al. 1984; Imaida et al. 1985; Wislocki et al. 1986; King 1987), 1,3-, 1,6-, and 1,6-dinitropyrene clearly induce tumors in experimental animals (Nesnow et al. 1984; Ohgaki et al. 1984, 1985; Tokiwa et al. 1984; Takayama et al. 1985; Imaida et al. 1986; Maeda et al. 1986; Wislocki et al. 1986; King 1987). Of these, 1,6-dinitropyrene appears to be the most potent tumorigen (Ohgaki et al. 1984, 1985; Imaida et al. 1986; Wislocki et al. 1986; King 1987).

It has been reported that after intraperitoneal administration of 1-nitropyrene to rats, DNA adducts were formed in the kidney, mammary gland, and liver (Hashimoto and Shudo 1985; Stanton et al. 1985). Since a substantial portion of these adducts appeared to be N-(deoxyguanosin-8-yl)-1-aminopyrene, it was suggested that 1-nitropyrene was activated to a DNA-binding derivative through nitroreduction to N-hydroxy-1-aminopyrene. Although 1-nitropyrene appears to bind to DNA in vivo, it is weakly tumorigenic when administered to rats intraperitoneally (Imaida et al. 1985; King 1987). In contrast, an equimolar dose of 1,6-dinitropyrene is clearly tumorigenic, inducing malignant fibrous histiocytomas, leukemias, and mammary gland tumors (Imaida et al. 1986; King 1987). This difference in tumorigenic response may be due to differences in the extent of adduct formation; however, other factors, including differential rates of DNA repair or differences in cytotoxicity between the two compounds, may be involved in modulating the tumorigenic response. In this research, my colleagues and I have examined factors that affect DNA adduct formation by nitropyrenes. To accomplish this objective, experiments were conducted in two specific areas. Initially, we compared the extents of DNA binding and the types of DNA adducts formed by 1-nitropyrene and 1,6-dinitropyrene after a single intraperitoneal injection of each compound. In addition, since nitro-PAHs have been reported to increase hepatic nitroreductase activity (Chou et al. 1987), and because nitroreduction has been implicated in the metabolic activation of nitropyrenes (Howard et al. 1983b; Djurić et al. 1985, 1986a; Hashimoto and Shudo 1985; Stanton et al. 1985), we compared the effect of 1-nitropyrene pretreatment upon the DNA binding of 1-nitropyrene and of 1,6-dinitropyrene both in vitro and in vivo. In the second series of experiments, we examined the importance of acetylation phenotype on the formation of DNA adducts from dinitropyrenes in vivo. These latter experiments followed from the suggestion that O-acetylation was a major factor in the extreme mutagenicity of dinitropyrenes in bacteria (reviewed in Beland et al. 1985; Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986), and from the observation that liver cytosol could catalyze the reduction of nitropyrenes to N-hydroxyamylamines and the subsequent O-acetylation of these intermediates (Djurić et al. 1985).
al. 1985). Synthetic \(N\)-(deoxyguanosin-8-yl)-1-aminoo-6-nitropyrene and \(N\)-(deoxyguanosin-8-yl)-1-aminoo-8-nitropyrene were prepared as previously described (Beland 1986).

**INSTRUMENTATION**

High-pressure liquid chromatography was conducted with an Altex (Beckman Instruments Inc., Fullerton, CA) 420 controller and two 110A pumps, a Waters Associates 440 ultraviolet monitor, a Hewlett-Packard 3380A and a Radiomatic FLO-Instruments Mass spectrometer. Ultraviolet and visible spectra and enzyme rates were obtained with a temperature-controlled Cary (Varian Associates, Inc., Sunnyvale, CA) 219 recording spectrophotometer.

**ANIMALS**

Male Sprague-Dawley rats (about 250 g) and male C57BL/6J mice (about 30 g; approximately six months old) were obtained from the breeding colony at the National Center for Toxicological Research, Jefferson, AR. Male A/J mice (about 30 g; approximately six months old) were purchased from Jackson Laboratories, Bar Harbor, ME. Male Bio. 1.5 and Bio. 82.20 hamsters (100 to 125 g; five to eight months old) were purchased from Bio Breeders, Watertown, MA. Male Bio. 82.73/H hamsters were obtained from Dr. David W. Hein, Morehouse School of Medicine, Atlanta, GA.

**TREATMENT OF ANIMALS**

Nine Sprague-Dawley rats were treated with a single intraperitoneal injection of 8 mg/kg body weight of unlabeled 1-nitropyrene dissolved in 20 percent dimethylsulfoxide (DMSO) and 80 percent trioxacain. Nine control animals received the vehicle only. After three days, groups of three animals were either killed for preparation of liver enzyme fractions, or treated with a single intraperitoneal injection of 200 \(\mu\)g/kg body weight of either radiolabeled 1-nitropyrene (4 Ci/mmol) or radiolabeled 1,6-dinitropyrene (1 Ci/mmol). All animals treated with radiolabeled nitropyrenes were killed 24 hours later, and their livers, lungs, kidneys, urinary bladders, and mammary glands were removed for DNA adduct analyses.

Hamsters (two to three animals per group) were treated with a single intraperitoneal injection of 1.5 mg/kg body weight of \(\{4.5.9.10\}^3\text{H}\)1,8-dinitropyrene (1 Ci/mmol) dissolved in 20 percent DMSO and 80 percent trioxacain. The animals were killed after 24 hours, and their livers, urinary bladders, and intestines were removed for DNA adduct analyses.

In an initial experiment, mice (two per group) were treated with a single intraperitoneal injection of 1.5 mg/kg body weight of \([4.5.9.10\}^3\text{H}]1,8\text{-dinitropyrene (5.1 \(\mu\)mol; 1 Ci/mmol) or 0.93 mg/kg body weight of \([ring-\text{3H}]2\text{-aminofluor}

**ENZYME ASSAYS**

Rat liver microsomes and cytosol (105,000 \(\times\) g supernatant) were prepared, as previously described (Djuric et al. 1986b), from livers perfused with ice-cold 10 mM Tris-HCl (pH 7.4), 250 mM sucrose buffer. Metabolites were quantified after incubation of 20 \(\mu\)M radiolabeled 1-nitropyrene, 1,6-dinitropyrene (1 Ci/mmol) with 0.2 to 1.0 \(\mu\)g/ml cytosolic or microsomal protein, 1 mM NADPH, 100 \(\mu\)M FMN, and 50 mM potassium phosphate (pH 7.4) in a total volume of 1 mL Anaerobic incubations were prepared as previously described (Djuric et al. 1986b). These 1-mL incubations were conducted under argon and contained an oxygen-scavenging system of 75 \(\mu\)mol of glucose, two units of glucose oxidase, and two units of catalase (Potter and Reed 1983). Some incubations contained NADH, 1-methylisocitramide, or hypoxanthine instead of NADPH, and were performed with and without 100 \(\mu\)M dicoumarol, menadione, or allo-purinol, respectively. Incubations were stopped after 10 minutes by the addition of two volumes of chloroform and methanol (1:1), and the chloroform-extractable metabolites (more than 95 percent of the radioactivity) were analyzed by HPLC. Separation of reduced metabolites was achieved with a Whatman (Clifton, NJ) Partisl 50DS3 RACII reverse-phase column, using an isocratic mobile phase of 76 percent methanol at 1 ml/min. Under these conditions, amino- pyrenes eluted at 5 minutes, nitropyrenes at 10 minutes, 1-nitrosopyrene at 12 minutes, and 1-nitro-6-nitrosopyrene at 15 minutes. Some incubations also contained 1 mM S-acetylcoenzyme A, and in these incubations, N-acetylamino- metabolites were detected at 3 minutes. Oxidized metabolites of 1-nitropyrene were resolved with a Waters C18 \(\times\)Bonda pak reverse-phase column using a linear gradient of 50 percent A (20 mM acetic acid) and 50 percent B (methanol and acetonitrile, 1:1) to 25 percent A and 75 percent B over 30
minutes (Djuric et al. 1986a). The identities of these metabolites were confirmed by coelution with synthetic standards as well as by ultraviolet, visible, and mass spectral analyses.

Nitrosoreductase and menadione reductase activities were measured by monitoring the cytosol- and microsomal-catalyzed reduction of succinylated cytochrome c, as previously described (Djuric et al. 1986b). Cytosolic DT-diaphorase activity was determined by monitoring the reduction of succinylated cytochrome c in incubations with menadione (Lind and Ernster 1974). Cytochrome P-450 and cytochrome bs levels were determined after dilution of the microsomes in 20 percent glycerol, 0.5 percent cholate, 0.4 percent Triton N-101, 1 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM potassium phosphate (pH 7.25), and reduction with dithionite and 200 mM NADH, respectively (Omura and Sato 1964). Cytosol P-455 reductase activity was measured at 37°C by monitoring reduction of 50 μM cytochrome c in the presence of 100 μM NADPH and 100 μg/ml microsomal protein (Potter and Reed 1963). Cytosol and cytochrome bs levels were measured as NADH:ferri-cyanide reductase using 100 μM potassium ferricyanide, 100 μM NADH, and 10 μg/ml microsomal protein at 37°C (Crane et al. 1956).

Epoxide hydrolase activity was assayed by incubating 10 μM 1-nitropyrene-4,5-oxide with 200 μg/ml microsomal protein and 50 mM potassium phosphate buffer (pH 7.4) at 37°C in a total volume of 500 μl. Incubations were stopped after 10 minutes by adding 500 μl of methanol, and 50-μl aliquots were analyzed directly by HPLC. 1-Nitropyrene-trans-4,5-dihydrodiol was separated from 1-nitropyrene-4,5-oxide using a Waters Novapak radial compression column and a mobile phase of 70 percent methanol at 2 ml/min. 1-Nitropyrene-trans-4,5-dihydrodiol was the only product formed, and it eluted at four minutes; the epoxide eluted at seven minutes. Epoxide hydrolase activity was calculated from the decrease in area of the epoxide peak. 1-Nitropyrene-trans-4,5-dihydrodiol was not formed in control incubations with heat-denatured microsomes. Hamster liver cytosol was prepared as previously described (Djuric et al. 1986b). Metabolites were quantified as outlined for the rat liver cytosol, after incubation of 20 μM [4,5,9,10-3H]1,8-dinitropyrene (1 Ci/mmol) with 1 mg/ml cytosolic protein, 1 mM NADPH, 1 mM S-acetylcysteine A, and 1 mM dithiothreitol in 50 mM sodium pyrophosphate buffer (pH 7.4) for 15 minutes at 37°C.

DNA ADDUCT ANALYSES

In vitro DNA binding assays with rat liver cytosol contained 200 μg/ml cytosolic protein, 2 mg/ml DNA, 1 mM NADPH, and 20 μM tritiated 1-nitropyrene or 1,6-dinitropyrene, with or without 1 mM S-acetylcysteine A, and were performed as previously described (Djuric et al. 1985). Hamster liver cytosol incubations contained 1 mg/ml cytosolic protein, 1 mM NADPH, 1 mM S-acetylcysteine A, and 20 μM [4,5,9,10-3H]1,8-dinitropyrene (1 Ci/mmol) or [ring-3H]2-aminofluorene (75 μCi/mmol) in 50 mM sodium pyrophosphate, 1 mM dithiothreitol buffer (pH 7.4), and were conducted for 15 minutes at 37°C. For the in vivo studies, liver, lung, kidney, and urinary bladder were removed and frozen at −20°C. Mammary gland epithelial cells were isolated from fresh tissue, as described by Moon and colleagues (1969). Intestinal epithelial cells were isolated as previously reported (Westra et al. 1985). Typically, tissue was stored for less than one month before the DNA was isolated.

Each tissue sample or cell preparation was suspended in 5 ml of 1 percent sodium dodecyl sulfate, 1 mM EDTA per gram of tissue, and was homogenized at room temperature with a Polytron (Brinkman Instruments, Inc., Westbury, CT) for 30 seconds at a speed setting of 4 to 5. The homogenates were incubated at 37°C with 2.5 mg of proteinase K per gram of tissue for 30 minutes (Beland et al. 1984). These samples, as well as the in vitro incubation mixtures, were sequentially extracted (Djuric et al. 1986a) with equal volumes of phenol, phenol:chloroform:isoamyl alcohol, and chloroform:isoamyl alcohol. The DNA in the aqueous layer was precipitated by adding 0.1 volume 5 M sodium chloride and 1.1 volume ethanol and storing at −20°C overnight. After centrifugation at 1,600 × g for five minutes, the DNA was redissolved in 1 ml of 1.5 mM sodium citrate, 15 mM sodium chloride per gram of tissue. The solutions were made 50 mM in Tris-HCl (pH 7.4) and incubated with heat-treated RNase A (200 μg per gram of tissue) and RNase T1 (100 units per gram of tissue) for 15 minutes at 37°C. The DNA was extracted again as described above, precipitated, and redissolved in 5 mM Bis-Tris buffer, 0.1 mM EDTA (pH 7.1). The isolated DNA was quantified by its absorbance at 260 nm, and binding levels were determined by liquid scintillation counting after digestion with DNase. The DNA was hydrolyzed to nucleosides, as described by Howard and co-workers (1983b), except that acid phosphatase was omitted. After hydrolysis, the adducts were partitioned into n-butanol, the n-butanol was evaporated, and the residue was dissolved in 50 percent methanol for analysis. Adducts were separated by HPLC, as described by Howard and colleagues (1983b). Additional aliquots of DNA were hydrolyzed with trifluoroacetic acid and analyzed by HPLC, as outlined by Helfich and colleagues (1986). In some instances, aliquots of the DNA obtained from the livers of rats treated with 1-nitropyrene were purified further by centrifugation through cesium chloride (Kadlubar et al. 1961).
STATISTICAL ANALYSES

Comparisons between groups were made by Student's t test (Simpson et al. 1960). Groups were considered to be significantly different when \( p < 0.05 \).

RESULTS

DNA BINDING IN VIVO IN RATS TREATED WITH 1-NITROPYRENE AND 1,6-DINITROPYRENE WITHOUT NITROREDUCTASE INDUCTION

The extent of DNA binding in vivo was determined in the mammary gland, liver, lung, kidney, and urinary bladder of rats 24 hours after a single intraperitoneal injection of 200 \( \mu \)g/kg body weight of \([4,5,9,10-^{3}\text{H}] 1\)-nitropyrene (4 Ci/mmol) or \([4,5,9,10-^{3}\text{H}] 1,6\)-dinitropyrene (1 Ci/mmol). DNA binding by 1-nitropyrene could not be detected in the urinary bladder or mammary gland epithelium (less than 0.04 pmol bound/mg DNA), but in the liver, lung, and kidney, radioactivity was associated with the DNA. However, upon either enzymatic or trifluoroacetic acid hydrolysis of the DNA, less than 15 percent of the radioactivity partitioned into n-butanol. High-pressure liquid chromatography analyses of both the n-butanol and aqueous phases were conducted. Radioactivity did not elute in the region normally observed for adducts (50 to 70 percent methanol). Instead, in all cases, the radioactivity eluted in the void volume (20 percent methanol) and methanol wash. Therefore, additional aliquots of the DNA were purified further by centrifugation through cesium chloride. This treatment resulted in the radioactivity banding close to the top of the gradient while the DNA banded in the middle. In additional experiments, rats were treated with 30 \( \mu \)g/kg body weight of \([4,5,9,10-^{3}\text{H}] 1\)-nitropyrene (60 mCi/mm), as described by Stanton and associates (1985). Although increased amounts of radioactivity were associated with the DNA, this binding was also judged to be noncovalent because the radioactivity did not remain associated with the DNA after centrifugation through cesium chloride.

In rats treated with \([4,5,9,10-^{3}\text{H}] 1,6\)-dinitropyrene, the highest amount of radioactivity was associated with the urinary bladder DNA, followed by the liver, mammary gland, kidney, and lung DNA (Table 1). DNA isolated from the liver, kidney, urinary bladder, and mammary gland had sufficient radioactivity for analysis of the 1,6-dinitropyrene DNA adducts by HPLC. Typically, 75 percent of the radioactivity could be recovered in n-butanol after enzymatic hydrolysis of the DNA and 85 percent of the recovered radioactivity coeluted with synthetic \( N \)-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene on reverse-phase HPLC (Figure 1). Additional aliquots of DNA were hydrolyzed with trifluoroacetic acid. Upon chromatography, 90 to 95 percent of the radioactivity coeluted with an ultraviolet marker obtained from treating \( N \)-hydroxy-1-amino-6-nitropyrene-modified DNA with trifluoroacetic acid, which confirmed the identity of the in vivo adduct.

DNA BINDING IN VIVO IN RATS TREATED WITH 1-NITROPYRENE AND 1,6-DINITROPYRENE AFTER NITROREDUCTASE INDUCTION

The extent of DNA binding in vivo by radioactive 1-nitropyrene and 1,6-dinitropyrene was determined after pretreatment of rats with 1-nitropyrene. The amount of radioactivity associated with the liver, lung, and kidney DNA after administration of 1-nitropyrene increased 50 to 80 percent in the nitroreductase-induced animals. However, this radioactivity did not appear to be covalently bound after we conducted analyses similar to the in vivo experiments described earlier. Pretreating rats with 1-nitropyrene did not increase significantly the binding of 1,6-dinitropyrene to

<table>
<thead>
<tr>
<th>Table 1. DNA Binding of 1,6-Dinitropyrene in Vivo&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DNA Binding of ([4,5,9,10-^{3}\text{H}] 1,6)-Dinitropyrene&lt;sup&gt;b&lt;/sup&gt; (pmol bound/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment of Rats</td>
<td>Bladder</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.28 ± 0.72</td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>6.17 ± 2.07</td>
</tr>
</tbody>
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<sup>a</sup> Male Sprague-Dawley rats were treated by a single intraperitoneal injection with 8 mg/kg unlabeled 1-nitropyrene dissolved in 20 percent DMSO and 80 percent trioctanoin. Control animals received the vehicle only. After three days, the animals were treated with a single intraperitoneal injection of 200 \( \mu \)g/kg \([4,5,9,10-^{3}\text{H}] 1,6\)-dinitropyrene (1 Ci/mmol) and were killed after an additional 24 hours.

<sup>b</sup> DNA was extracted from tissues, as described in the Methods section, and quantified by its ultraviolet absorbance at 260 nm. Binding levels were determined by liquid scintillation counting. Results are the average of three animals ± SD.

<sup>c</sup> Significantly different from vehicle control.
DNA Binding by 1-NP and DNPs: Effects of Nitroreductase Induction

Figure 1. High-pressure liquid chromatogram of DNA isolated from the mammary gland epithelium of rats treated with intraperitoneal injection with 200 μg/kg body weight of [4,5,9,10-3H]1,6-dinitropyrene. DNA was hydrolyzed enzymatically and the adducts were extracted into n-butanol. The n-butanol was evaporated and the residues were dissolved in 50 percent methanol with synthetic N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, which served as an ultraviolet marker. The N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene was prepared by reacting N-hydroxy-1-amino-6-nitropyrene with calf thymus DNA at pH 5. Its characterization is described in Beland (1986b). The separation was accomplished using a C_{18} Bondapak reverse-phase column with a nonlinear gradient (Waters No. 2) of 20 to 65 percent methanol over 20 minutes. At 25 minutes, the column was washed with 100 percent methanol. dR = deoxyribose.

DNA from liver, lung, urinary bladder, or mammary gland (Table 1); however, in the kidney, there was a 60 percent increase.

RAT LIVER MICROSONAL ENZYMES

Pretreatment of male Sprague-Dawley rats with a single intraperitoneal injection of 8 mg/kg body weight of 1-nitropyrene did not alter the levels of hepatic microsomal cytochrome P-450 (about 0.8 nmol/mg protein) or cytochrome b_{5} (approximately 0.4 nmol/mg protein), nor the NADPH-cytochrome P-450 reductase activity (about 120 nmol/mg protein/min). NADPH-cytochrome b_{5} reductase activity was increased from 9.0 ± 0.05 to 12.0 ± 0.5 μmol/mg protein/min (p < 0.05), and microsomal epoxide hydride was elevated approximately 50 percent, from 1.73 ± 0.15 to 2.85 ± 0.07 nmol/mg protein/min (p < 0.02). 1-Nitropyrene pretreatment did not increase the rate of oxidative microsomal metabolism of 1-nitropyrene in aerobic incubations. For example, with both types of microsomes, the rate of formation of 3-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene was about 170 to 180 and 110 to 120 pmol/mg protein/min, respectively. Oxidative microsomal metabolism of 1,6-dinitropyrene was not detected.

Nitroreductase activity of liver microsomes, as assayed by the formation of 1-aminopyrene and 1-amino-6-nitropyrene, was increased approximately twofold by the pretreatment of rats with 1-nitropyrene (Table 2). In the anaerobic incubations with 1-nitropyrene, formation of 1-nitosoprene was not detected. With 1,6-dinitropyrene, formation of 1-nitro-6-nitrosoprene was detected, and this decreased by 40 percent in incubations with microsomes from rats pretreated with 1-nitropyrene.

Adding 100 μM DPEA to incubations with both types of microsomes decreased the nitroreduction of 1-nitropyrene with about 100 percent inhibition of 1-nitro-6-nitrosoprene formation with control microsomes (Table 2). Carbon monoxide (CO) strongly inhibited the formation of 1-amino-6-nitropyrene with both types of microsomes, and caused approximately 50 percent inhibition of 1-nitro-6-nitrosoprene formation with control microsomes (Table 2). Air decreased formation of all reduced metabolites from both 1-nitropyrene and 1,6-dinitropyrene, as reported previously (Djuric et al. 1986b). Menadione and allopurinol inhibited with liver cytosol from rats pretreated with 1-nitropyrene nitroreduction in the presence of 1-methylnicotinamide and hypoxanthine, respectively. The NADH-supported reduction of these compounds.

RAT LIVER CYTOSOLIC ENZYMES

The rate of 1-aminopyrene formation was increased approximately twofold in anaerobic incubations conducted with liver cytosol from rats pretreated with 1-nitropyrene (Table 3). As shown in Table 3, NADH was the most effective electron donor, followed by NADPH, 1-methylnicotinamide, and hypoxanthine. Menadione and allopurinol inhibited nitroreduction in the presence of 1-methylnicotinamide and hypoxanthine, respectively. The NADH-supported nitroreduction of 1-nitropyrene was not inhibited by dicoumarol. The addition of 1 mM S-acetylcoenzyme A to the incubations with NADH resulted in the quantitative conversion of 1-aminopyrene to N-acetyl-1-aminopyrene with both types of cytosol. The formation of 1-nitrosoprene in anaerobic incubations was near the limit of detection of 1 pmol/mg protein/min, while in aerobic incubations, the concentration of 1-nitrosoprene increased with a concomitant decrease in 1-aminopyrene (data not shown; this has
Table 2. Reductive Rat Liver Microsomal Metabolism of Nitrated Pyrenesa

<table>
<thead>
<tr>
<th>Pretreatment of Rats</th>
<th>Inhibitor</th>
<th>1-Nitropyrene</th>
<th>1-Nitrosopyrene</th>
<th>1,6-Dinitropyrene</th>
<th>1-Nitro-6-nitrosopyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-Aminopyrene</td>
<td>1-Nitrosopyrene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>–</td>
<td>0.27 ± 0.06</td>
<td>&lt; 0.005</td>
<td>2.41 ± 0.78</td>
<td>1.57 ± 0.03</td>
</tr>
<tr>
<td>DPEA</td>
<td></td>
<td>0.19 ± 0.04</td>
<td>&lt; 0.005</td>
<td>0.23 ± 0.10b</td>
<td>1.19 ± 0.31</td>
</tr>
<tr>
<td>SKF-525A</td>
<td></td>
<td>0.26 ± 0.01</td>
<td>0.01 ± 0.00</td>
<td>&lt; 0.005b</td>
<td>&lt; 0.005b</td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>–</td>
<td>0.52 ± 0.04e</td>
<td>0.01 ± 0.00</td>
<td>4.01 ± 0.75</td>
<td>0.93 ± 0.20e</td>
</tr>
<tr>
<td>DPEA</td>
<td></td>
<td>0.23 ± 0.06b</td>
<td>&lt; 0.005</td>
<td>0.90 ± 0.06b</td>
<td>1.54 ± 0.44</td>
</tr>
<tr>
<td>COc</td>
<td>ND</td>
<td></td>
<td></td>
<td>0.11 ± 0.03b</td>
<td>1.38 ± 0.37</td>
</tr>
<tr>
<td>SKF-525A</td>
<td></td>
<td>0.45 ± 0.08</td>
<td>0.01 ± 0.01</td>
<td>&lt; 0.005b</td>
<td>&lt; 0.005b</td>
</tr>
</tbody>
</table>

a Incubations were conducted under argon (1 ml total volume) and contained 1 mM NADPH; 100 μM FMN; 50 mM potassium phosphate (pH 7.4); 1 mM EDTA; an oxygen-scavenging system of glucose, glucose oxidase, and catalase; 20 μM [4,5,9,10-3H]1-nitropyrene or [4,5,9,10-3H]1,6-dinitropyrene; and 0.2 mg/ml (for 1,6-dinitropyrene) or 1 mg/ml (for 1-nitropyrene) microsomal protein. Where indicated, 100 μM DPEA or 1 mM SKF-525A was also included in the incubations. Reactions were stopped after 10 minutes by adding 2 ml of a mixture of chloroform and methanol (1:1). The chloroform-extractable metabolites, accounting for more than 95 percent of the radioactivity, were analyzed by HPLC as described in the Methods section. The results are presented as the mean ± mean variance of duplicate incubations.

b Significantly different from incubations conducted in the absence of an inhibitor (p < 0.05).

c In these incubations, CO was substituted for argon.

d ND = not done.

e Significantly different from vehicle control (p < 0.05).

Table 3. Reduction of 1-Nitropyrene by Rat Liver Cytosol in the Presence of FMNa

<table>
<thead>
<tr>
<th>Pretreatment of Rats</th>
<th>Electron Donor</th>
<th>Inhibitor</th>
<th>1-Aminopyrene Formation (pmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>NADPH</td>
<td>–</td>
<td>93 ± 7</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>–</td>
<td>46 ± 7b</td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>NADPH</td>
<td>–</td>
<td>208 ± 21c</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>–</td>
<td>50 ± 7b</td>
</tr>
<tr>
<td>Vehicle</td>
<td>NADH</td>
<td>–</td>
<td>173 ± 28</td>
</tr>
<tr>
<td></td>
<td>Dicoumarol</td>
<td>–</td>
<td>125 ± 37</td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>NADH</td>
<td>–</td>
<td>325 ± 40c</td>
</tr>
<tr>
<td></td>
<td>Dicoumarol</td>
<td>–</td>
<td>322 ± 6c</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1-Methyl nicotinamide</td>
<td>Menadione</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>1-Methyl nicotinamide</td>
<td>Menadione</td>
<td>8 ± 4</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>4 ± 1b</td>
</tr>
<tr>
<td>Vehicle</td>
<td>Hypoxanthine</td>
<td>–</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>Hypoxanthine</td>
<td>–</td>
<td>11 ± 4</td>
</tr>
<tr>
<td></td>
<td>Allopurin</td>
<td>–</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

a Incubations, under argon, contained 20 μM 1-nitropyrene, 1 mg/ml cytosolic protein, 100 μM FMN, 1 mM electron donor, 50 mM potassium phosphate (pH 7.4), and an oxygen-scavenging system of glucose, glucose oxidase, and catalase. Inhibitors were added at a concentration of 100 μM. Incubations were stopped after 10 minutes by adding two volumes of a mixture of chloroform and methanol (1:1). The results are presented as the mean ± mean variance of duplicate incubations.

b Significantly different from incubations conducted in the absence of an inhibitor (p < 0.05).

c Significantly different from vehicle control (p < 0.05).
been described in more detail previously by Djurić and coworkers (1986b).

In anaerobic incubations with 1,6-dinitropyrene, rat liver cytosol, and NADPH or NADH, the formation of 1-amino-6-nitropyrene, but not of 1-nitro-6-nitrosopyrene, was increased approximately twofold by 1-nitropyrene pretreatment (Table 4). Nitroreduction was detected in the absence of electron donors, but this was only approximately 15 percent of that observed with added NADPH or NADH. Hypoxanthine and 1-methylnicotinamide did not stimulate the reduction of 1,6-dinitropyrene above background levels. In aerobic incubations with NADPH, the decrease in 1-amino-6-nitropyrene formation in the presence of air roughly equaled the increase in 1-nitro-6-nitrosopyrene formation (Table 4). Dicoumarol caused a slight, although statistically insignificant, decrease in the formation of both reduced metabolites in incubations with NADH. As was observed with 1-nitropyrene, the addition of 1 mM S-acetylcoenzyme A to the incubations in the presence of NADH resulted in the quantitative conversion of 1-amino-6-nitropyrene to N-acetyl-1-amino-6-nitropyrene.

Cytosolic nitrosoreductase activity, as measured by the succinoylated cytochrome c assay using 1-nitrosopyrene and 1-nitro-6-nitrosopyrene (Djuric et al. 1986b), was increased by about 20 percent in rats pretreated with 1-nitropyrene (Table 5). However, the reduction of succinoylated cytochrome c in cytosolic incubations with menadione, which can be used as a measure of DT-diaphorase activity

### Table 4. Reduction of 1,6-Dinitropyrene by Rat Liver Cytosol

<table>
<thead>
<tr>
<th>Pretreatment of Rats</th>
<th>Electron Donor (pmol/mg protein/min)</th>
<th>Inhibitor</th>
<th>1-Amino-6-nitropyrene</th>
<th>1-Nitro-6-nitrosopyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>NADPH</td>
<td>-</td>
<td>100 ± 30</td>
<td>460 ± 130</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>50 ± 10</td>
<td>490 ± 100</td>
<td></td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>NADPH</td>
<td>-</td>
<td>210 ± 10b</td>
<td>460 ± 130</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>50 ± 10c</td>
<td>660 ± 80</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>NADH</td>
<td>-</td>
<td>110 ± 10</td>
<td>390 ± 60</td>
</tr>
<tr>
<td>1-Nitropyrene</td>
<td>NADH</td>
<td>Dicoumarol</td>
<td>90 ± 10b</td>
<td>300 ± 70</td>
</tr>
</tbody>
</table>

\[a\] Incubations were conducted with 20 \(\mu\)M 1,6-dinitropyrene, 200 \(\mu\)g/ml cytosolic protein, 1 mM NADPH or NADH, and 50 mM potassium phosphate (pH 7.4), and were stopped after 10 minutes by adding two volumes of a mixture of chloroform and methanol (1:1). Anaerobic incubations were conducted under argon and contained an oxygen-scavenging system of glucose, glucose oxidase, and catalase. Some incubations also contained 100 \(\mu\)M dicoumarol. The results are presented as the mean ± mean variance of duplicate incubations.

\[b\] Significantly different from vehicle control \((p < 0.05)\).

\[c\] Significantly different from incubations conducted in the absence of an inhibitor \((p < 0.05)\).

### Table 5. Reduction of Succinoylated Cytochrome c in Rat Liver Cytosolic Incubations

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pretreatment of Rats</th>
<th>Rate of Succinoylated Cytochrome c Reduction (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Nitrosopyrene</td>
<td>Vehicle</td>
<td>102.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>1-Nitropyrene</td>
<td>121.3 ± 16.5</td>
</tr>
<tr>
<td>1-Nitro-6-nitrosopyrene</td>
<td>Vehicle</td>
<td>39.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>1-Nitropyrene</td>
<td>52.4 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>Menadione</td>
<td>123.6 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>1-Nitropyrene</td>
<td>110.8 ± 10.0</td>
</tr>
<tr>
<td>Menadioneb</td>
<td>Vehicle</td>
<td>109.5 ± 13.4</td>
</tr>
<tr>
<td></td>
<td>1-Nitropyrene</td>
<td>98.8 ± 11.7</td>
</tr>
</tbody>
</table>

\[a\] Incubations were conducted at 37°C and 550 nm, essentially as described by Kuthan and coworkers (1982), and consisted of 100 \(\mu\)g/ml cytosolic protein, 100 \(\mu\)M NADPH, 30 \(\mu\)M succinoylated cytochrome c, and 20 \(\mu\)M 1-nitrosopyrene or 1-nitro-6-nitrosopyrene, or 50 \(\mu\)M menadione in a total volume of 1 ml. The results are presented as the mean ± mean variance of duplicate incubations.

\[b\] In these incubations, 100 \(\mu\)M NADH was substituted for NADPH.
was similar in both control and 1-nitropyrene-pretreated cytosols with either NADPH or NADH as electron donors.

**RAT LIVER CYTOSOL CATALYZED DNA BINDING IN VITRO**

The increase in nitroreductase activity of hepatic cytosol was also assayed by quantifying the in vitro DNA binding of 1-nitropyrene and 1,6-dinitropyrene. Pretreating rats with 1-nitropyrene caused a 1.5-fold increase in the cytosol-catalyzed binding of 1-nitropyrene to DNA and a 3-fold increase with 1,6-dinitropyrene (Table 6). In previous experiments, we found that the addition of S-acetylcoenzyme A to cytosolic incubations resulted in much higher levels of DNA binding by the dinitropyrenes, due to O-acetylation of the N-hydroxyarylamine intermediates (Djuric et al. 1985). Thus, the binding to DNA by 1,6-dinitropyrene was also quantified in the presence of S-acetylcoenzyme A (Table 6). The binding was twofold higher when using cytosol from rats pretreated with 1-nitropyrene than when using cytosol from control rats, which corresponded to the twofold increase in nitroreductase activity (Table 4).

**HAMSTER LIVER CYTOSOL CATALYZED DNA BINDING IN VITRO**

The extent of DNA binding by 1,8-dinitropyrene was quantified in incubations conducted with cytosol from the livers of hamsters. In the presence of S-acetylcoenzyme A, cytosol from the slow-acetylator phenotype hamster strains, Bio. 1.5 and 87.20, catalyzed two-to-three times the binding of 1,8-dinitropyrene to DNA as was observed with the fast-acetylator phenotype strain, Bio. 87.20 (Table 7). In the absence of S-acetylcoenzyme A, the binding was less than 10 pmol of 1,8-dinitropyrene per milligram of DNA. The adducts from the incubations supplemented with S-acetylcoenzyme A were analyzed by HPLC after hydrolysis with trifluoroacetic acid. In each instance, a single peak of radioactivity was observed. When \( \text{N-2-aminofluorene} \) was used as the substrate, cytosol from strain Bio. 87.20 catalyzed two-to-four times more binding than did the other two strains (Table 7). As shown in Table 8, the extent of metabolism of 1,8-dinitropyrene was similar with strains Bio. 1.5 and 87.20; thus, the difference in the extent of cytosol-catalyzed DNA binding of 1,8-dinitropyrene was not a result of differences in nitroreduction.

**DNA BINDING IN HAMSTERS IN VIVO**

Bio. 1.5 and 87.20 hamsters were given a single intraperitoneal injection of 1.5 mg/kg body weight of 1,8-dinitropyrene, and after 24 hours the extent of DNA binding was assayed in the liver, bladder, and intestinal epithelium (Table 9). As was observed with the liver cytosol incubations, there was more binding to hepatic DNA in strain Bio. 1.5 than in strain Bio. 87.20; however, these differences were not statistically significant. With bladder DNA, there was more binding in strain Bio. 1.5, but again, this was not statistically significant. The same extent of binding was observed in the intestine DNA from both strains.

**DNA BINDING IN MICE IN VIVO**

In an initial experiment, A/J and C57BL/6j mice were given a single intraperitoneal dose of 5.1 \( \mu \text{mol/kg body weight of} \)

---

**Table 6. DNA Binding Catalyzed by Rat Liver Cytosol in Vitro**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pretreatment of Rats</th>
<th>Without S-Acetylcoenzyme A</th>
<th>With S-Acetylcoenzyme A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Nitropyrene</td>
<td>Vehicle</td>
<td>0.8 ± 0.1</td>
<td>ND^b</td>
</tr>
<tr>
<td></td>
<td>1-Nitropyrene</td>
<td>1.2 ± 0.0^c</td>
<td>ND</td>
</tr>
<tr>
<td>1,6-Dinitropyrene</td>
<td>Vehicle</td>
<td>5 ± 1</td>
<td>52 ± 4</td>
</tr>
<tr>
<td></td>
<td>1-Nitropyrene</td>
<td>15 ± 6</td>
<td>90 ± 11^c</td>
</tr>
</tbody>
</table>

^a DNA binding was determined in incubations conducted under argon with 200 \( \mu \text{g cytosolic protein, 1 mM NADPH, 100 mM FMN, 20 mM tritiated 1-nitropyrene or 1,6-dinitropyrene (1 Ci/mmol), and an oxygen-scavenging system consisting of glucose, glucose oxidase, and catalase in a total volume of 1 ml. Incubations were stopped after 15 minutes by adding phenol. The DNA was extracted further and the binding was quantified, as described in the Methods section. Some incubations also contained 1 mM S-acetylcoenzyme A. The results are presented as the mean ± mean variance of duplicate incubations.}

^b ND = not done. Previous experiments (Djuric et al. 1985) have indicated that the binding of 1-nitropyrene to DNA is not increased by adding S-acetylcoenzyme A.

^c Significantly different from vehicle control (\( p < 0.05 \)).
Table 7. DNA Binding Catalyzed by Hamster Liver Cytosol in Vitro

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Strain</th>
<th>1,8-Dinitropyrene (pmol/mg DNA)</th>
<th>N-hydroxy-2-aminofluorene (pmol/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bio. 1.5</td>
<td>299 ± 53c (2)</td>
<td>199 ± 34c (2)</td>
</tr>
<tr>
<td></td>
<td>Bio. 82.73</td>
<td>254 ± 16c (2)</td>
<td>320 ± 44c (2)</td>
</tr>
<tr>
<td></td>
<td>Bio. 87.20</td>
<td>94 ± 20 (3)</td>
<td>714 ± 80 (3)</td>
</tr>
<tr>
<td>2</td>
<td>Bio. 1.5</td>
<td>332 ± 59d (3)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Bio. 87.20</td>
<td>171 ± 38 (3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

DNA binding was determined in incubations conducted under argon at 37°C with 1 mg cytosolic protein, 1 mM NADPH, 1 mM S-acetylcoenzyme A, and 20 μM tritiated 1,8-dinitropyrene (1 Ci/mmol) or N-hydroxy-2-aminofluorene (100 Ci/mmol) in 50 mM sodium phosphate and 1 mM dithiothreitol (pH 7.4). Incubations were stopped after 15 minutes by adding phenol. The DNA was extracted further and the binding was quantified, as described in the Methods section. In the absence of S-acetylcoenzyme A, the binding was less than 10 pmol/mg DNA. The results are presented as the mean ± SD.

DISCUSSION

In this study we have compared the extents of DNA adduct formation by 1-nitropyrene and 1,6-dinitropyrene to determine if this difference could account for the reported differences in tumorigenesis between these two compounds. When rats were treated in vivo with [4,5,9,10-3H]1-nitropyrene, we found substantial radioactivity associated with liver, lung, and kidney DNA. However, this radioactivity did not migrate with the DNA upon centrifugation through cesium chloride, and HPLC analyses of DNA hydrolysates did not indicate the presence of DNA adducts such as N-(deoxyguanosin-8-yl)-1-nitropyrene. These results do not agree with data presented by Stanton and colleagues (1985) and Hashimoto and Shuda (1985). The discrepancy does not appear to be due to differences in the doses administered (200 μg/kg body weight in our experiment compared to 25 to 30 mg/kg body weight in the previ-

Table 8. Metabolism of 1,8-Dinitropyrene by Hamster Liver Cytosol in Vitro

<table>
<thead>
<tr>
<th>Strain</th>
<th>S-Acetylcoenzyme A</th>
<th>1-Acetylaminoo-8-nitropyrene</th>
<th>1-Amino-8-nitropyrene</th>
<th>1-Nitro-8-nitrosopyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio. 1.5</td>
<td>+</td>
<td>47.9 ± 3.8</td>
<td>–</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>48.0 ± 2.8</td>
<td>13.3 ± 3.7</td>
</tr>
<tr>
<td>Bio. 87.20</td>
<td>+</td>
<td>50.6 ± 9.3</td>
<td>2.7 ± 1.8</td>
<td>23.3 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>57.3 ± 5.7</td>
<td>–</td>
</tr>
</tbody>
</table>

 Incubations were conducted under argon at 37°C with 1 mg cytosolic protein, 1 mM NADPH, with or without 1 mM S-acetylcoenzyme A, and 20 μM tritiated 1,8-dinitropyrene (1 Ci/mmol) in 50 mM sodium phosphate and 1 mM dithiothreitol (pH 7.4). Incubations were stopped after 15 minutes by adding two volumes of a mixture of chloroform and methanol (1:1). The metabolites were analyzed as described in the Methods section. The results are presented as the mean ± mean variance of duplicate incubations expressed in pmol/mg protein/min.
ous studies) because we obtained essentially the same results in a second experiment using a dosage of 30 mg/kg body weight of 1-nitropyrene. One possible explanation for these differences in results is the purity of the 1-nitropyrene, which has been a problem for both mutagenesis assays (Beland et al. 1985) and tumorigenesis assays (Ohgaki et al. 1985). With the HPLC conditions usually used, we have found that C8-deoxyguanosine adducts obtained from dinitropyrenes are difficult to resolve from the analogous adduct of 1-nitropyrene, that is, N-(deoxyguanosin-8-yl)-1-aminopyrene. On the basis of the level of binding we detected from 200 μg/kg body weight of 1,6-dinitropyrene (Table 1), if a sample of 1-nitropyrene contained as little as 0.2 percent dinitropyrene contamination, the binding by the dinitropyrene could account for the adducts attributed to 1-nitropyrene when the latter compound was administered at a dose of 30 mg/kg body weight.

In contrast to 1-nitropyrene, a single major DNA adduct was clearly present in rats treated with 1,6-dinitropyrene (Figure 1). This adduct appeared to be N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, which was the same adduct we observed previously in incubations with rat liver cytosol that had been fortified with S-acetylcoenzyme A (Djuric et al. 1985). This suggests that nitroreduction, and perhaps O-acetylation, is involved in the metabolic activation of 1,6-dinitropyrene in vivo. The highest levels of 1,6-dinitropyrene-DNA binding were detected in the bladder, followed by the liver, but there was also substantial binding to the DNA from the mammary gland, a target for 1,6-dinitropyrene tumorigenesis after intraperitoneal administration (Imaiida et al. 1986; King 1987). Thus, the differences in binding between 1-nitropyrene and 1,6-dinitropyrene correlate with the relative tumorigenicity of the two compounds.

Since the adduct detected in vivo from 1,6-dinitropyrene was indicative of nitroreduction involvement in the metabolic activation, additional experiments were conducted in which nitroreductases were induced to determine if this induction could affect the subsequent DNA binding of 1-nitropyrene and 1,6-dinitropyrene. In these experiments, rats were pretreated with 1-nitropyrene because it has been reported to induce nitroreductase activity (Chou et al. 1987) and is the major nitro-PAH in diesel emissions (Beland et al. 1985; Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986). Liver microsomal and cytosolic nitroreductase activities were increased twofold in rats treated intraperitoneally with 1-nitropyrene. In the microsomal fraction, cytochrome P-450 appeared to play a central role, and our results are in agreement with the findings of Saito and colleagues (1984) in that microsomal nitroreduction of 1-nitropyrene could be inhibited by DPEA but not by SKF-525A. This DPEA-mediated inhibition was more pronounced with 1-nitropyrene-induced microsomes.

The microsomal nitroreduction with either control or in-

Table 10. Binding of 2-Aminofluorene, 1,6-Dinitropyrene, and 1,8-Dinitropyrene to Liver DNA in Mice in Vivo

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Compound</th>
<th>Dose (μmol/kg)</th>
<th>Strain</th>
<th>Adducts per 10⁶ DNA Nucleotides²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-Aminofluorene</td>
<td>5.1</td>
<td>A/J</td>
<td>0.29 ± 0.07 (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C57BL/6J</td>
<td>0.30 ± 0.03 (2)</td>
</tr>
<tr>
<td>1,8-Dinitropyrene</td>
<td>5.1</td>
<td>A/J</td>
<td>2.32 ± 0.27 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C57BL/6J</td>
<td>1.66 ± 1.53 (4)</td>
</tr>
<tr>
<td>2</td>
<td>2-Aminofluorene</td>
<td>51</td>
<td>A/J</td>
<td>2.67 ± 2.20 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C57BL/6J</td>
<td>0.79 ± 0.29 (4)</td>
</tr>
<tr>
<td>1,6-Dinitropyrene</td>
<td>51</td>
<td>A/J</td>
<td>2.67 ± 2.20 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C57BL/6J</td>
<td>0.79 ± 0.29 (4)</td>
</tr>
</tbody>
</table>

² Mice were treated intraperitoneally with a single dose of [ring-¹H₂]2-aminofluorene (75 mCi/mmol), [4,5,9,10-²H]1,6-dinitropyrene (1,200 mCi/mmol), or [4,5,9,10-²H]1,8-dinitropyrene (966 mCi/mmol) at the dose indicated. After 24 hours, the animals were killed and the binding to hepatic DNA was analyzed, as indicated in the Methods section. The results are presented as the mean ± SD.

b Number of animals is given in parentheses.

c Insufficient radioactivity for binding measurement.
duced microsomes of 1,6-dinitropyrene differed in several respects from that of 1-nitropyrene (Table 2). During the metabolism of 1,6-dinitropyrene, the intermediate reduction product, 1-nitro-6-nitrosopyrene, was detected, while with 1-nitropyrene only the terminal reduction product, 1-amino-6-nitropyrene, was found. This difference may be due to differences in the reduction potentials of these compounds. The initial reduction of 1-nitropyrene, as measured by its half-wave potential, is considerably more difficult ($\varepsilon_{1/2} = -1.03$ V; Djurić and Beland, unpublished data) than the initial reduction of 1-nitrosopyrene ($\varepsilon_{1/2} = -0.46$ V). In contrast, the reduction potentials of 1,6-dinitropyrene and 1-nitro-6-nitrosopyrene are similar ($-0.50$ and $-0.55$ V, respectively), which could allow significant concentrations of 1-nitro-6-nitrosopyrene to be formed. Another difference between the microsomal nitroreduction of 1,6-dinitropyrene and 1-nitropyrene is that SKF-525A completely inhibited the reduction of the former but not the latter. This suggests that there may be different cytochrome P-450 isozymes involved in the nitroreduction of these two nitropyrenes. The isozyme selectivity could result from differences in the electron densities of the respective nitro groups, which is reflected in the higher reduction potential of 1-nitropyrene. Similar differences in isozyme specificity are important for the metabolism of 2-nitrofluorene and nitroquinoline oxide to mutagens (Kawano et al. 1985).

There also appeared to be differences between the microsomal reduction of 1,6-dinitropyrene and 1-nitro-6-nitrosopyrene. If it is assumed that 1-amino-6-nitropyrene was formed through a 1-nitro-6-nitrosopyrene intermediate, then it can be calculated from Table 2 that DPEA and CO inhibited the formation of 1-amino-6-nitropyrene to a greater extent (80 to 100 percent) than the formation of 1-nitro-6-nitrosopyrene (70 to 80 percent). This difference may be due to the fact that the concentration of the nitro substrate is greater than that of the nitroso metabolite. In addition, different cytochrome P-450 isozymes may be involved in the reduction of the nitro and nitroso derivatives, and these may have different Michaelis constants ($K_m$) for CO and DPEA, as well as for the substrate. NADPH-cytochrome P-450 reductase may also be involved in the reduction process, and this may be more important for nitroreduction than for nitrosoreduction, as has been suggested for the reduction of nitrobenzene (Harada and Omura 1980).

The cytosolic nitroreductase activity toward 1-nitropyrene has been shown to involve xanthine oxidase, aldehyde oxidase, and other unknown NADPH- and NADH-dependent enzymes (Nachtmann and Wei 1982; Tatsumi et al. 1986). Our results indicated that 1-nitropyrene pretreatment increased nitroreductase activity of hepatic cytosol (using both 1-nitropyrene and 1,6-dinitropyrene as substrates) about twofold with either NADH or NADPH as electron donors. This did not appear to be due to an increase in DT-diaphorase, since the NADH-dependent nitroreduction was not inhibited significantly by dicoumarol (Tables 3 and 4) and there was no increase in cytosolic reduction of menadione (Table 5). Thus, the nature of the NADH-dependent nitroreductase remains unknown. A twofold increase in nitroreductase activity was also observed when quantifying the reduction of 1-nitropyrene with electron donors specific for xanthine oxidase (hypoxanthine) and aldehyde oxidase (1-methylnicotinamide), as shown in Table 3. 1,6-Dinitropyrene, however, did not appear to be a good substrate for these latter two enzymes. Nitrosoreductase activity of both microsomes and cytosol was measured using the succinoylated cytochrome c assay (Djurić et al. 1986b). Microsomal nitrosoreductase activity was not increased by 1-nitropyrene pretreatment, while cytosolic nitrosoreductase activity was only slightly elevated (approximately 20 percent) (Table 5). Thus, the twofold increase in 1-amino-6-nitropyrene and 1-amino-6-nitrosopyrene formation in both microsomal and cytosolic incubations from rats pretreated with 1-nitropyrene (Tables 2 through 4) is more likely due to an increase in nitroreductase activity, which suggests that while 1-nitropyrene pretreatment did not increase the binding of a mixture of...
dinitropyrene to lung DNA (Howard et al. 1986). Therefore, while the DNA adduct formed by 1,6-dinitropyrene in vivo indicates that nitroreduction is involved in its metabolic activation, the lack of increase in DNA binding by 1,6-dinitropyrene upon the induction of nitroreductases suggests that other factors (for example, O-acetylation of N-hydroxyarylamine intermediates) limit the extent of DNA binding by this compound in vivo.

The importance of acetylation in the metabolic activation of dinitropyrene was addressed using two animal models, hamsters and mice. Hamsters were selected for our initial experiments because, like humans, they are polymorphic for acetylation, with both rapid and slow phenotypes being observed (Weber and Hein 1985). In addition, both hamsters and humans have slightly acidic urine and are susceptible to the induction of bladder cancer by aromatic amines. In vitro incubations were conducted with hamster liver cytosol and 1,8-dinitropyrene in the presence and absence of S-acetylcoenzyme A. Three strains were examined: Bio. 87.20, which has been characterized as a "rapid" acetylator, and Bio. 1.5 and 82.73, which have been designated as "slow" acetylators (Weber and Hein 1985). With N-hydroxy-2-aminofluorene, there was two-to-three times more binding with the cytosol from strain Bio. 87.20 than from the other two strains (Table 7), which is consistent with the data reported by Hein and colleagues (1987). With 1,8-dinitropyrene, the opposite relationship was observed; two-to-three times more binding was detected with cytosol from strains Bio. 1.5 and 82.73. A similar difference was observed in vivo, although the magnitude was not as great; when single doses of 1,8-dinitropyrene were administered, there was 1.5-fold more binding to the liver and bladder DNA of strain Bio. 1.5 than of strain Bio. 87.20 (Table 9). The reasons for the difference between N-hydroxy-2-aminofluorene and 1,8-dinitropyrene are presently not known. However, it should be noted that the acetylation phenotype in these hamsters has been characterized by their ability to N-acetyl-p-aminobenzoic acid. Both polymorphic and monomorphic enzymes exist, and their relative ability to N- and O-acetylate appears to depend upon the particular substrate being examined (Hein et al. 1987). The mutation that causes the polymorphism of N- and O-acetylase activities has not been characterized, but it is conceivable that with certain substrates decreased activity will be observed, while with other compounds increased catalytic activity will be found.

While these experiments were being conducted, Levy and Weber (1988) reported marked differences in the ability of certain strains of mice to activate 2-aminofluorene to a DNA-binding derivative. In particular, C57BL/6j mice, which are characterized as rapid acetylators, formed five times as many hepatic 2-aminofluorene DNA adducts as A/J mice, which are slow acetylators. We considered this to be an interesting observation in light of the recent reports that certain nitro-PAHs are tumorigenic in mice (El-Bayoumy et al. 1984; Wislocki et al. 1986). If confirmed, this finding would suggest that mice are an appropriate animal model to elucidate the role of acetylation in aromatic amine and nitro-PAH tumorigenesis. In preliminary experiments, we were not able to repeat the results of Levy and Weber (1988) with 2-aminofluorene in that we obtained slightly higher binding to hepatic DNA of A/J mice administered 2-aminofluorene (Table 10). Likewise, in mice administered single doses of 1,6-dinitropyrene, there was threefold more binding to the hepatic DNA of A/J mice than to the hepatic DNA of C57BL/6j mice. This may be due to differences in the experimental protocols; Levy and Weber used considerably higher doses of 2-aminofluorene and killed their animals two hours after treatment. Nevertheless, these preliminary results support the hypothesis that acetylation is a critical factor in the activation of dinitropyrenes in vivo.

ACKNOWLEDGMENTS

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REFERENCES


ABOUT THE AUTHOR

Frederick A. Beland received a Ph.D in chemistry from Montana State University in 1974 and was a postdoctoral fellow with Dr. Ronald G. Harvey at the University of Chicago. He is currently Director of the Division of Biochemical Toxicology at the National Center for Toxicological Research, and Adjunct Associate Professor of Biochemistry at the University of Arkansas for Medical Sciences. Dr. Beland's primary research interests are the mechanisms of chemical carcinogenesis and mutagenesis.

PUBLICATION RESULTING FROM THIS RESEARCH


ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DPEA</td>
<td>2-[(2,4-dichloro-6-phenylphenoxy)-ethylamine</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>nitro-PAHs</td>
<td>nitro-derivatives of polycyclic aromatic hydrocarbons</td>
</tr>
</tbody>
</table>
INTRODUCTION

In the fall of 1985, the Health Effects Institute (HEI) issued a Request for Preliminary Applications (RFPA 85–3) to solicit proposals on "Health Effects of Automotive Emissions." In response to this request, Dr. Frederick A. Beland, from the University of Arkansas for Medical Sciences, sent a preliminary letter and, subsequently, a full proposal for a study entitled, "Importance of O-Acetylation and Ring Oxidation in the Metabolic Activation of Nitrated Pyrenes." After the proposal was reviewed by experts in the field and by the HEI Research Committee, the HEI asked Dr. Beland to modify his original two-year proposal to a one-year pilot study by deleting some sections of the proposed study. The modified study was approved in July 1986. The project began in October 1986, and total expenditures for the study were $61,185. The Investigator's Report for the project was submitted in August 1987. The final Investigator's Report, which included a title change to "DNA Binding by 1-Nitropyrene and Dinitropyrenes in Vitro and in Vivo: Effects of Nitroreductase Induction," was accepted by the Health Review Committee in July 1988.

During the review of the Investigator's Report, the Review Committee and the investigator had the opportunity to exchange comments and to clarify issues in the Investigator's Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigator's Report in perspective, as an aid to the sponsors of the HEI and to the public.

THE CLEAN AIR ACT

The U.S. Environmental Protection Agency (EPA) sets standards for diesel (and other) emissions under Section 202 of the Clean Air Act, as amended in 1977. Section 202(a)(1) directs the Administrator to "prescribe (and from time to time revise) . . . standards applicable to the emissions of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a)(3)(A)(i) specifically directs the Administrator to "prescribe regulations . . . applicable to emissions of carbon monoxide, hydrocarbons, and oxides of nitrogen from classes . . . of heavy-duty vehicles or engines." Section 202(a)(3)(A)(iii), similarly, requires regulations applicable to emissions of particulate matter from classes or categories of vehicles.

Under these provisions, the EPA has taken regulatory actions with respect to diesel engines. In 1980, the EPA set light-duty diesel particulate matter standards, and, in 1984, granted a two-year delay in their effective date. The EPA established emissions averaging in 1983, and it set nitrogen oxides standards in 1985. In 1988, the agency established revised particulate standards for certain light-duty diesel trucks. In May 1989, the EPA proposed new certification programs for banking and trading of oxides of nitrogen and particulate credits for diesel-, gasoline-, and methanol-fueled heavy-duty engines. For heavy-duty diesel engines, the EPA set hydrocarbon and carbon monoxide standards in 1985. In addition, under Section 109 of the Act, the EPA has established National Ambient Air Quality Standards for particulate matter. Those standards were most recently revised in July 1997.

Research on the mutagenic and carcinogenic effects of diesel-engine emission components contributes to an increased understanding of the risks to humans from exposure to these components. This research can thereby contribute to informed decision making for standards within the Clean Air Act.

BACKGROUND

Exhaust emissions from diesel engines contain a diverse variety of compounds adsorbed to the soot particles as well as the gases that are produced by the combustion process. During the oil crisis of the mid-1970s, concern arose that increased use of vehicles equipped with the more fuel-efficient diesel engines would increase the levels of airborne particulates and gases produced by these engines. During this same time, several investigations showed that organic solvent extracts of diesel particles, particularly the nitroaromatic fraction containing nitropyrenes, were mutagenic to bacteria (Lewtas 1982). Subsequent studies indicated that these extracts also produced mutagenicity and chromosomal damage in eukaryotic cells (Claxton 1983; Lewtas 1983). More recent studies have shown that chronic inhalation of high levels of diesel-engine emissions induced tumors in the lungs of laboratory rodents (Brightwell et al. 1986; Heinrich et al. 1986; Ishinishi et al. 1986; Iwai et al. 1986; Mauderly et al. 1986). Several epidemiological studies have also suggested an association between chronic exposure to diesel exhaust and an increased risk of lung and bladder cancer in humans (Silverman et al. 1986; Steenland 1986; Garshick et al. 1987, 1988). These findings have prompted research to elucidate the potential mechanisms of toxicity of components in diesel-engine exhaust,
and how these mechanisms may contribute to the induction of cancer.

This study focuses on nitropyrenes, a group of genotoxic compounds present in diesel-engine exhaust. Nitropyrenes are polycyclic aromatic hydrocarbons that contain one or more nitrogen groups. They are products of reactions between the aromatic hydrocarbons and nitrogen oxides that are produced during fuel combustion in diesel engines. Although they can exist in crystalline form in the exhaust, nitropyrenes are predominantly adsorbed onto the diesel soot particles. These particles range in size from 0.1 to 0.3 µm (Tokiwa and Ohnishi 1986), and it is estimated that, after inhalation, 25 to 35 percent of the particles in this size range deposit in the deep alveolar regions of the lungs (McClellan 1987). Particle retention is believed to increase the bioavailability and potential toxicity of nitropyrenes bound to the particles. Natural defenses in the lungs against deposited materials include ciliated cells that line airway epithelia and sweep these particles out of the lungs, and phagocytosis of the particles by airway and alveolar macrophages. However, the increased incidence of cancer in the lungs of animals after chronic diesel-exhaust exposure indicates that these pulmonary defenses are not adequate to completely protect the lungs against this disease.

Amounts of 1-nitropyrene in diesel-engine exhaust exceed those of the dinitropyrenes, and, although 1-nitropyrene is mutagenic in bacterial assays, in vivo tests indicate that it may be only a weak carcinogen or a noncarcinogen. In contrast, several nitropyrenes, particularly 1,6-dinitropyrene and 1,8-dinitropyrene, are both potent mutagens and carcinogens (Rosenkranz and Howard 1986). The types of tumors produced by nitropyrenes depend upon the animal species and route of administration (Tokiwa and Ohnishi 1986; Hecht 1988; King 1988). 1-Nitropyrene has been shown to produce tumors in newborn rats at the site of subcutaneous injection and in the mammary gland, but it has not consistently produced tumors when tested in several species of adult animals. Dinitropyrenes produce a variety of tumors, including malignant fibrous histiocytomas, sarcomas, adenocarcinomas, and squamous cell carcinomas. Lung tumors and leukemias have been induced by 1,6-dinitropyrene.

Unrepaired or incorrectly repaired DNA damage is regarded as a potential first step in the transformation of a normal cell to a cancer cell. DNA adducts may be important progenitors of the mutations produced by xenobiotic chemicals such as nitropyrenes. After nitropyrenes enter cells, enzymatic biotransformation reactions produce arylamine derivatives that covalently bind to DNA to form DNA adducts. These adducts induce mutations and may activate oncogenes (Tahira et al. 1986). Therefore, DNA adducts may serve as valuable indicators of the effective dose of a particular compound to a target tissue (Hecht 1988).

It is unclear whether or not the extent of DNA adduct formation detected in a particular tissue correlates with tumors induced by a particular nitropyrene in that same tissue. The formation and subsequent detection of DNA adducts in an organ or tissue depend upon several interconnected factors. In whole-animal studies, the delivery of nitropyrenes to tissues is influenced by both the route of administration and the physiological state of the animal. Bioactivation of nitropyrenes by intestinal flora can also play a role (Rosenkranz and Mermelstein 1985). Additional factors must be considered at the cellular level (Shaikh et al. 1988). First, what are the types and the extent of the enzymatic biotransformation systems in a particular tissue, and is the compound detoxified or activated by these systems? Second, if the compound is metabolized to an electrophilic reactant, what is its reactivity with DNA nucleophiles? Third, how effective are that tissue's enzyme systems for DNA repair in detecting and removing specific DNA adducts?

Two biotransformation reactions, nitroreduction and acetylation, appear to be critical steps in the activation of nitropyrenes. Nitroreductases, which are present in both bacterial and animal cells, produce potentially reactive forms of nitropyrenes by reducing the nitro group to the corresponding arylhydroxylamine (Tokiwa and Ohnishi 1986). Nitroreduction is a pivotal reaction in the biotransformation of nitropyrenes, which is illustrated by the fact that nitroreductase-deficient strains of bacteria are much less susceptible to mutagenesis (Rosenkranz and Mermelstein 1983). Acetylation, a reaction that adds an acetyl group to a nitrogen or oxygen atom in a compound, appears to be the important sequential step in biotransformation, particularly for dinitropyrenes. McCoy and coworkers (1982, 1983) reported that, in the Salmonella typhimurium assay system, O-acetylation follows nitroreduction in the process by which dinitropyrenes, but not 1-nitropyrene, are bioactivated into mutagens. Similar biotransformations have been described in mammalian systems (Đurić et al. 1985). In addition, ring oxidation of 1-nitropyrene, but not 1,6- and 1,8-dinitropyrenes, occurs; this, however, appears to be a potential detoxification pathway.

Although N- and O-acetylation, as well as N,O-acetyltransfer, can occur, it is not clear which of these pathways is important in the metabolism of nitropyrenes in different mammalian systems. Humans, as well as other species such as hamsters, mice, rats, and rabbits, have been categorized as “fast” or “slow” acetylator phenotypes on the basis of their N-acetyltransferase activity (Weber and Hein 1985). Humans, like these other species, exhibit polymorphic distri-
bution between these phenotypes, and some evidence suggests that hereditary differences in N-acetylator status may be related to certain cancers induced by aromatic amines. Studies with bladder cancer patients who have a history of exposure to aromatic amines, compounds that structurally resemble metabolites of nitropyrenes, indicate that these patients are more likely to be slow acetylaters (Cartwright et al. 1982). Specific animal strains that have been identified as fast or slow acetylaters can be useful research models with which to examine the importance of this enzymatic reaction in the biotransformation of toxic compounds, which potentially include nitropyrenes.

There is, as yet, no conclusive evidence that either air pollution or specific components of air pollution, such as the nitropyrenes in diesel-engine exhaust, produces cancer in humans. However, as previously discussed, animal and epidemiological studies suggest that chronic exposure to diesel-engine exhaust may be associated with an increased risk of cancer development. Nitropyrenes are important suspects in this link because of their potent genotoxicity in vitro and in vivo test systems. It has been suggested that, in addition to acting as substrates for biotransformation enzymes such as cytochrome P-450 isozyme, nitropyrenes may also induce synthesis of those same enzymes and potentiate the production of the reactive metabolites (Rosenkranz and Mermelstein 1985). Metabolic activation of compounds to DNA-reactive intermediates is currently regarded as the initial event that may lead to mutation and, potentially, to neoplastic transformation. Because it is not known whether or not the mechanisms for induction of mutations in bacteria and for neoplastic changes in mammalian tissues by nitropyrenes are similar, detection and measurement of DNA adducts in target tissues and evaluation of factors that may affect their formation are important areas for research.

JUSTIFICATION FOR THE STUDY

In 1985, the HEI solicited, by a preliminary application process, proposals for studies on novel and important aspects of the health effects of automotive emissions, including the potential health effects caused by exposure to diesel-engine exhaust. Because nitropyrenes are potent mutagens and carcinogens both in vitro and in vivo, studies to elucidate their role in the toxicity of diesel-engine emissions were of particular interest. The Research Committee viewed the study proposed by Dr. Beland concerning the biochemistry and metabolism of nitropyrenes, and the associated production of DNA adducts as an important link to understanding the genotoxic mechanisms of these compounds.

OBJECTIVES OF THE STUDY

The overall objectives of the study by Dr. Beland were to detect DNA adducts that are produced in the tissues of laboratory animals by exposure to nitropyrenes and to identify the biotransformation reactions that are important for the formation of such adducts. Dr. Beland also wanted to determine whether or not the extent of DNA adduct formation by a given nitropyrene correlated with its tumorigenicity in vivo. His specific aims were stated as follows: (1) to determine and compare the extent of DNA adduct formation in vivo by 1-nitropyrene and dinitropyrenes and to determine the effect of nitropyrene pretreatment on the subsequent metabolism and DNA binding of these compounds; and (2) to determine the importance of the acetylation phenotype in the metabolic activation of dinitropyrenes to DNA-binding derivatives in vivo.

STUDY DESIGN

To evaluate the effect of nitropyrene pretreatment on DNA adduct formation, groups of rats were injected intraperitoneally either with 1-nitropyrene in a vehicle or with the vehicle only. After three days, animals from each of these groups were killed for DNA adduct analyses. Remaining animals from these groups were injected with either radiolabeled 1-nitropyrene or radiolabeled 1,6-dinitropyrene, and then killed one day later for analyses of DNA adducts in various organs.

To examine the role of the acetylation phenotype in DNA adduct formation, three strains of hamsters and two strains of mice (two to four animals per group) that had been identified as slow or fast acetylator phenotypes were used. Hamsters were injected with radiolabeled 1,8-dinitropyrene and killed one day later for DNA adduct analyses in several different tissues. Mice were injected with radiolabeled 1,6-dinitropyrene or radiolabeled 2-aminofluorene and killed one day later for adduct analyses in liver tissue only.

SUMMARY OF INVESTIGATOR'S CONCLUSIONS

Dr. Beland reported that no covalently bound radiolabeled DNA, which would indicate the presence of adducts, was found in any of the tissue samples from animals injected with radiolabeled 1-nitropyrene, whether or not the animals had been pretreated with 1-nitropyrene. In contrast, after radiolabeled 1,6-dinitropyrene treatment, DNA from several different tissues coeluted with the synthetic
adduct marker \(N\)-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, indicating DNA adduct formation. Pretreatment with 1-nitropyrene increased the extent of this binding in several organs compared with vehicle-treated control animals. In addition to the kidney, bladder, and lungs, this adduct was found in the mammary gland of rats, one site of tumor formation when 1,6-dinitropyrene is injected intraperitoneally. Dr. Beland concludes that these findings correlate with the tumorigenicity, or the lack thereof, for these two nitropyrenes.

Enzymatic analyses were used to evaluate the role of nitroreduction in the in vivo bioactivation of nitropyrenes. After 1-nitropyrene pretreatment, enzyme assays showed that nitroreductase activity increased twofold in both the microsomal and cytosolic fractions of rat liver. On the basis of enzyme inhibition data, the activities toward 1-nitropyrene and 1,6-dinitropyrene appeared to be dissimilar. This increase in nitroreductase activity was accompanied by an apparently enhanced binding of nitropyrene to DNA.

Comparison of DNA adduct formation between fast and slow acetylator strains of hamsters and mice showed that the slow acetylator phenotypes of both species formed more adducts with dinitropyrenes than the fast acetylator strains. Slightly more binding of 2-aminofluorene was also detected in mice with the slow acetylator phenotype.

**TECHNICAL EVALUATION**

**ATTAINMENT OF STUDY OBJECTIVES**

The major goals of Dr. Beland’s study were met successfully. This investigation provided information that adds to our understanding of the metabolism of nitropyrenes and how they are activated to form DNA adducts.

**ASSESSMENT OF METHODS AND STUDY DESIGN**

The experiments presented in this report were well designed and well conducted. The data generated are based upon sound experimental procedures; state-of-the-art methods were used in the study, some of which were developed in Dr. Beland’s laboratory.

The author had proposed to study DNA binding in hamsters that had received radiolabeled 1-nitropyrene or 1,3-, 1,6-, or 1,8-dinitropyrene. Although both 1,6- and 1,8-dinitropyrene were used in the preliminary DNA binding studies in fast and slow acetylator mice, only 1,8-dinitropyrene was studied in hamsters and only 1,6-dinitropyrene was studied in mice. Dr. Beland reported that decreased availability of the radiolabeled 1,8-dinitropyrene accounted for this change. In addition, although the time constraints of this study precluded their inclusion in the study design, dose-response studies with the nitropyrenes for the different animal species would have been instructive.

Strains of hamsters and mice that were categorized as fast and slow acetylators were used to evaluate the importance of acetylation phenotype in the formation of DNA adducts. Dr. Beland supported his selection of hamsters on the grounds that their acetylation polymorphism is similar to that of humans and that both humans and hamsters are susceptible to bladder cancer caused by aromatic amines.

**STATISTICAL AND DATA ANALYSIS**

Two or three animals were used per treatment group, and the appropriate parametric test, the Student’s \(t\) test, was used to compare experimentally treated animals with vehicle-treated control animals. However, comparisons among such small sample sizes produce severe power problems unless there is essentially no variability attributable to laboratory errors and differences among animals. The lack of statistical significance in several assays, despite 50 to 300 percent changes produced by the experimental conditions, illustrates this problem. Because this was a pilot study and the number of animals was small, it would have been appropriate either to use a less stringent statistical test than the conventional \(p < 0.05\), or simply to report the statistical test values that were calculated from the data.

**INTERPRETATION OF RESULTS**

The interpretations and conclusions drawn by Dr. Beland from his study are appropriate. However, alternative interpretations are possible for some of the results, particularly with regard to DNA adduct formation and the choice of the hamster model for the acetylation studies.

Dr. Beland reported the unexpected finding that, in rats treated with radioactive 1-nitropyrene that was greater than 99.5 percent pure, he was unable to demonstrate the presence of \(N\)-(deoxyguanosin-8-yl)-1-aminopyrene or other DNA adducts. This result contrasts with the earlier findings by Dr. Beland and his co-workers (Heftich et al. 1985) that this adduct was formed in bacteria and cultured mammalian cells exposed to 1-nitropyrene. Dr. Beland’s current results also vary from the results of Stanton and coworkers (1985) and Hashimoto and Shudo (1985), who also administered radioactive 1-nitropyrene to rats and identified the putative adducts. On the basis of his findings with radioactive 1,6-dinitropyrene and the difficulty of separating the DNA adducts of 1-nitropyrene and 1,6- and 1,8-dinitro-
pyrene by high-pressure liquid chromatography. Dr. Beland argues that the results reported by these other investigators could be due to contamination of the 1-nitropyrene by as little as 0.2 percent dinitropyrene. This is a plausible explanation in view of the methods for synthesis and purification of 1-nitropyrene.

Overall, this negative finding is significant in light of the weak or borderline carcinogenicity of 1-nitropyrene compared with the clearcut carcinogenicity of 1,6- and 1,8-dinitropyrene. No covalent binding to DNA was found with radiolabeled 1-nitropyrene, whereas radiolabeled 1,6-dinitropyrene adducts were detected in the bladder, liver, mammary gland, kidneys, and lungs of rats. Dr. Beland reports that his data for DNA adduct formation correlate with the reported differences in carcinogenicity for these compounds. An alternative interpretation is that the initial metabolism of 1-nitropyrene in the cells produced DNA adducts that were not detected by the methods used in this study. The absence of DNA adducts after 1-nitropyrene treatment could also be attributed to inadvertent loss or degradation during the isolation procedures, or to spontaneous hydrolysis.

Several strains of hamsters and mice identified as fast or slow acetylators were used as model systems for the studies on O-acetylation. As previously discussed, the metabolism of aromatic amines by cytosolic enzymes in the liver is complex. As noted by Dr. Beland in his discussion, the acetylation polymorphisms of these strains of animals were defined by the ability of their hepatic N-acetyltransferase to acetylate p-aminobenzoic acid; however, this is only one of three acetyltransfer activities that could participate in the metabolism of nitropyrenes. Because acetylator phenotype is related only to certain N-acetylation reactions, the relevance of these animal models for studying the O-acetylation of nitropyrenes is debatable.

It is surprising that the data in Table 7 indicate that, after addition of 2-aminoﬂuorene to hamster liver cytosol, DNA binding by this compound was greater in fast acetylators, while 1,8-dinitropyrene binding was greater in slow acetylators. Dr. Beland does not comment on a mechanism for the increased DNA adduct formation in the slow acetylators. Experiments with the progeny of a cross between these two strains would be needed to ascribe these conflicting results to acetylator genotype. The investigator acknowledges that, although the mutation that causes the polymorphism of the N- and O-acetyltransferase activity has not been characterized, catalytic activity could vary with different substrates. This explanation may be an oversimplification because it seems unlikely that the mutation would produce an enzyme or enzymes with decreased activity toward some substrates but increased activity toward others.

Dr. Beland also noted that the results of his in vivo mouse studies with different acetylator phenotypes contrasted with other studies (Levy and Weber 1988) in that his slow rather than fast acetylators formed more adducts with 2-aminoﬂuorene. He suggests that these differences are related to variations in experimental protocols. An alternative hypothesis is that acetyltransferase competes with the acceptor of N-acetylation rather than catalyzing the activation pathways of 2-aminoﬂuorene that produce DNA adducts. In addition, both in vitro and in vivo, slow hamster phenotypes formed more adducts with 1,8-dinitropyrene than fast phenotypes. It is possible that the two hamster strains differ in nitroreductase activity, but the data Dr. Beland presented in Table 8 suggest that this is not the case. The question of why slow acetylators produced more dinitropyrene DNA adducts in vivo remains unanswered.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

The results of this study suggest that further research is needed to elucidate better the in vivo metabolism of nitropyrenes and, particularly, the acetylation of these compounds. Currently, little is known about the detailed enzymology, biochemistry, physiology, or genetics of the specific acetylases that are involved in these reactions.

Further research is needed not only to characterize DNA adduct formation by 1,6- and 1,8-dinitropyrene and the role of O-acetylation in this process, but also to resolve whether or not 1-nitropyrene forms DNA adducts. Finally, it is also important to elucidate the role of other components of diesel-engine emissions in the formation of DNA adducts before adducts can be used as chemical dosimeters for human environmental or occupational exposures to genotoxic compounds (Wogan and Gorelick 1985).

CONCLUSIONS

The carcinogenicity of 1-nitropyrene has been tested by several investigators, but the results of their experiments have been inconsistent. It is possible that these inconsistencies are related to contamination of 1-nitropyrene with the more potent dinitropyrenes. Dr. Beland's finding that 1-nitropyrene does not form DNA adducts in animal tissues is significant in that it provides a mechanistic explanation for the lack of carcinogenicity of this compound. However, these findings are preliminary and should be confirmed with other model systems and other experimental approaches.
The metabolism of dinitropyrenes involves an acetylation step; in this respect, they are similar to aromatic amines. However, susceptibility to aromatic-amine-induced bladder cancer in humans and hamsters may be a function of the individual acetylator phenotype. It should be noted that acetylation of aromatic amines may occur by one or more acetyl transfer reactions. The significance of each of these enzymatic reactions in determining the overall carcinogenicity of aromatic amines in humans or in animals is not known. In addition, interpretation of some of the results reported in this study was difficult because the behavior of 2-aminofluorene was not consistent with the results of other studies. Thus, the role of the acetylator phenotype, if any, in determining the risk from exposure to nitropyrenes is not clear. This topic deserves further study.

It is not yet known whether or not the carcinogenicity of nitropyrenes in tissues has the same structural basis as their mutagenicity in bacteria. Although short-term assays with bacteria and mammalian cells are useful and provide relatively rapid screens for genotoxicity, they do not provide critical information about the mechanism of damage to DNA at the molecular level. Measurement and characterization of DNA adducts in target tissues and comparisons between in vitro and in vivo results, as presented by Dr. Beland in this study, provide pertinent research approaches for addressing this issue.

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