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The Effects of Copollutants on the Metabolism and DNA Binding of Carcinogens

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

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HEI Statement

Synopsis of Research Report Number 66

Interactive Effects of Nitropyrenes in Diesel Exhaust

BACKGROUND

High doses of inhaled diesel engine exhaust produce lung tumors in laboratory animals and may cause cancer in humans. Because of the potential risks to human health, diesel engine emissions are regulated by the Clean Air Act.

Over the last decade, scientists have made extensive progress in understanding the nature of the individual constituents in diesel exhaust and their biological activity. Diesel exhaust is an extremely complex mixture of small respirable particles and thousands of organic compounds, some of which are adsorbed onto the particles. Among the most extensively studied compounds in diesel exhaust are the nitropyrenes. These chemicals can be activated by the body's metabolism to form highly reactive products that interact with DNA to form DNA adducts. These adducts can interfere with the normal processes of DNA replication and can lead to genetic mutations that may result in carcinogenesis.

The problem with interpreting much of the experimental work on nitropyrenes is that, in diesel exhaust, these compounds do not occur in isolation, but are part of a highly complex mixture. Nevertheless, most of the laboratory studies have involved separate exposures to individual nitropyrenes. Little is known about how the presence of one nitropyrene may change the metabolism and the ability of a second nitropyrene to react with DNA and form adducts. Furthermore, little is known about possible effects of the parent compound, pyrene, a common environmental pollutant, on the biological activity of the nitropyrenes found in diesel exhaust. HEI supported the study described here in order to understand the way that metabolism and adduct formation of nitropyrenes may be affected by the presence of pyrene.

APPROACH

Dr. Howard and colleagues conducted a pilot study using two approaches to examine the effects of pyrene on the metabolism of nitropyrene. First, test tube studies were performed to determine the extent to which 1-nitropyrene metabolism was inhibited by pyrene and other pollutants. In the second series of experiments, laboratory mice were exposed to 1-nitropyrene or 1,6-dinitropyrene alone or in the presence of possible copollutants such as pyrene or other nitropyrenes. The urine and feces of the mice were examined for metabolites of 1-nitropyrene or 1,6-dinitropyrene. In addition, mouse liver DNA was examined using two techniques for measuring the presence of DNA adducts.

RESULTS AND IMPLICATIONS

The test tube studies showed that pyrene inhibited some of the enzymes involved in 1-nitropyrene metabolism. Other compounds, closely related in chemical structure to pyrene, were found to be even stronger inhibitors. However, different results were obtained in the mice. Coadministration of pyrene in the animals had little effect on the overall metabolism of 1-nitropyrene or 1,6-dinitropyrene, or on the formation of DNA adducts from 1-nitropyrene. Pyrene decreased, whereas 1-nitropyrene increased, the amount of DNA adducts formed from 1,6-dinitropyrene. However, these effects were not of a large magnitude. Moreover, the two techniques for measuring DNA adducts did not give a consistent pattern. The inconsistencies between the results from the test tube studies and those from the animals, as well as the small effects observed in the animals, suggest that studying the interactive effects of two pollutants by using selected individual compounds is probably not the optimal way of addressing the problem of interactions among components of complex mixtures. This study leaves unanswered the question of whether the toxicity of individual nitropyrenes in diesel exhaust is affected by the presence of other nitropyrenes or closely related chemicals such as pyrene.

This Statement is a summary, prepared by the Health Effects Institute and approved by the Board of Directors, of a research project sponsored by the HEI from 1990 to 1992. This study was conducted by Dr. Paul C. Howard of Case Western Reserve University, Cleveland, OH. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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The Effects of Copollutants on the Metabolism and DNA Binding of Carcinogens

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ABSTRACT

Copollutants found in air samples and other complex chemical mixtures may alter the metabolism, and thus the biological activity, of chemical carcinogens. As an initial step to determine whether the metabolism and DNA binding of carcinogenic nitrated polycyclic aromatic hydrocarbons found in diesel exhaust particles are altered by copollutants, we studied the effect of pyrene on the metabolism and DNA binding of 1-nitropyrene, and the effect of pyrene and 1-nitropyrene on the metabolism and DNA adduct formation of 1,6-dinitropyrene in male $B_6C_3F_1$ mice. In in vitro incubations using liver microsomes from untreated mice, pyrene was a mixed-type inhibitor, with a 6.42-μM $^{app}K_i$. 2-Aminofluorene and 3-amino-2-methoxydibenzofuran were also effective inhibitors of 1-nitropyrene metabolism. Pyrene did not affect the total in vivo excretion of 1-nitropyrene when coadministered to mice at either a 20-fold or a 250-fold molar excess. At the higher dose of pyrene, however, the urinary excretion of 1-nitropyrene metabolites decreased by approximately 20%, whereas the concentration of fecal metabolites increased by the same amount. Similar in vivo experiments were conducted using 2-aminofluorene as the inhibitor. The excretion of 1-nitropyrene was not significantly affected by 2-aminofluorene treatment. Treatment-related DNA adducts were not detected by ^{32}P-postlabeling analyses of liver DNA when 1-nitropyrene was administered by itself or with a 20- or 250-fold molar excess of pyrene. The coadministration of pyrene or 1-nitropyrene had no effect on the total excretion of 1,6-dinitropyrene metabolites. A single major adduct that coeluted with *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene was detected in hepatic DNA from mice treated with 1,6-dinitropyrene. The concentration of this adduct was significantly decreased by the coadministration of a 25-fold molar excess of pyrene and was significantly increased by simultaneous treatment with a 25-fold molar excess of 1-nitropyrene. These results demonstrate the effect of copollutants on potentially carcinogenic components of diesel exhaust and urban air.

INTRODUCTION

Humans are exposed to complex chemical mixtures that arise from anthropogenic sources including urban air, tobacco smoke, internal combustion engine exhausts, coal tar, water and soil in urban areas, air in work environments, and toxic wastes in and around waste generators and dumps. Contained within some of these mixtures are nitrated polycyclic aromatic hydrocarbons (nitro-PAHs), a group of chemicals that arise from incomplete combustion processes in the presence of nitrogen oxides and from the photochemical oxidation of PAHs in the presence of nitrogen pentoxide (Pitts et al. 1982; Ramdahl et al. 1982; Arey et al. 1989). Of the nitro-PAHs that have been detected from various sources, 1-nitropyrene, 2-nitrofluoranthene, and 3-nitrofluoranthene appear to be the most abundant. In diesel exhausts, for instance, the concentrations of these compounds can approach 100 ng per gram of particulate.

Diesel exhausts have been shown to be carcinogenic in some laboratory animal models (Brightwell et al. 1986; Heinrich et al. 1986; Iwai et al. 1986; Mauderly et al. 1987, 1990) and are suspected of being carcinogenic in humans (International Agency for Research on Cancer 1989). Of the compounds detected in diesel exhaust, 1-nitropyrene and 1,6-dinitropyrene have significant genotoxic activity that may be important in the tumorigenic properties of this mixture. The metabolic activation of 1-nitropyrene to a mutagen involves nitroreduction to an *N*-hydroxyarylamine intermediate that reacts with DNA to give a C-8-substituted deoxyguanosine adduct, *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG-C8-AP) (Howard et al. 1983a). This pathway has been demonstrated in the *Salmonella typhimurium* histidine reversion assay (Howard et al. 1983a). It also appears to be important in the metabolism of 1-nitropyrene to a mutagen in Chinese ham-

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report Number 66, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Paul C. Howard, HFT, National Center for Toxicological Research, Jefferson, AR 72079.

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ster V79 and ovary cells (Takayama et al. 1983; Heflich et al. 1985, 1986a,b), in human HepG2 cells (Eddy et al. 1987), and in the transformation of human diploid fibroblasts and Syrian hamster embryo cells (DiPaolo et al. 1983; Howard et al. 1983b; Patton et al. 1986). By comparison, the oxidation of 1-nitropyrene to yield phenol and vicinal dihydrodiol derivatives (C-oxidation) of 1-nitropyrene is generally regarded as a detoxification pathway that does not lead to DNA adducts other than dG-C8-AP (Smith et al. 1990b). However, 1-nitropyrene K-region epoxides and phenols are mutagenic in *S. typhimurium* (Fifer et al. 1986; Consolo et al. 1989) and Chinese hamster ovary cells (Smith et al. 1990a). The metabolism of 1,6-dinitropyrene to a mutagen also involves nitroreduction; however, 1,6-dinitropyrene differs from 1-nitropyrene in two important aspects. First, the *N*-hydroxyarylamine of 1,6-dinitropyrene is further activated by *O*-acetylation (Djurić et al. 1985; Watanabe et al. 1990; Einistö et al. 1991), and second, 1,6-dinitropyrene does not appear to undergo C-oxidation, as evidenced by high-performance liquid chromatographic (HPLC) analysis of the metabolites and by the DNA adduct profile (Djurić et al. 1985, 1988; Helfich et al. 1986c; Smith et al. 1990b).

In addition to being mutagenic, 1-nitropyrene and 1,6-dinitropyrene are carcinogenic, with 1,6-dinitropyrene being significantly more mutagenic and tumorigenic than 1-nitropyrene (Ohgaki et al. 1985; Tokiwa and Ohrishi 1986; Imaida et al. 1991). Although the pathways by which 1-nitropyrene and 1,6-dinitropyrene are metabolized in vivo to tumorigens have been studied less extensively than their metabolism to mutagens, it appears that in at least some target tissues, nitroreduction to *N*-hydroxyarylamine intermediates is important (Beland and Kadlubar 1990). The metabolism and biological activity of chemicals can be affected by the coadministration of other chemicals. One example involves the *N*-nitrosamines *N'*-nitrosonornicotine (NNN) and 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), which induce lung and oral tumors when applied to the oral cavities of rats. However, when they are coadministered with aqueous snuff extracts, the tumor incidence decreases. This suggests that the snuff extracts inhibit the metabolic activation of NNN or NNK, increase the detoxification of the *N*-nitrosamines, decrease the absorption of the *N*-nitrosamines, or inhibit the progression of the neoplasia that develops (Brunneman et al. 1987).

Van Duuren and associates (1973) reported the effects of pyrene and fluoranthene on the DNA adduct formation and tumorigenicity of benzo[*a*]pyrene in mice. They reported that including pyrene as a cocarcinogen with topical application of benzo[*a*]pyrene to ICR/Ha Swiss mice resulted in an approximately twofold increase in the number of skin papillomas and squamous carcinomas. In the same study, benzo[*e*]pyrene and catechol had a greater cocarcinogenic effect than pyrene, whereas phenol and benzo[*ghi*]perylene were less effective as cocarcinogens. Rice and associates (1984) painted the backs of shaved CD-1 mice with [^{3}H]benzo[*a*]pyrene with and without approximately a 10-fold molar excess of pyrene, fluoranthene, or phenanthrene. The cocarcinogens pyrene and fluoranthene increased [^{3}H]benzo[*a*]pyrene's binding to DNA by approximately 60%, whereas phenanthrene slightly decreased [^{3}H]benzo- [*a*]pyrene's DNA binding. Phenanthrene had been shown previously to inhibit benzo[*a*]pyrene's tumorigenicity (Hoffman and Wynder 1963).

The nitro-PAHs in diesel exhausts occur in association with a multitude of other chemicals that could modify their genotoxic potency. Pyrene, for example, which is the parent nucleus of 1-nitropyrene and 1,6-dinitropyrene, is approximately 100 times more prevalent than 1-nitropyrene and 5,000 times more prevalent than 1,6-dinitropyrene. If pyrene competes for the enzymes that activate or detoxify 1-nitropyrene, 1,6-dinitropyrene, or both, the biological activity of these nitro-PAHs should differ from the activity estimated for the pure compounds administered by themselves. As an initial step to determine whether the metabolism and DNA binding of carcinogenic nitro-PAHs found in diesel exhaust particles are altered by copollutants, we studied the effect of pyrene on the metabolism and DNA binding of 1-nitropyrene and the effect of pyrene and 1-nitropyrene on the metabolism and DNA adduct formation of 1,6-dinitropyrene.

Unexpectedly, we found that 2-aminofluorene inhibited 1-nitropyrene metabolism in vitro to a greater extent than pyrene or fluoranthene. Therefore, we used 2-aminofluorene as a model copollutant and determined its effect on 1-nitropyrene and 1,6-dinitropyrene metabolism in vivo.

SPECIFIC AIMS

The specific aims of this one-year study were twofold: (1) to determine the effect of pyrene on the in vitro and in vivo metabolism and DNA adduct formation of 1-nitropyrene in male $B_6C_3F_1$ mice; and (2) to determine the effect of pyrene and 1-nitropyrene on the in vivo metabolism and DNA adduct formation of 1,6-dinitropyrene in male $B_6C_3F_1$ mice.

METHODS AND STUDY DESIGN

MATERIALS

1-Nitropyrene (99% pure), 1,6-dinitropyrene, [4,5,9,10-^{3}H]1-nitropyrene ([^{3}H]1-nitropyrene, 20.2 Ci/mmol, radiochemical purity 98%), and [4,5,9,10-^{3}H]1,6-dinitropyrene ([^{3}H]1,6-dinitropyrene, 10.1 Ci/mmol, radiochemical purity 98%) were obtained from Chemsyn Science Laboratories

(Lenexa, KS). The following compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI): 3-amino-2-methoxydibenzofuran (purity 97%); 2-amino-9-fluorenone; 7-ethoxycoumarin (purity>99%); 1-aminofluorene (purity 99%); 2-amino-5-chlorobenzoxazole (zoxazolamine) (purity 97%); 3-amino-9-fluorenone (purity 98%); cyclohexylamine (purity>99%); *exo*-2-aminonorboronane (purity 99%); 2-nitrofluorene (purity 98%); 2-amino-9-hydroxyfluorene (purity 97%); 4-amino-1,2,4-triazole; 1-amino-7-nitrofluorene (purity>99%); 2-aminofluorene (purity 98%); 9-aminophenanthrene (purity 96%); and pyrene (gold-label purity greater than 98%). 3-Aminofluoranthene and 2-aminofluorene obtained from Aldrich Chemical Co. were purified over silica with benzene and hexane before use. The 2-aminofluorene was monitored by thin-layer chromatography (silica with benzene) and found to be free of contaminating oxidized products. Nicotinamide adenine dinucleotide phosphate, reduced (NADPH), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, testosterone, and warfarin (sodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). The following were synthesized by techniques previously described: 1-nitrosopyrene, 1-nitropyren-3-ol, 1-nitropyren-6-ol, and 1-nitropyren-8-ol (Howard et al. 1983c); 1-nitropyrene-4,5-oxide and 1-nitropyrene-9,10-oxide (Fifer et al. 1986); and 1-nitropyrene-4,5-dihydrodiol, and 1-nitropyrene-9,10-dihydrodiol (Silvers et al. 1992). Dimethylsulfoxide (silylation grade, under nitrogen) was obtained from Pierce Chemical Co. (Rockford, IL); and trioctanoin (stored at 2°C to 4°C) was obtained from Pfaltz and Bauer (Waterbury, CT). All other chemicals were of the highest grade available.

EQUIPMENT

The metabolites of [^{3}H]1-nitropyrene and [^{3}H]1,6-dinitropyrene were separated using a Varian (Walnut Creek, CA) 5500 series HPLC equipped with a Varian UV-200 UV-VIS multiple-wavelength detector, a 5-µm µBondapak-C_{18} semi-preparative reverse-phase HPLC column (Waters Associates, Milford, MA), and a Radiomatic Flo-One β flow-through scintillation counter (Radiomatic Instruments, Meriden, CT) with a 2.5-mL flow cell. Either a Varian 9090 HPLC autosampler or a Rheodyne 7125 manual HPLC injector (Rheodyne Instruments, Cotati, CA) was used. The Radiomatic flow-through scintillation counter was operated by an IBM PC-XT computer (IBM Corp., Armonk, NY). The flow-through scintillation counter had an efficiency of 43% with ^{3}H.

ANIMALS

The use and care of the animals in these studies conformed to the guidelines established by the American Association for the Accreditation of Laboratory Animal Care, as monitored by the Case Western Reserve University Institutional Animal Care and Use Committee. The mice were housed three per cage in model 2100M metabolism cages (Lab Products Inc., Federalsburg, MD). For acclimation, the mice were housed for one week in the cages prior to dosing and were fed Purina Lab Chow 5001 meal (Ralston Purina Co., St. Louis, MO) and tap water ad libitum.

PREPARATION OF MICROSOMES

Liver microsomes were prepared from 6- to 8-week-old male $B_6C_3F_1$ mice (Jackson Laboratories, Bar Harbor, ME). Following asphyxiation from carbon dioxide and cervical dislocation, the livers were removed. After the excision of the gall bladders, the livers were minced and allowed to stand on ice for 10 minutes in 250 mM sucrose, 100 mM potassium phosphate, pH 7.4 (sucrose-PO_4 buffer). The livers then were weighed, and three volumes of sucrose-PO_4 buffer were added. The livers were homogenized for 15 seconds using a Brinkman Polytron PT30 probe (Brinkman Instruments, Westbury, CT), and centrifuged at 12,000 × *g* for 20 minutes at 2°C to 4°C. The supernatant then was centrifuged at 100,000 × *g* for one hour at 2°C to 4°C. The microsomal pellets were resuspended in 150 mM potassium chloride using Potter-Elvehjem homogenizers, and centrifuged at 100,000 × *g* for one hour at 2°C to 4°C. The resultant pellets were resuspended in sucrose-PO_4 buffer using Potter-Elvehjem homogenizers and stored in aliquots of 500 µL at −75°C. Each preparation represents the combined livers from three mice prior to homogenization.

IN VITRO METABOLISM OF 1-NITROPYRENE

[^{3}H]1-Nitropyrene (1 Ci/mmol) was incubated at a concentration of 0.21 to 40 µM in 25-mL Erlenmeyer flasks in a 2-mL mixture containing the following: 1 mM NADPH, 4 mM glucose-6-phosphate, 1 mM magnesium chloride, 0.1 units/mL glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (type XXIV, Sigma Chemical Co.), 50 mM potassium phosphate (pH 7.4), and 70 to 200 µg microsomal protein. The amount of microsomal protein and time of incubation were adjusted to ensure zero-order kinetics; typically, the incubations contained 80 µg/mL microsomal protein and lasted for 10 minutes. Following incubation, the substrate and metabolites were extracted using an equal volume of chloroform and methanol (2:1). After centrifugation (200 × *g*, 10 minutes), the chloroform layer was removed, and the aqueous layer was reextracted with 2 mL chloroform. The organic layers were combined and stored at −20°C.

Potential inhibitors of mouse liver microsomal metabolism of [^{3}H]1-nitropyrene were added before microsomes

were added. Both substrates and inhibitors were added in 5 μL dimethylsulfoxide.

QUANTIFICATION OF IN VITRO [^{3}H]1-NITROPYRENE METABOLISM

The in vitro metabolites of [^{3}H]1-nitropyrene were separated by HPLC. Typically, 50 nCi was analyzed for each sample. Separation was accomplished using a 10-μm Waters μBondapak C_{18} HPLC column (0.78 × 30 cm) and the following mobile phase at 1.8 mL/min, with linear gradient changes: 0 minutes, 45% ethanol in water; 20 minutes, 55% ethanol; 44 minutes, 62% ethanol; 48 minutes, 100% ethanol. The [^{3}H]1-nitropyrene and ^{3}H-labeled metabolites were quantified using a flow-through scintillation counter (Flo-One β, Radiomatic Instruments, Meriden, CT), and the total metabolism of 1-nitropyrene was determined by the sum of the individual metabolites. The HPLC conditions resulted in the following retention times: 1-nitropyrene-9,10-dihydrodiol, 5 minutes; 1-nitropyrene-4,5-dihydrodiol, 10.5 minutes; a mixture of 1-nitropyrene K-region epoxides, 27.5 minutes; 1-nitropyren-6-ol and 1-nitropyren-8-ol, 36 minutes; 1-nitropyren-3-ol, 44 minutes; 1-nitropyrene, 49 minutes. Assignments of the retention times were verified using nonradiolabeled standards. Statistical analysis of the kinetics of pyrene inhibition of [^{3}H]1-nitropyrene metabolism was performed with commercially available software (Enzfitter, Elsevier Biosoft, Cambridge, United Kingdom), fitting nonlinear regression analysis to the curve of velocity versus substrate concentration.

IN VIVO METABOLISM OF 1-NITROPYRENE AND 1,6-DINITROPYRENE

Three separate experiments were performed to determine whether the in vivo metabolism of 1-nitropyrene and 1,6-dinitropyrene could be altered by the administration of a copollutant. In these experiments, all of the compounds were administered to the mice intraperitoneally. In a separate study using $B_6C_3F_1$ mice (P.C. Howard, M.C. Consolo, K.L. Dooley, F.A. Beland, unpublished results), we found little difference in the pharmacokinetics of [^{3}H]1-nitropyrene when administered at 4 μmol/kg of body weight either by gavage or intraperitoneally. Because the intent of the experiments described was to determine whether a copollutant could alter the metabolism of 1-nitropyrene or 1,6-dinitropyrene, and not to mimic routes of environmental exposure to the nitro-PAH, we chose intraperitoneal administration as the route of introduction of the compounds to the animals.

The mice were housed three per cage. The feces and urine were collected at 8- to 12-hour intervals, with the urine and feces being separated by the cage diffuser and collected in 50-mL conical centrifuge tubes. Fecal and urine samples were collected at room temperature and stored at −20°C until analysis.

Table 1. Experimental Design for Testing the Effect of Pyrene on [^{3}H]1-Nitropyrene Metabolism In Vivo

Group[a]	[^{3}H]1-Nitropyrene Administered per Mouse	Pyrene Administered per Mouse
1	10 nmol (10 μCi)	None
2	10 nmol (10 μCi)	200 nmol
3	10 nmol (10 μCi)	2,500 nmol

[a] Each group comprised three cages with three mice per cage (n = 9).

In the first experiment (Table 1), each mouse was administered 10 nmol [^{3}H]1-nitropyrene (1 μCi/nmol) with no pyrene or a 20-fold or a 250-fold molar excess of pyrene in a total volume of 400 μL trioctanoin. In the second experiment (Table 2), each mouse was administered 10 nmol [^{3}H]1-nitropyrene (1 μCi/nmol) with no 2-aminofluorene or a 25-fold or a 250-fold molar excess of 2-aminofluorene in a total of 100 μL trioctanoin. In the third metabolic experiment (Table 3), the mice were administered 100 nmol [^{3}H]1,6-dinitropyrene (0.1 μCi/nmol) with no 1-nitropyrene or pyrene, or a 25-fold molar excess of 1-nitropyrene, or a 25-fold molar excess of pyrene in a total volume of 100 μL trioctanoin. In the experiments outlined in Table 3, the trioctanoin contained 14% dimethylsulfoxide to facilitate the initial dissolution of the 1,6-dinitropyrene. The urine and feces from each of the cages were collected for up to 100 hours and stored at −20°C.

EXTRACTION OF [^{3}H]1-NITROPYRENE AND [^{3}H]1,6-DINITROPYRENE METABOLITES FROM THE URINE AND FECES

The urine samples were thawed and clarified by centrifugation at 500 × g, and the volume and ^{3}H content were quantified. The feces were thawed and weighed, and 3 mL water was added to rehydrate the fecal matrix. After approx-

Table 2. Experimental Design for Testing the Effect of 2-Aminofluorene on [^{3}H]1-Nitropyrene Metabolism in Vivo

Group[a]	[^{3}H]1-Nitropyrene Administered per Mouse	2-Aminofluorene Administered per Mouse
4	10 nmol (10 μCi)	None
5	10 nmol (10 μCi)	250 nmol
6	10 nmol (10 μCi)	2,500 nmol

[a] Each group comprised three cages with three mice per cage (n = 9).

Table 3. Experimental Design for Testing the Effect of Pyrene and 1-Nitropyrene on [^{3}H]1,6-Dinitropyrene Metabolism in Vivo

Group[a]	[^{3}H]1,6-Dinitropyrene Administered per Mouse	Copollutant Administered per Mouse
7	100 nmol (10 μCi)	None
8	100 nmol (10 μCi)	2,500 nmol 1-Nitropyrene
9	100 nmol (10 μCi)	2,500 nmol Pyrene

[a] Each group comprised three cages with three mice per cage (n = 9).

imately 30 minutes at 40°C to 45°C to allow swelling, 15 mL acetonitrile was added, and the mixture was vigorously agitated at 45°C to 50°C for 30 minutes. The solution was clarified by centrifugation at 800 × g and the aqueous soluble metabolites were removed in the water and acetonitrile. Further sequential extractions were accomplished with 15 mL acetonitrile, 10 mL of 100 mM acetic acid, and 10 mL of 100 mM sodium hydroxide. The residual ^{3}H content of the fecal matrix was determined by liquid scintillation counting using Insta-Gel (Packard Instruments Co., Downers Grove, IL), following acidification with acetic acid. This material is referred to as "nonextractable" ^{3}H-labeled metabolites that did not extract under the protocol used. Because this material is associated with the remaining solid fecal material, its chemical nature is unknown. The ^{3}H content in each of the extracts was quantified by liquid scintillation counting. Three drops of triethylamine were added to the combined acetonitrile extracts to retard oxidation of the metabolites.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF RADIOLABELED METABOLITES EXTRACTED FROM URINE AND FECES

The HPLC analysis of the metabolites was accomplished using a 10-μm Waters μBondapak semipreparative C_{18} reverse-phase column (0.78 × 30 cm) and a mobile phase at 1.8 mL/min, with a gradient as follows: 0 to 25 minutes, isocratic at 35% ethanol in 20 mM acetic acid; 25 to 37 minutes, linear change from 35% ethanol in 20 mM acetic acid to 65% ethanol in 20 mM acetic acid; 37 to 65 minutes, isocratic at 65% ethanol in 20 mM acetic acid; 65 to 75 minutes, linear change from 65% ethanol in 20 mM acetic acid to 100% ethanol. With this gradient, 1-nitropyren-6-*O*-glucuronide and 1-nitropyren-8-*O*-glucuronide (44 minutes), 1-nitropyren-6-*O*-sulfate and 1-nitropyren-8-*O*-sulfate (50 minutes), 1-nitropyren-6-ol and 1-nitropyren-8-ol (62 minutes), 1-nitropyren-3-ol (71 minutes), and 1-nitropyrene (73 minutes) eluted with the retention times indicated in parentheses. The amounts of metabolites eluting from the HPLC column were quantified using a flow-through scintillation counter (Flo-One β Radiomatic Instruments) equipped with a 2.5-mL flow cell. The counting efficiency for ^{3}H was 43% when Ecolite (+) (ICN Radiochemicals, Cosa Mesa, CA) was used as the scintillant at a 2.5:1 ratio to the HPLC eluate (vol:vol).

IN VIVO FORMATION OF DNA ADDUCTS WITH 1-NITROPYRENE AND 1,6-DINITROPYRENE

The experimental design for testing the effect of copollutants on DNA adduct formation is given in Tables 4 and 5. Four mice were used in each treatment group for each experimental point. In the experiments outlined in Table 4, the compounds were administered in 100 μL trioctanoin; in those outlined in Table 5, the compounds were administered in 100 μL trioctanoin containing 14% dimethylsulfoxide to facilitate initial 1,6-dinitropyrene dissolution. At the times indicated, the animals were killed by cervical dislocation, and the livers and lungs were wrapped in aluminum foil, labeled, frozen in liquid nitrogen, and stored at −75°C.

ISOLATION OF TISSUE DNA AND ^{32}P-POSTLABELING ANALYSIS OF DNA ADDUCTS

DNA adducts were assayed by ^{32}P-postlabeling on DNA isolated from liver nuclei. The isolation of nuclei and purification of the DNA were conducted according to the

Table 4. Experimental Design for Testing the Effect of Coadministration of Pyrene on the in Vivo Formation of 1-Nitropyrene–DNA Adducts

Group[a]	1-Nitropyrene Administered per Mouse	Pyrene Administered per Mouse	Length of Experiment (days)
10	10 nmol	None	1, 2, 4
11	10 nmol	200 nmol	1, 2, 4
12	10 nmol	2,500 nmol	1, 2, 4
13	None	2,500 nmol	1, 2, 4
14	None	None	2, 4

[a] Four mice were used in each treatment group for each length of experiment (at which time all mice were killed); n = 12 for groups 10 through 13; n = 8 for group 14.

Table 5. Effect of Coadministration of Pyrene and 1-Nitropyrene on 1,6-Dinitropyrene–DNA Adduct Formation in Vivo[a]

Group[b]	[^{3}H]1,6-Dinitropyrene Administered per Mouse	Copollutant Administered per Mouse	DNA Adducts Determined by ^{3}H Labeling (fmol/μg DNA)	DNA Adducts Detected by ^{32}P-Postlabeling (fmol/μg DNA)
15	100 nmol	None	0.41 ± 0.06	0.46 ± 0.05
16	100 nmol	250 nmol 1-Nitropyrene	0.50 ± 0.13	0.49 ± 0.07
17	100 nmol	2,500 nmol 1-Nitropyrene	0.73 ± 0.29	0.59 ± 0.07[c]
18	100 nmol	2,500 nmol Pyrene	0.41 ± 0.12	0.34 ± 0.04[d]
19	None	2,500 nmol 1-Nitropyrene	0[e]	0.04 ± 0.00[f]
20	None	2,500 nmol Pyrene	0[e]	0.04 ± 0.01[f]
21	None	None	< 0.03[g]	0.07 ± 0.04[f]

[a] The experiment lasted two days, at which time the mice were killed.

[b] Four mice were used in each treatment group.

[c] Statistical analyses were performed using Student's t test comparing the groups with the DNA adduct levels obtained in group 15; $p < 0.05$.

[d] $p < 0.01$.

[e] These levels of adducts were approximately equal to background levels.

[f] $p < 0.001$.

[g] Based on a limit of detection of 100 dpm.

method described by Poirier and associates (1991). The ^{32}P-postlabeling was conducted by the n-butanol enrichment and contact transfer procedures described by Smith and coworkers (1990b). The adducts were quantified by comparing them with DNA standards that were modified to a known extent with the adducts of interest. In some instances, adducts were quantified by measuring the amount of radioactivity associated with the DNA following the administration of [^{3}H]1-nitropyrene and [^{3}H]1,6-dinitropyrene. These samples were also analyzed by ^{32}P-postlabeling.

STATISTICAL ANALYSIS

In the in vivo metabolism studies, one-way analysis of variance with pairwise comparisons using the Scheffe multiple comparison test was used at each time point of evaluation to identify differences associated with copollutant administration. Repeated-measures two-factor analysis of variance was used to identify a trend in response over time measured and to identify any trend in the differences associated with copollutant administration during the period of observation. Statistical analysis was run using SPSS/PC+ V5.0 (Chicago SPSS, Chicago, IL) on an AT&T 6386 WGS microcomputer. Significance for all tests was set at $p<0.05$.

RESULTS

IN VITRO INHIBITION OF 1-NITROPYRENE METABOLISM BY PYRENE AND OTHER CHEMICALS

The inhibition of [^{3}H]1-nitropyrene metabolism was investigated using liver microsomal protein from untreated male $B_6C_3F_1$ mice and varying concentrations of pyrene (Figure 1). The results indicated that pyrene is a mixed-type inhibitor of [^{3}H]1-nitropyrene metabolism. The apparent Michaelis-Menken kinetic constant ($^{app}K_m$) of [^{3}H]1-nitropyrene metabolism was 1.2 ± 0.4 μM with an apparent maximal velocity ($^{app}V_{max}$) of 0.62 ± 0.13 nmol/min/mg protein (n = 4 microsomal preparations; mean ± SD). Additional analyses using the slopes of the pyrene inhibition lines revealed a 6.4-μM apparent inhibition constant ($^{app}K_i$) (Segel 1975).

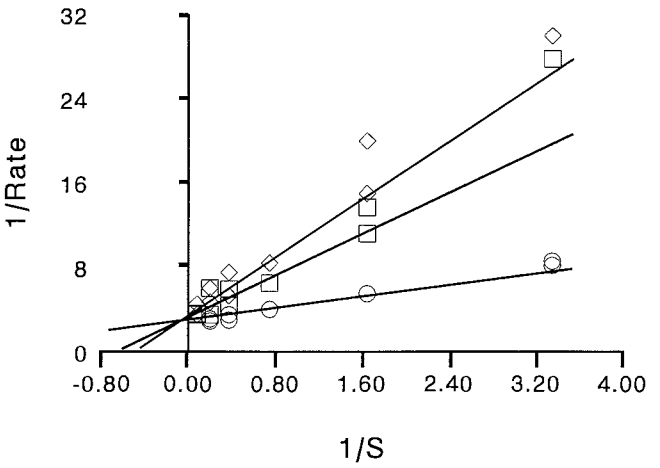

Figure 1. Kinetic analysis of the effect of pyrene on the metabolism of 0.3- to 10.1-μM [^{3}H]1-nitropyrene in $B_6C_3F_1$ mouse liver microsomes. Pyrene concentrations were 0 μM (○), 10 μM (□), and 20 μM (△). The lines were determined from the $^{app}K_m$ and $^{app}V_{max}$ values obtained from the nonlinear regression curve of the velocity (1/rate) versus the substrate concentration (1/S) and were replotted using these values on a double-reciprocal plot.

Table 6. The Effect of Various Xenobiotics on the Metabolism of 10-μM [^{3}H]1-Nitropyrene by Untreated Mouse Liver Microsomes in Vitro[a]

Compound	Concentration (μM)	Percentage of Control
2-Aminofluorene	30	23.4
3-Amino-2-methoxydibenzofuran	30	28.5
2-Amino-9-fluorenone	30	44.9
7-Ethoxycoumarin	30	74.4
1-Aminofluorene	30	78.2
Zoxazolamine	500	80
3-Amino-9-fluorenone	30	83.9
1-Amino-2-nitrofluorene	30	84.3
Testosterone	120	90
3-Aminoflouranthene	30	94
Warfarin	300	100
Cyclohexylamine	3,000	100
2-Aminonorboronane	200	100
2-Nitrofluorene	30	106.8
2-Amino-9-hydroxyfluorene	30	122.2
4-Amino-1,2,4-triazole	30	133.8

[a] Compounds are ranked in order of inhibition. The data are presented as the percentage of control metabolism of 1-nitropyrene (10 μm) in the presence of 5 μL of the solvent dimethylsulfoxide. All xenobiotics were added in 5 μL dimethylsulfoxide. Each value represents at least duplicate analyses from duplicate assays.

Although pyrene is the parent nucleus of 1-nitropyrene and 1,6-dinitropyrene, other compounds that are also metabolized by cytochrome P-450 isozymes may be greater inhibitors of 1-nitropyrene metabolism. This could result from a lower $^{app}K_i$ or from a mechanism-based inhibition of the cytochrome. For this reason, we investigated the ability of several other compounds to inhibit 1-nitropyrene metabolism in vitro. Testosterone, zoxazolamine, warfarin, cyclohexylamine, and 4-amino-1,2,4-triazole were selected on the basis of their metabolism by specific cytochrome P-450 isozymes. The other compounds were selected because they were arylamine or nitroaryl compounds bearing structural similarity to 1-nitropyrene (except for cyclohexylamine and *exo*-2-aminonorboronane). Table 6 shows these results. 2-Aminofluorene and 3-amino-2-methoxydibenzofuran were the most effective inhibitors. At concentrations of 30 μM, both of these compounds inhibited approximately 75% of the metabolism of 10-μM [^{3}H]1-nitropyrene. Other derivatives of 2-aminofluorene and other specific substrates of cytochrome P-450 isozymes were not as effective as inhibitors. In fact, several compounds, including 2-nitrofluorene, 2-amino-9-hydroxyfluorene, and 4-amino-1,2,4-triazole, stimulated the metabolism of 1-nitropyrene. Therefore, we were able to utilize two inhibitors of 1-nitropyrene metabolism for the in vivo studies: pyrene as the parent nucleus and 2-aminofluorene as a more effective inhibitor.

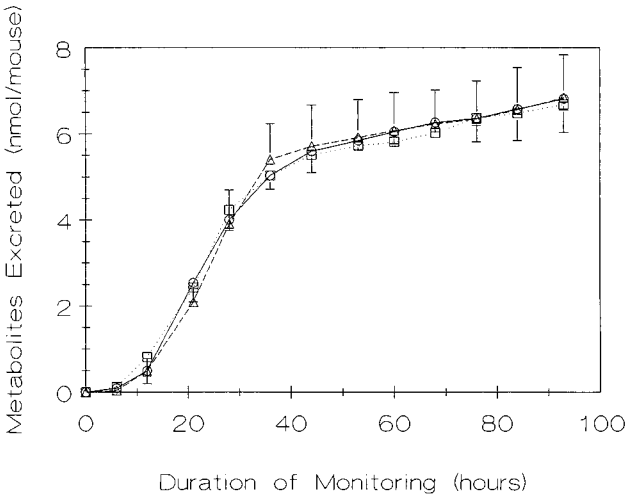

Figure 2. Total excretion of [^{3}H]1-nitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 10 nmol [^{3}H]1-nitropyrene per mouse for group 1 (O, solid line), **10 nmol [^{3}H]1-nitropyrene and 200 nmol pyrene per mouse** for group 2 (□, dotted line) **and 10 nmol [^{3}H]1-nitropyrene and 2,500 mnol pyrene per mouse** for group 3 (△, dashed line) **was monitored for up to 93 hours.** Values represent the average value ± SD for three cages with three mice per cage.

IN VIVO METABOLISM OF [^{3}H]1-NITROPYRENE AND THE EFFECT OF THE COADMINISTRATION OF PYRENE

The effect of the coadministration of pyrene on the total cumulative excretion of 1-nitropyrene metabolites is shown in Figure 2. Approximately 70% of the administered dose of [^{3}H]1-nitropyrene was excreted over four days; this amount was not affected either by a 20-fold or by a 250-fold molar excess of pyrene. Figure 3 shows a decrease in the urinary excretion of [^{3}H]1-nitropyrene, with the 250-fold dose of py-

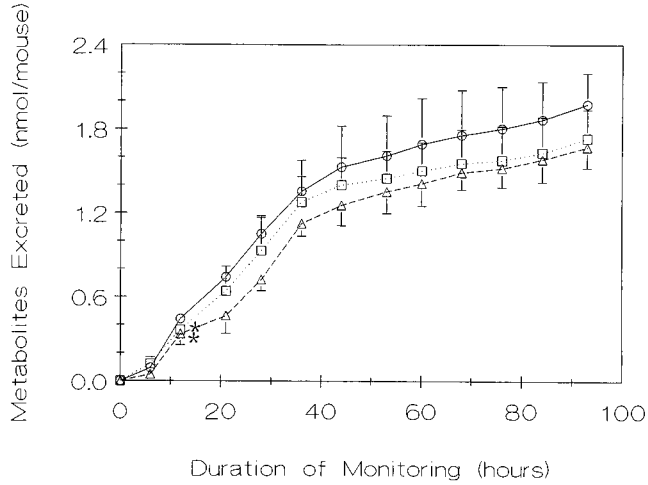

Figure 3. Urinary excretion of [^{3}H]1-nitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 10 nmol [^{3}H]1-nitropyrene per mouse for group 1 (○, solid line), **10 nmol [^{3}H]1-nitropyrene and 200 nmol pyrene per mouse** for group 2 (□, dotted line), **and 10 nmol [3H]1-nitropyrene and 2,500 nmol pyrene per mouse** for group 3 (△, dashed line) **was monitored for up to 93 hours.** Values represent the average value ± SD for three cages with three mice per cage. The results designated by the asterisk (*) are significantly different from the control group's values at that time ($p < 0.05$).

rene causing approximately a 20% decrease in cumulative urinary 1-nitropyrene metabolites; however, statistical analysis revealed that the doses did not cause a significant ($p < 0.05$) change in the excretion of metabolites. Cumulative excretion of the fecal metabolites increased to a similar extent (approximately 20%) at the highest pyrene doses (Figure 4), although these changes were not statistically significant at the 95% confidence level. The feces contained [^{3}H]1-nitropyrene metabolites that were not extractable with the protocol used in these studies. Figure 5 shows the percentage of the excreted fecal metabolites that were not extractable under the protocol used (see the Methods section). This was calculated by dividing the cumulative amount of nonextractable metabolites by the cumulative total excreted. The pyrene had no effect on the nonextractable [^{3}H]1-nitropyrene metabolites in the feces, with all treatments resulting in approximately 50% of the metabolites being nonextractable. At present, the chemical nature of this nonextractable material is unknown.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE URINARY AND FECAL [^{3}H]1-NITROPYRENE METABOLITES AFTER COADMINISTRATION OF PYRENE

In order to determine whether pyrene coadministration affected the profile of 1-nitropyrene metabolites, urine and fecal extracts were subjected to HPLC analyses. As shown in Figure 6, there were essentially no differences between group 1, which received only [^{3}H]1-nitropyrene, and groups 2 and 3, which were coadministered a 20-fold and a 250-fold molar excess of pyrene.

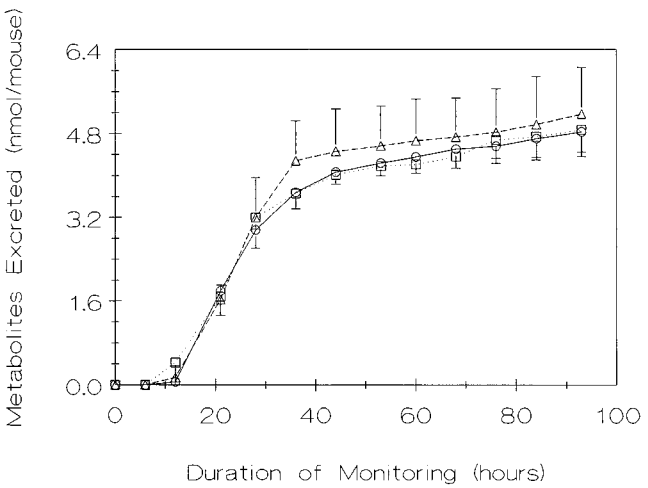

Figure 4. Fecal excretion of [^{3}H]1-nitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 10 nmol [^{3}H]1-nitropyrene per mouse for group 1 (○, solid line), **10 nmol [$_3$H]1-nitropyrene and 200 nmol pyrene per mouse** for group 2 (□, dotted line), **and 10 nmol [^{3}H]1-nitropyrene and 2,500 nmol pyrene per mouse** (△, dashed line) **was monitored for up to 93 hours.** Values represent the total [^{3}H]1-nitropyrene and [^{3}H]-labeled metabolites in the feces as the average value ± SD for three cages with three mice per cage.

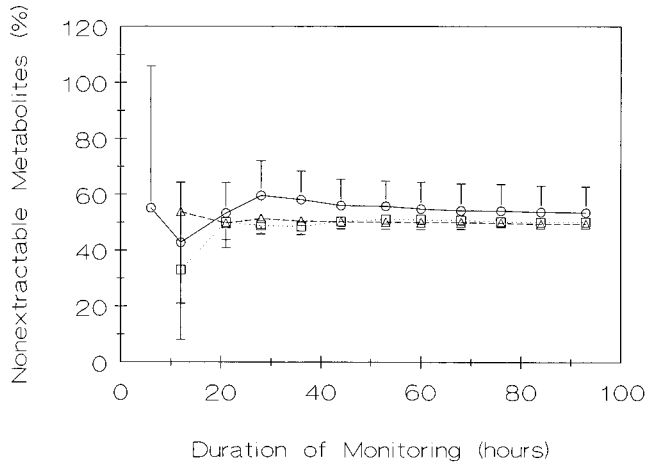

Figure 5. The percentage of fecal [^{3}H]1-nitropyrene metabolites that were not extractable from the fecal matrix by the methods used (acetonitrile, acetic acid, base) was monitored. The feces were from male $B_6C_3F_1$ mice intraperitoneally administered 10 nmol [^{3}H]1-nitropyrene per mouse for group 1 (○, solid line), 10 nmol [^{3}H]1-nitropyrene and 200 nmol pyrene per mouse for group 2 (□, dotted line), and 10 nmol [^{3}H]1-nitropyrene and 2,500 nmol pyrene per mouse for group 3 (△, dashed line) and monitored for up to 93 hours. Values represent the average value ± SD for three cages with three mice per cage.

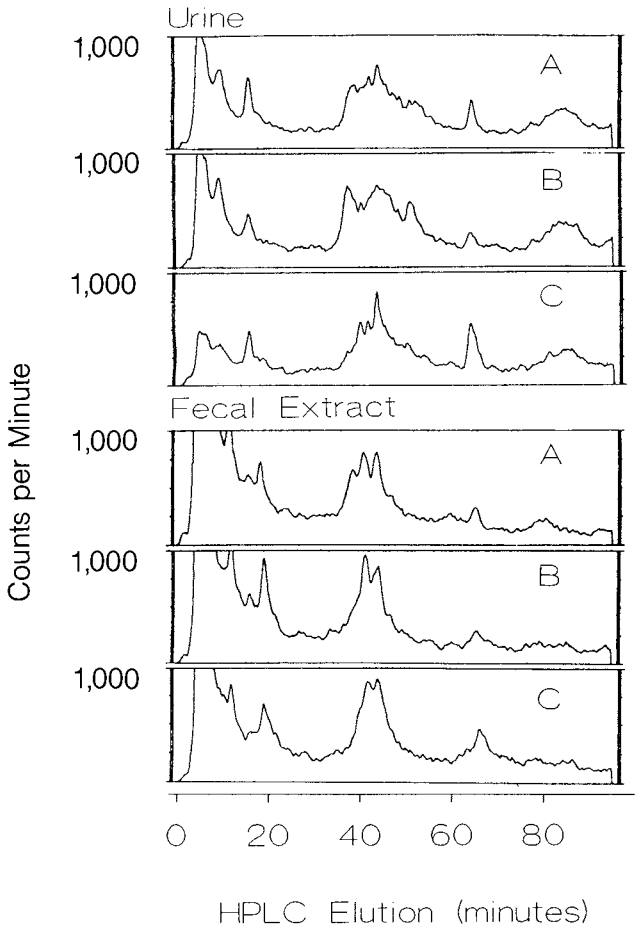

Figure 6. HPLC analysis of the [^{3}H]1-nitropyrene metabolites was performed on the urine and fecal extracts from male $B_6C_3F_1$ mice treated intraperitoneally with (A) 10 nmol [^{3}H]1-nitropyrene per mouse for group 1, **(B) 10 nmol [^{3}H]1-nitropyrene and 200 nmol pyrene per mouse** for group 2, **and (C) 10 nmol [^{3}H]1-nitropyrene and 2,500 nmol pyrene per mouse** for group 3. (HPLC conditions are presented in the Methods section.) The urine and fecal samples are from the 21-hour fraction. Glucuronide and sulfate standards were synthesized using microsomes and a mixture of 1-nitropyren-3-ol, 1-nitropyren-6-ol, and 1-nitropyren-8-ol and had the following retention times: 1-nitropyren-6-*O*-glucuronide and 1-nitropyren-8-*O*-glucuronide, 44 minutes; 1-nitropyren-6-*O*-sulfate and 1-nitropyren-8-*O*-sulfate, 50 minutes; 1-nitropyren-6-ol and 1-nitropyren-8-ol, 62 minutes; 1-nitropyren-3-ol, 71 minutes; and 1-nitropyrene, 73 minutes.

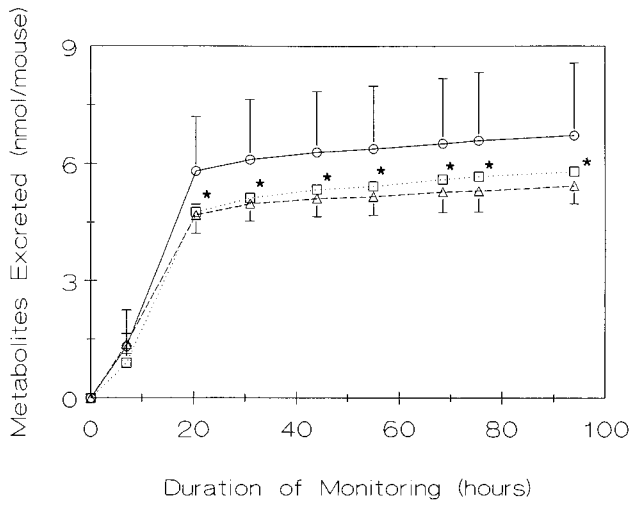

Figure 7. Total excretion of [^{3}H]1-nitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 10 nmol [^{3}H]1-nitropyrene per mouse for group 4 (○, solid line), **10 nmol [^{3}H]1-nitropyrene and 250 nmol 2-aminofluorene per mouse** for group 5 (□, dotted line), **and 10 nmol [^{3}H]1-nitropyrene and 2,500 nmol 2-aminofluorene per mouse** for group 6 (△, dashed line) **was monitored for up to 94 hours.** Values represent the average value ± SD for three cages with three mice per cage. The results designated by the asterisk (*) are significantly different from the control group's values at that time ($p<0.05$).

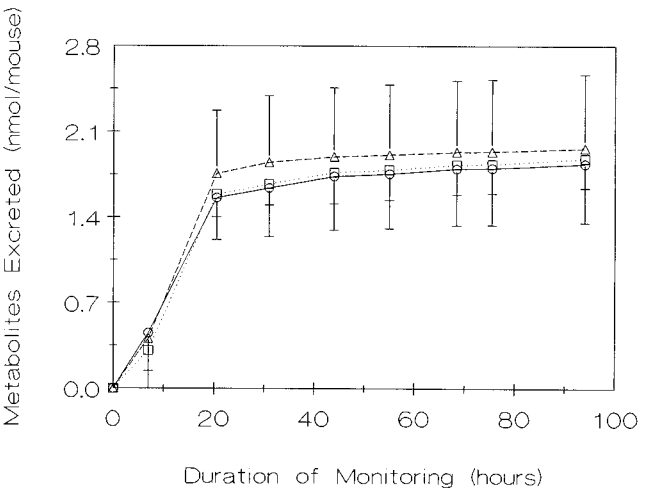

Figure 8. Urinary excretion of [^{3}H]1-nitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 10 nmol [^{3}H]1-nitropyrene per mouse for group 4 (○, solid line), **10 nmol [^{3}H]1-nitropyrene and 250 nmol 2-aminofluorene per mouse** for group 5 (□, dotted line), **and 10 nmol [^{3}H]1-nitropyrene and 2,500 nmol 2-aminofluorene per mouse** for group 6 (△, dashed line) **was monitored for up to 94 hours.** Values represent the average value ± SD for three cages with three mice per cage.

IN VIVO METABOLISM OF [^{3}H]1-NITROPYRENE AND THE EFFECT OF THE COADMINISTRATION OF 2-AMINOFLUORENE

The overall effect of the administration of 2-aminofluorene on [^{3}H]1-nitropyrene metabolism in vivo is shown in Figure 7. Although a dose-response effect seems apparent, and several values for the 25-fold group are significantly different from the control values at the same time points, at the 95% confidence level, there is no dose-related trend.

Figure 8 illustrates the cumulative excretion of the metabolites in the urine of the mice. Approximately 18% of the administered [^{3}H]1-nitropyrene was excreted in the urine. The administration of increasing amounts of 2-aminofluorene resulted in no change in the total excretion of the [^{3}H]1-nitropyrene metabolites into the urine. Figure 9 dem-

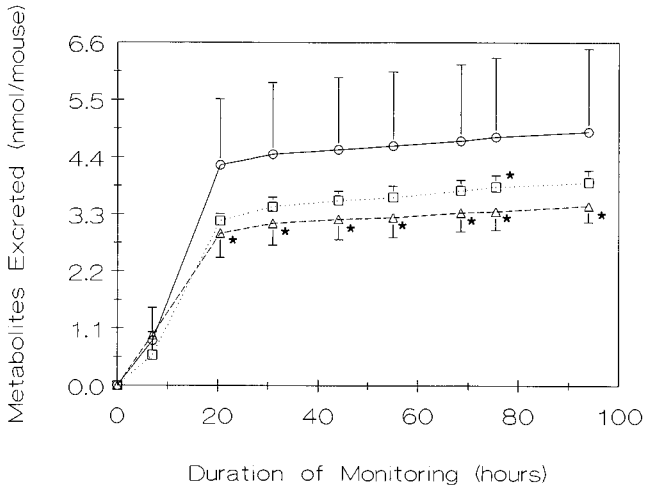

Figure 9. Fecal excretion of [^{3}H]1-nitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 10 nmol [^{3}H]1-nitropyrene per mouse for group 4 (O, solid line), **10 nmol [^{3}H]1-nitropyrene and 250 nmol 2-aminofluorene per mouse** for group 5 (□, dotted line), **and 10 nmol [^{3}H]1-nitropyrene and 2,500 nmol 2-aminofluorene per mouse** for group 6 (△, dashed line) **was monitored for up to 94 hours.** Values represent the total [^{3}H]1-nitropyrene and ^{3}H-labeled metabolites in the feces as the average value ± SD for three cages with three mice per cage. The results designated by the asterisk (*) are significantly different from the control group's values at that time ($p<0.05$).

onstrates the cumulative excretion of [^{3}H]1-nitropyrene metabolites in the feces of the mice. Approximately 50% of the administered [^{3}H]1-nitropyrene was excreted in the feces. A trend was observed whereby the increasing amounts of 2-aminofluorene resulted in a decrease in the excretion of

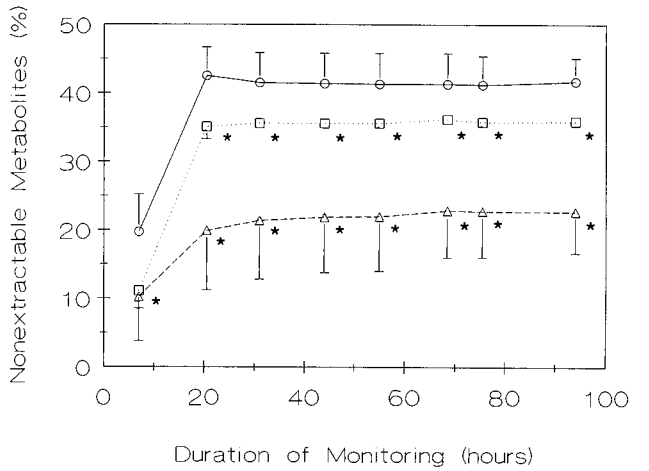

Figure 10. The percentage of fecal [^{3}H]1-nitropyrene metabolites not extractable from the fecal matrix. The feces were from male $B_6C_3F_1$ mice intraperitoneally administered 10 nmol [^{3}H]1-nitropyrene per mouse for group 4 (O, solid line), 10 nmol [^{3}H]1-nitropyrene and 250 nmol 2-aminofluorene per mouse for group 5 (□, dotted line), and 10 nmol [^{3}H]1-nitropyrene and 2,500 nmol 2-aminofluorene per mouse for group 6 (△, dashed line) and monitored for up to 94 hours. Values represent the average value ± SD for three cages with three mice per cage. The results designated by the asterisk (*) are significantly different from the control group's values at that time ($p<0.05$).

[^{3}H]1-nitropyrene metabolites into the feces. The high dose of 2-aminofluorene induced a statistically significant change in the excretion ($p<0.05$).

A strong dose-response relationship was observed ($p<0.05$) between the coadministration of 2-aminofluorene and the amount of [^{3}H]1-nitropyrene metabolites that could not be extracted from the feces (Figure 10). In the control group, the nonextractable [^{3}H]1-nitropyrene metabolites reached a plateau at approximately 42% of the total [^{3}H]1-nitropyrene metabolites in the feces. As the 2-aminofluorene concentration was increased from a 25-fold molar excess to a 250-fold molar excess, the nonextractable radioactivity decreased from 36% to 23%, respectively.

IN VIVO DNA ADDUCT FORMATION BY 1-NITROPYRENE AND THE EFFECT OF THE COADMINISTRATION OF PYRENE

Mice were each administered 10 nmol 1-nitropyrene, along with a 25-fold or 250-fold molar excess of pyrene. DNA was isolated from liver nuclei two days after treatment and analyzed by ^{32}P-postlabeling for the presence of DNA adducts. Treatment-related adducts were not detected.

IN VIVO METABOLISM OF [^{3}H]1,6-DINITROPYRENE AND THE EFFECT OF THE COADMINISTRATION OF PYRENE AND 1-NITROPYRENE

Figure 11 shows the total excretion of [^{3}H]1,6-dinitropyrene metabolites from mice coadministered a 25-fold molar excess of pyrene or 1-nitropyrene with 100 nmol [^{3}H]1,6-dinitropyrene. The coadministration of these compounds had no statistically significant effect on the total excretion of the [^{3}H]1,6-dinitropyrene metabolites. As shown in Figures 12 and 13, pyrene and 1-nitropyrene had no statistically significant effect on the excretion of [^{3}H]1,6-dinitropyrene metabolites in the urine or feces. The effect of the coadministration of 1-nitropyrene on the formation of nonextractable [^{3}H]1,6-dinitropyrene metabolites in the feces (Figure 14) was similar to the effect of 2-aminofluorene and [^{3}H]1-nitropyrene (Figure 10). Approximately 44% of the [^{3}H]1,6-dinitropyrene metabolites in the feces were nonextractable by the extraction regimen, which included acetonitrile, acetic acid, and sodium hydroxide at 50°C. The addition of a 25-fold molar excess of pyrene had no statistically significant effect on the formation of this material; however, the administration of a 25-fold molar excess of 1-nitropyrene decreased the nonextractable [^{3}H]1,6-dinitropyrene metabolites by 27%.

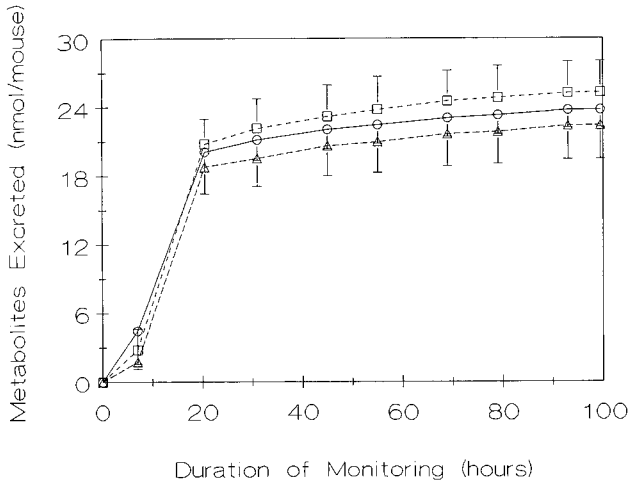

Figure 11. Total excretion of [^{3}H]1,6-dinitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 100 nmol [^{3}H]1,6-dinitropyrene per mouse for group 7 (○, solid line), **100 nmol [^{3}H]1,6-dinitropyrene and 2,500 nmol 1-nitropyrene per mouse** for group 8 (□, dotted line), **and 100 nmol [^{3}H]1,6-dinitropyrene and 2,500 nmol pyrene per mouse** for group 9 (△, dashed line) **was monitored for up to 99.5 hours.** Values represent the average value ± SD for three cages with three mice per cage.

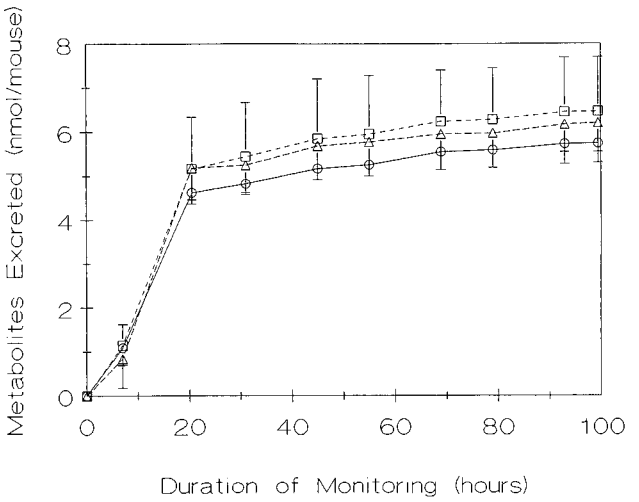

Figure 12. Urinary excretion of [^{3}H]1,6-dinitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 100 nmol [^{3}H]1,6-dinitropyrene per mouse for group 7 (○, solid line), **100 nmol [^{3}H]1,6-dinitropyrene and 2,500 nmol 1-nitropyrene per mouse** for group 8 (□, dotted line), **and 100 nmol [^{3}H]1,6-dinitropyrene and 2,500 nmol pyrene per mouse** for group 9 (△, dashed line) **was monitored for up to 99.5 hours.** Values represent the average value ± SD for three cages with three mice per cage.

IN VIVO DNA ADDUCT FORMATION BY 1,6-DINITROPYRENE AND THE EFFECT OF THE COADMINISTRATION OF PYRENE AND 1-NITROPYRENE

Mice were each exposed to 100 nmol [^{3}H]1,6-dinitropyrene, and then killed after two days. The DNA from liver nuclei was isolated, and the presence of DNA adducts was quantified by the presence of nonextractable ^{3}H. The results are shown in Table 5. Administering [^{3}H]1,6-dinitropyrene to mice at 100 nmol/mouse resulted in the formation of the C-8 deoxyguanosyl DNA adduct in the liver at levels of 0.41 ± 0.06 fmol/μg DNA. Including 1-nitropyrene at a 2.5-fold and a 25-fold excess resulted in increases of adducts to 0.50 ± 0.13 and 0.73 ± 0.29 fmol/μg DNA, respectively.

Aliquots of the liver DNA also were analyzed by ^{32}P-postlabeling (Figure 15). In each instance, a single major adduct with the same elution characteristics as an adduct obtained from DNA modified with *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene was detected. When the adduct concentrations were determined on the basis of this standard, they compared quite favorably with values estimated from measuring the ^{3}H content (Table 5). The inclusion of a 25-fold excess of pyrene over the [^{3}H]1,6-dinitropyrene resulted in a statistically significant ($p<0.01$) 26% decrease in the 1,6-dinitropyrene DNA adducts, as measured by the ^{32}P-postlabeling technique. The inclusion of a 25-fold excess of 1-nitropyrene significantly ($p<0.05$) increased the level of 1,6-dinitropyrene DNA adducts in the livers of the mice by 28%. The ^{32}P-postlabeling technique was unable to detect any 1-nitropyrene DNA adducts in either of the DNA adduct studies, even at doses of 2,500 mnol per mouse.

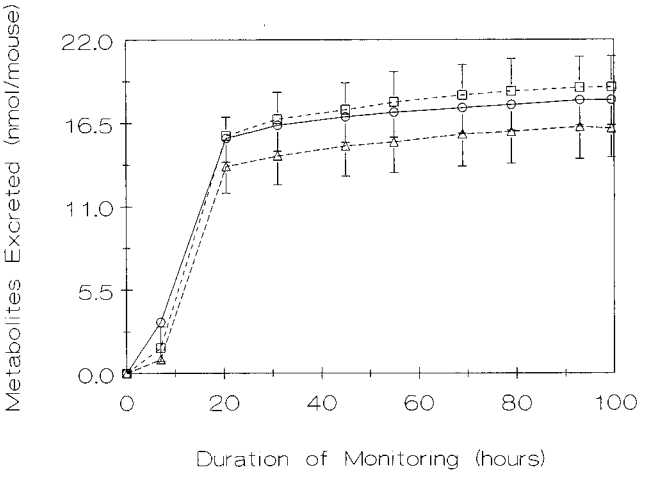

Figure 13. Fecal excretion of [^{3}H]1,6-dinitropyrene metabolites from male $B_6C_3F_1$ mice intraperitoneally administered 100 nmol [^{3}H]1,6-dinitropyrene per mouse for group 7 (○, solid line), **100 nmol [^{3}H]1,6-dinitropyrene and 2,500 nmol 1-nitropyrene per mouse** for group 8 (□, dotted line), **and 100 nmol [^{3}H]1,6-dinitropyrene and 2,500 nmol pyrene per mouse** for group 9 (△, dashed line) **was monitored for up to 99.5 hours.** Values represent the total [^{3}H]1,6-dinitropyrene and ^{3}H-labeled metabolites in the feces as the average value ± SD for three cages with three mice per cage.

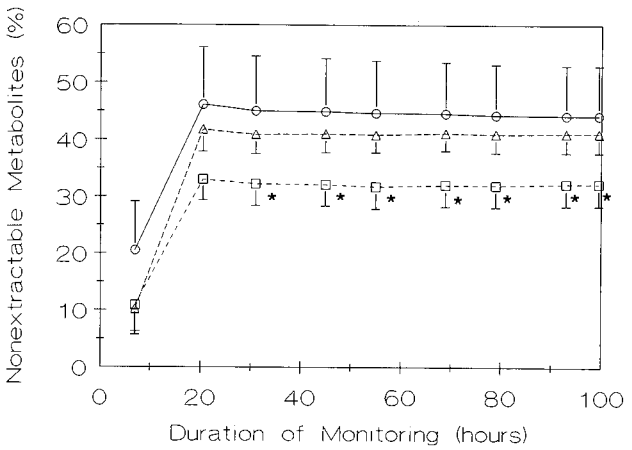

Figure 14. The percentage of fecal [^{3}H]1,6-dinitropyrene metabolites not extractable from the fecal matrix. The feces were from male $B_6C_3F_1$ mice intraperitoneally administered 100 nmol [^{3}H]1,6-dinitropyrene per mouse for group 7 (○, solid line), 100 nmol [^{3}H]1,6-dinitropyrene and 2,500 nmol 1-nitropyrene per mouse for group 8 (□, dotted line), and 100 nmol [^{3}H]1,6-dinitropyrene and 2,500 nmol pyrene per mouse for group 9 (△, dashed line) and monitored for up to 99.5 hours. Values represent the average value ± SD for three cages with three mice per cage. The results designated by the asterisk (*) are significantly different from the control group's values at that time ($p < 0.05$).

DISCUSSION

The studies described in this report were designed to test the hypothesis that copollutants present in mixtures at concentrations much higher than those of suspected carcinogens will alter the metabolism and bioactivation of the carcinogens. This one-year pilot project was designed to determine whether these effects could be detected in a limited-design study. In view of the time and design limitations, we could not ask specific questions concerning the effects of long-term exposure of animals to copollutants or complex mixtures (as copollutants) or the effects such exposure would have on enzyme levels, the carcinogen metabolism, and DNA adduct formation. Nor were the experiments designed to address the likelihood that continued exposure of animals to genotoxic compounds would result in altered states of DNA repair, or that altered routes of administration would affect the pharmacokinetic distribution of the compounds. Rather, as presented above in the Results section and as discussed below, the experiments were designed to allow a preliminary investigation of the effect of likely copollutants on two nitro-PAH environmental copollutants.

A number of studies provide support for this hypothesis. Huang and colleagues (1981) demonstrated a differential effect of flavone and 7,8-benzoflavone on the in vitro metabolism of benzo[*a*]pyrene. The metabolism of 80-nM benzo[*a*]pyrene with purified cytochrome P-450 isozymes LM3c and LM4 was stimulated more than 5-fold by the addition of approximately 500-μM flavone. Including 7,8-benzoflavone at 50- to 100-μM concentrations stimulated LM3c-mediated benzo[*a*]pyrene metabolism 10-fold, but had only marginal effects with LM4. Including 7,8-benzoflavone inhibited the metabolism of benzo[*a*]pyrene by isozymes LM2, LM3b, and LM6. The metabolism of benzo[*a*]pyrene was principally catalyzed (in decreasing order) by LM6>LM3b> all other isozymes. When flavone was added, the principal metabolism switched to LM4>LM6>LM3b. Likewise, when 7,8-benzoflavone was added, the principal metabolism switched to LM3b≈LM3c≈LM4>LM6. Similar effects were noted in another study with α-naphthoflavone and LM2 and LM4 (Johnson et al. 1979). Therefore, the addition of compounds that are metabolized through the cytochrome P-450 system is capable of altering the metabolism of a chemical carcinogen, in this case benzo[*a*]pyrene. We have re-

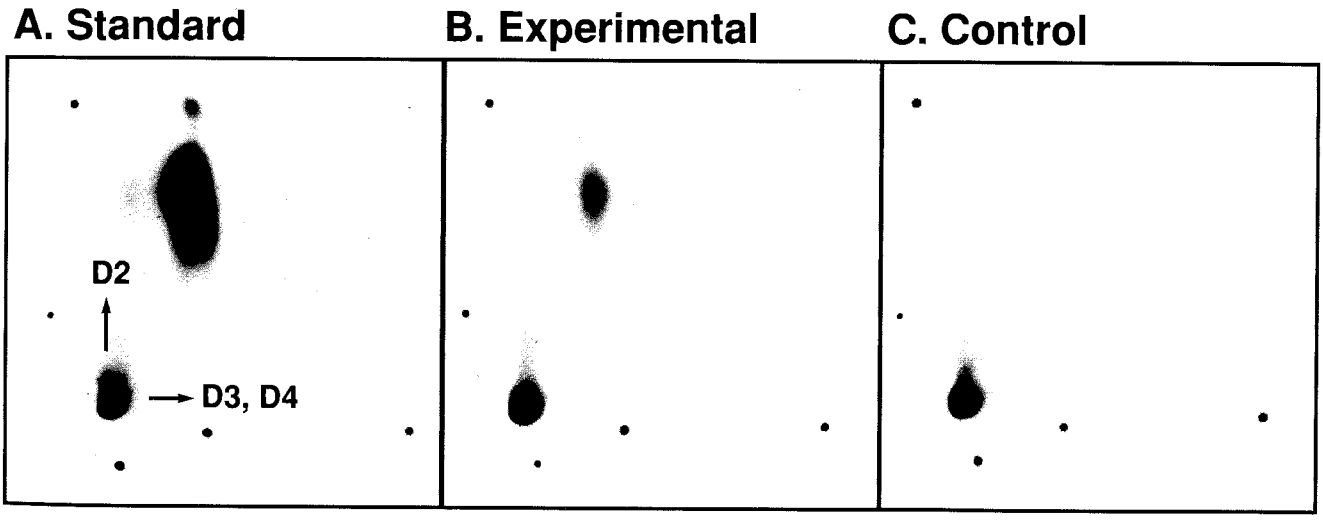

Figure 15. ^{32}P-Postlabeling autoradiograms of (A) a DNA standard modified with *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene; (B) liver DNA from a male $B_6C_3F_1$ mouse treated with 100 nmol 1,6-dinitropyrene; and (C) liver DNA from a male $B_6C_3F_1$ mouse treated with the solvent (100 μL dimethylsulfoxide and trioctanoin [14:86]).

ported similar results with the rabbit microsomal metabolism of 1-nitropyrene (Howard et al. 1988).

The antihypertensive drug debrisoquine is metabolized by the cytochrome P-450 isozymes to 4-hydroxydebrisoquine, and exhibits polymorphism in humans (Relling et al. 1989). Other medicinals, including ajmalicine (an antihypertensive), α-lobeline (a respiratory stimulant), haloperidol (a neuroleptic drug), and phenylcyclopropylamine (an antidepressant), have been shown to compete for the polymorphically expressed debrisoquine P-450, which results in a prolonged serum half-life for the debrisoquine (Fonné-Pfister and Meyer 1988; Relling et al. 1989). Therefore, if a carcinogen is metabolized through the debrisoquine P-450, the simultaneous presence of the above drugs can alter the carcinogen's activity. Similarly, because Raucey and associates (1989) have shown that acetaminophen is metabolized by P-450 2E1 (rabbit form, 3a; rat form, j) and P-450 1A2 (rabbit form, 4; rat form, d), an analogous situation could exist with the simultaneous presence of acetaminophen and carcinogens that are activated or detoxified by P-450 2E1 or 1A2. In fact, Raucey and colleagues (1989) attributed ethanol toxicity to the cometabolism of acetaminophen and ethanol by P-450 2E1.

We have shown in these experiments that the coadministration of 2-aminofluorene alters the metabolism of [^{3}H]1-nitropyrene in vivo. This alteration in metabolism was accomplished not through the formation of new or different metabolites, but apparently through affecting the distribution of [^{3}H]1-nitropyrene. This had the net result of increasing the total [^{3}H]1-nitropyrene excreted through the feces. Pyrene was ineffective in altering the metabolism of [^{3}H]1-nitropyrene. In the in vitro experiments, 2-aminofluorene was a better inhibitor of mouse liver microsomal metabolism of [^{3}H]1-nitropyrene, thus possibly explaining the greater effect of 2-aminofluorene than pyrene on [^{3}H]1-nitropyrene metabolism in vivo. In each of the in vivo studies, the inability of either 2-aminofluorene or pyrene to inhibit the metabolism of [^{3}H]1-nitropyrene demonstrated that the intrinsic hepatic clearance rates for [^{3}H]1-nitropyrene must be quite high and not saturable at these concentrations. This is made evident by the first-order elimination of [^{3}H]1-nitropyrene metabolites in the feces and urine (data not shown). Saturation of the metabolic systems in the mice would have resulted in a zero-order elimination of [^{3}H]1-nitropyrene metabolites from the mice during the initial excretion.

In the experiments designed to determine the level of 1-nitropyrene–DNA adducts and the effect of pyrene administration on these levels, DNA adducts were not detected. This result is in agreement with results of earlier studies demonstrating a general lack of DNA adduct formation by 1-nitropyrene in vivo (Beland 1991). There are several metabolic steps at which 1-nitropyrene can form genotoxic metabolites. The first pathway is nitroreduction to an *N*-hydroxyarylamine. This would result in the formation of a C-8-guanyl adduct. The lack of this adduct in previous studies (Beland 1991) and in this study indicates that this is not an important genotoxic pathway in vivo for 1-nitropyrene at these concentrations. The principal route for detoxifying 1-nitropyrene is through cytochrome P-450–mediated oxidation. Metabolites of cytochrome P-450 oxidation include phenolic derivatives of 1-nitropyrene at C-3, C-4, C-5, C-6, C-8, C-9, and C-10. With the mouse liver microsomes in vitro, the predominant metabolites were 1-nitropyren-6-ol and 1-nitropyren-8-ol (not shown). The *O*-sulfates of 1-nitropyren-6-ol and 1-nitropyren-8-ol were present in the urine and feces of the mice treated with [^{3}H]1-nitropyrene, whereas the *O*-sulfate of 1-nitropyren-3-ol was not present (Figure 6). The cytochrome P-450 isozymes metabolize 1-nitropyrene to two K-region epoxides (C-4 to C-5, C-9 to C-10). Both of these epoxides have been shown to be mutagenic and form DNA adducts in Chinese hamster ovary cells in vitro (Smith et al. 1990a). However, the absence of any DNA adducts in the in vivo studies with 1-nitropyrene suggests that amounts of the epoxides formed in vivo are insufficient to facilitate DNA adduct formation. However, this study was not designed to determine the adducts that could have been formed at higher doses of 1-nitropyrene. It has been shown that animal epoxide hydrolases and glutathione transferases have differing affinities for the two K-region epoxides of 1-nitropyrene (Kataoka et al. 1991). Therefore, at higher doses of 1-nitropyrene and, hence, higher levels of the epoxides, DNA adduction may occur.

One interesting aspect of the in vivo studies was the formation of the nonextractable [^{3}H]1-nitropyrene and [^{3}H]1,6-dinitropyrene fecal metabolites. The origin of these metabolites is unclear at present, and it is not known whether these are protein adducts or some other metabolites of [^{3}H]1-nitropyrene or [^{3}H]1,6-dinitropyrene that resist extraction by our protocol. However, in both cases, the presence of higher levels of copollutants resulted in decreases in the formation of this material. In the case of the studies with [^{3}H]1,6-dinitropyrene, this decrease in nonextractable metabolites from the feces was accompanied by increased numbers of [^{3}H]1,6-dinitropyrene–DNA adducts. Although the two may not be metabolically related, we cannot explain this phenomenon without further investigation into the identity of the nonextractable fecal metabolites.

The bioactivation of 1,6-dinitropyrene has been shown to proceed through nitroreduction of one of the nitro groups to an *N*-hydroxyarylamine (Djurić et al. 1985; Watanabe et al. 1990; Einistö et al. 1991). Owing to the stability of this *N*-hydroxyarylamine, DNA binding is enhanced by *O*-acetylation to 1-(*N*-acetoxy)-amino-6-nitropyrene, which forms DNA adducts following decomposition to a reactive arylnitrenium ion. In the absence of any cytochrome P-450 C-oxidation of 1,6-dinitropyrene (Djurić et al. 1985, 1988; Heflich

et al. 1986c; Smith et al. 1990b), the only route for metabolic detoxification is through nitroreduction to an *N*-hydroxyarylamine, then further to an arylamine. This implies that the same metabolic pathway is responsible for the detoxification and for the bioactivation, which may explain the differences in the tumorigenicity of 1,6-dinitropyrene and 1-nitropyrene. Although 1-nitropyrene has several pathways for detoxification, the only pathway available for 1,6-dinitropyrene is nitroreduction.

In our experiments with [^{3}H]1,6-dinitropyrene metabolism, we found that the presence of 1-nitropyrene affected the excretion of [^{3}H]1,6-dinitropyrene metabolites. It increased the amounts of [^{3}H]1,6-dinitropyrene metabolites that were excreted, decreased the nonextractable metabolites that were in the feces, and increased the 1,6-dinitropyrene–DNA adduct formation. Shimada and Guengerich (1990) reported that human cytochrome P-450 3A4 is responsible for the nitroreduction of 1,6-dinitropyrene in vitro. We reported that the same human cytochrome P-450 is responsible for the human liver microsomal C-oxidation of 1-nitropyrene (Howard et al. 1990). Therefore, if a similar situation exists in mice, in which the same cytochrome P-450 is responsible for the activation of 1,6-dinitropyrene and the detoxification of 1-nitropyrene, the DNA adduct formation should have decreased in the mice treated with 1,6-dinitropyrene and 1-nitropyrene. The data at present do not allow us to determine the mechanism for the effect of 1-nitropyrene on 1,6-dinitropyrene metabolism and DNA adduct formation.

The data reported herein support our initial hypothesis that the presence of certain copollutants will affect the metabolism and DNA adduct formation of suspected carcinogens in complex mixtures. In these experiments, we used a simple mixture of two components. In the environment, we are exposed to complex mixtures that contain hundreds or thousands of different chemicals. These chemicals may affect the metabolism of the carcinogens in the complex mixture, which are usually present in minor concentrations. These results suggest that many of the tumorigenic studies that have been performed to date with single carcinogens may have to be reevaluated in light of the components of the complex mixtures in which they are present in the environment, and that studies must be designed to look more closely at the effects of classes of chemicals and individual components of complex mixtures on carcinogen metabolism.

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Paul C. Howard is currently a Staff Scientist at the National Center for Toxicological Research in Jefferson, AR. During the course of the research reported here, he was an Associate Professor in the Department of Environmental Health Sciences at Case Western University, Cleveland, OH. His studies for the past 16 years have focused on the metabolic activation, metabolic detoxification, and DNA binding of chemicals. During his graduate studies at the University of Arkansas for Medical Sciences, he investigated the formation and repair of 2-acetylaminofluorene–DNA adducts in primary cultures of rat hepatocytes. As a postdoctoral fellow at the National Center for Toxicological Research, he studied the metabolism of 1-nitropyrene, and identified and studied the formation and repair of the 1-nitropyrene–DNA adduct in in vitro systems. As a faculty member of the School of Medicine at Case Western Reserve University, he focused his efforts on understanding the metabolic activation and

cytochrome P-450 metabolism of several nitrated polynuclear aromatic hydrocarbons including 1-nitropyrene and 3-nitrofluoranthene.

Frederick A. Beland is Director of the Division of Biochemical Toxicology at the National Center for Toxicological Research, Jefferson, AR. For the past 25 years, he has applied the concepts of bioorganic and theoretical chemistry to elucidate the mechanisms of action of organic toxicants, mutagens, and carcinogens. As an undergraduate student at Colorado College, he examined the accumulation of organochlorine pesticides in animals and humans at various levels of the food chain. During his graduate studies at Montana State University, he investigated the degradation of organochlorine pollutants using three different systems: electrochemistry, molecular orbital calculations, and a model reductive biological environment. While a postdoctoral fellow at the University of Chicago, he prepared and studied the reactivity of polynuclear aromatic hydrocarbon metabolites. During the last 15 years, he has concentrated his efforts on understanding the mechanisms of chemical carcinogenesis, with particular emphasis on aromatic amines and nitropolynuclear hydrocarbons.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Howard PC, Smith BA, Fullerton NF, Beland FA. 1993. Modulations of the in vivo DNA binding of 1,6-dinitropyrene by pyrene and 1-nitropyrene in male $B_6C_3F_1$ mice. Proc Am Assoc Cancer Res 34:940.

ABBREVIATIONS

$^{app}K_i$	apparent kinetic inhibition constant
$^{app}K_m$	apparent Michaelis-Menten kinetic constant
$^{app}V_{max}$	apparent maximal velocity
cpm	counts per minute
dG-C8-AP	*N*-(deoxyguanosin-8-yl)-1-aminopyrene
dpm	disintegrations per minute
HPLC	high-performance liquid chromatography
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
nitro-PAHs	nitrated polycyclic aromatic hydrocarbons
NNK	4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone
NNN	*N'*-nitrosonornicotine
PO_4	phosphate

INTRODUCTION

The environment, particularly in heavily industrialized regions, contains a wide variety of pollutants, including many that are mutagenic, carcinogenic, or both. The toxicological effects are not completely understood, especially when these substances occur as components of complex mixtures.

Diesel exhaust is one example of a complex pollutant mixture. From an environmental health perspective, diesel exhaust is of particular concern because it contains a large number of polynuclear aromatic hydrocarbons (PAHs)* and nitro-PAHs, compounds known to be mutagenic and carcinogenic in cells and in laboratory animals. Although a number of individual PAHs and nitro-PAHs have been characterized in various bioassays, it is likely that their biological effects are altered when they are part of a complex mixture. However, it is not known why, under what conditions, or through what mechanisms these alterations occur.

The Health Effects Institute (HEI) has issued several Requests for Applications (RFAs) to solicit research proposals aimed at better understanding the health effects of diesel engine exhaust and individual constituents of diesel emissions. Fully understanding these health effects, and those of other mobile-source emissions, requires methods specific to the study of the health effects of complex mixtures. Therefore, HEI has funded a small theoretical research program on complex mixtures; in addition, HEI has issued a Request for Preliminary Applications (RFPA) that provides a mechanism for considering proposals that fall outside the focused areas of inquiry of individual RFAs. Dr. Paul Howard's proposal, "The Effect of Copollutants on the Carcinogenicity of Chemicals," was submitted through this process in response to RFPA 89–3, "Motor Vehicle Emissions Health Effects Studies: Exposure Assessment Methods," and was considered in the winter of 1989. The Health Research Committee encouraged Dr. Howard to submit a full proposal, which he did in January 1990. His study, which was intended to be a one year study, began in September 1990. However, the Research Committee approved a four-month no-cost extension requested by the investigator to clarify some of the disparity between the in vivo and in vitro findings. Total costs for the study were $111,186. Dr. Howard's final report was received in March 1992; a revised version was received in December 1992, and was accepted for publication by the Health Review Committee in February 1993. 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.

* A list of abbreviations appears at the end of the Investigators' Report.

REGULATORY BACKGROUND

Diesel engines are more efficient and emit 10% to 20% less carbon dioxide than gasoline engines. Despite these advantages, their use has potential environmental and health consequences. Diesel engines operating without emissions control technology emit higher levels of oxides of nitrogen than gasoline engines, and a highly complex mixture of gases and carbonaceous particles. The particles in diesel exhaust are particularly troublesome because they are of respirable size and have hundreds of toxic chemicals, including PAHs, adsorbed onto their surfaces.

Polynuclear aromatic hydrocarbons and their chemical derivatives are well known to be mutagenic in bacterial and mammalian cells and carcinogenic in laboratory animals (International Agency for Research on Cancer 1989). Moreover, several long-term animal bioassays have shown that chronic inhalation of high concentrations of diesel exhaust causes lung tumors in rats, and some evidence suggests that workers exposed to high concentrations of diesel exhaust have a higher incidence of lung cancer than their nonexposed counterparts (reviewed by Mauderly 1992). Because of concerns about the potential health consequences of inhaling diesel exhaust, the U.S. Environmental Protection Agency (EPA) sets emissions standards for diesel engines and vehicles under Section 202 of the Clean Air Act, as amended in 1990.

Section 202(a)(1) of the Act directs the Administrator of the EPA to "prescribe (and from time to time to revise) . . . standards applicable to the emissions of any air pollutant from any class or classes of 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) of the Act specifically directs the Administrator to set standards for the "emissions of carbon monoxide, hydrocarbons, oxides of nitrogen and particulate matter from classes of heavy-duty vehicles and engines. . . ."

The EPA has taken a variety of regulatory actions with respect to diesel engines and vehicles under the authority given it by Sections 202(a)(1) and 202(a)(3)(A)(i). For example, the EPA has set emission standards for hydrocarbons, carbon monoxide, oxides of nitrogen, and particulate matter for heavy-duty and light-duty trucks. These emission standards are initially made applicable to all engines and vehicles produced in a given model year. Engines and vehi-

cles of the same class that are produced in succeeding years must also comply with these existing standards, unless the EPA establishes a new set of standards.

Determining appropriate emission standards for particles and hydrocarbons depends, in part, on assessing the health risks they present. That process requires a better understanding of the interactive effects of pollutants found in the emissions than currently exists. Dr. Howard's study was funded by HEI in order to develop an approach to measure interactions of compounds found in diesel exhaust that may pose health risks.

SCIENTIFIC BACKGROUND

A large number of pollutants to which humans are exposed are complex mixtures of thousands of chemical compounds. Typical examples include cigarette smoke, toxic waste, emissions from industrial sources, and fossil fuel combustion products including diesel exhaust. Although it has long been known that exposure to many of these mixtures poses health risks, it has been difficult to quantify the risks associated with specific compounds found within the mixtures, or to quantify or predict the risks of whole mixtures. To understand the potential health risks of the mixture as a whole we need to understand more than the individual effects of compounds known to be present in the mixture. When individual compounds are combined in a mixture, they can be expected to interactively affect a number of processes, including metabolism, repair mechanisms, clearance, or other activities that modify the biologically effective dose of a toxic chemical. Thus, it is important to find a feasible way to address the toxicity of mixtures: both to assign risk to particular compounds within the mixture, and to understand their altered risks when they are present as part of a complex mixture. Dr. Howard investigated the interactive effects of constituents of diesel engine emissions; he chose as model compounds three nitropyrenes that are thought to contribute to the carcinogenicity of diesel engine exhaust.

NITROPYRENES IN DIESEL EXHAUST

Nitropyrenes, a class of nitro-PAHs containing one or more nitro groups covalently bound to the carbon atoms of pyrene, account for approximately half of the total mutagenic activity of diesel exhaust in bacterial assays (Huisingh et al. 1978). 1-Nitropyrene (1-NP) is mutagenic in hamster and human cells, and in bacteria (Takayama et al. 1983; Heflich et al. 1985, 1986 a,b; Patton et al. 1986; Eddy et al. 1987). However, most of the mutagenic activity of the nitropyrenes in bacteria is produced by 1,6-dinitropyrene (1,6-DNP) and 1,8-dinitropyrene (1,8-DNP), compounds found in much lower concentrations than 1-NP in diesel particle extracts (Mermelstein et al. 1981; Rosenkranz 1982; Salmeen et al. 1984). In addition to being more mutagenic than 1-NP, the dinitropyrenes are also substantially more tumorigenic (Tokiwa and Ohnishi 1986, Imaida et al 1991). Although many studies of mutagenicity and tumorigenicity have examined individual nitropyrenes, it is well known that the nitropyrenes occur as complex mixtures in diesel extracts, and may compete for similar metabolic pathways. Thus, it is possible that the mutagenic or carcinogenic response to any of these compounds changes when it is part of a mixture.

METABOLIC FATE OF NITROPYRENES

Metabolizing foreign compounds usually involves enzymatically converting them to more water-soluble polar derivatives. Polar groups can be formed in several ways. The most common process involves adding hydroxyl groups to the molecule through an oxidative process that involves cytrochromes P-450. In aromatic compounds that contain a substituent group, this also could involve adding hydroxyl groups to the ring, which is called ring oxidation. Hydroxyl groups introduced through oxidation can serve as sites for enzymatic addition of glucuronic acid or sulfate, to form water-soluble products that can be excreted readily in urine or bile (Sipes and Gandolfi 1986).

In certain cases, converting foreign compounds to more water-soluble derivatives also can occur through reduction. (Both 1-NP and 1,6-DNP have been shown to undergo nitroreduction.) Oxidation and reduction both may produce compounds that form easily excreted conjugates. However, both processes also may convert foreign compounds to more toxic species; for example, electrophilic metabolites that can react covalently with various nucleophilic sites in DNA, RNA, and proteins to form adducts (Miller 1978). The relative importance of oxidation and reduction in the formation of more toxic metabolites of the nitropyrenes is complex. Results of in vitro studies implicate the role of nitroreduction as a pathway for converting 1-NP and 1,6-DNP to intermediate forms that react with DNA in bacteria and mammalian tissues (Djurić et al. 1986a, 1988). In vivo studies of 1-NP have been less consistent in demonstrating nitroreduction as the activation pathway for 1-NP. DNA adducts of 1-NP reduction products have been identified in lungs, kidneys, and mammary glands of rats and mice treated with 1-NP (Stanton et al. 1985; Mitchell 1988; Roy et al. 1989). However, when rats were treated with 1-NP at doses that cause tumors, an unidentifiable adduct that does not appear to be a reduction product also is found in DNA obtained from rat mammary gland (Smith et al. 1990).

The structural similarity of the nitropyrenes suggests that they may be metabolized by the same enzymes. Thus, when both 1-NP and 1,6-DNP are present together, it is possible that changes in their metabolism will occur, resulting in a shift in the pattern of formation of DNA adducts, or a shift in excretory patterns of metabolites. In a similar way, the parent compound, pyrene, an environmental pollutant from numerous sources, may influence the direction of metabolism of 1-NP or 1,6-DNP, possibly increasing or decreasing the formation of certain DNA adducts.

JUSTIFICATION FOR THE STUDY

We have limited understanding of the interactive effects of components of diesel exhaust, and of the interactive effects of exposure to diesel exhaust and other environmental pollutants such as cigarette smoke or ozone. This is partly due to a lack of appropriate methods to provide quantitative information on the risk to human health from inhaling whole emissions from motor vehicles. Because these emissions are mixtures of thousands of potentially toxic compounds, the HEI Health Research Committee was interested in developing better methods to assess the toxicity of compounds found in such mixtures.

Dr. Howard proposed to investigate the effects of exposure to one or more pollutants with closely related chemical structures. Specifically he proposed a study to test the effect of pyrene, a ubiquitous environmental pollutant, on the in vivo and in vitro metabolism and potential carcinogenicity of 1-NP. He hypothesized that pyrene would inhibit the ring oxidation of 1-NP, reducing the amount of 1-NP metabolized through the pathway that is generally regarded as being a detoxification pathway. This could lead to an increase in metabolizing of 1-NP through the nitroreductive pathway, and thus to an increase in the formation of DNA adducts. The Health Research Committee thought that Dr. Howard's proposed study would provide a foundation for building quantitative methodologies to assess the risk of human exposure to complex mixtures. Because the study was designed to be a pilot study, it would be conducted at a relatively modest cost, and was expected to provide information about the feasibility of using this methodology for evaluating interactions in other complex mixtures.

OBJECTIVES AND STUDY DESIGN

The overall objective of Dr. Howard's study was to test the hypothesis that pyrene alters 1-NP metabolism and thereby increases 1-NP–DNA adduct formation. The study was extended to examine the effects of pyrene or 1-NP on the metabolism of 1,6-DNP. To test these hypotheses, the study had three specific aims:

1. To establish in vitro kinetic parameters of 1-NP metabolism by mouse liver cytochromes P-450 alone and in the presence of a 20 mol and 200 mol excess of pyrene.
2. To establish the effect of pyrene on the in vivo metabolism of 1-NP, and the effect of pyrene or 1-NP on the in vivo metabolism of 1,6-DNP. The in vivo effects were to be determined by examining the pattern of urinary and fecal excretion of radiolabeled metabolites of the administered xenobiotic.
3. To establish the effect of pyrene on in vivo formation of DNA adducts after the administration of 1-NP or 1,6-DNP. In addition, the effect of 1-NP on in vivo formation of DNA adducts from 1,6-DNP was to be tested. The formation of DNA adducts was to be determined by (a) quantifying ^{3}H-labeled DNA adducts formed in liver and lung after the administration of the radiolabeled xenobiotic; and (b) identifying adducts in these same tissues using ^{32}P-postlabeling analyses (Randerath et al. 1981).

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The initial objectives of this one-year study were largely met. The investigators determined the in vitro parameters of 1-NP metabolism, determined excretory patterns of animals treated with nitropyrenes, and demonstrated the formation of nitropyrenes-DNA adducts. However, it was not possible to develop from the study results a cohesive picture of the interactive effects of pyrene and nitropyrenes, or of nitropyrenes on each other.

ASSESSMENT OF METHODS AND STUDY DESIGN

This investigation represents a reasonable starting point to address the issues outlined in their proposal. The biochemical methods employed in this investigation were clearly outlined and were appropriate for this pilot study of the effects of copollutants. The choice of the copollutant to be administered with a nitropyrene as the primary pollutant appears to be logical. Pyrene was selected because of its ubiquitous occurrence, and because it is the parent compound of the genotoxic agents 1-NP and 1,6-DNP. Furthermore, close chemical association are apparent among pyrene, 1-NP, and 1,6-DNP: All three are similar in structure, and it is reasonable to assume that pyrene, 1-NP, and 1,6-DNP would be present together as environmental pollutants. Finally, pyrene represents one of the more abundant PAH pollutants in the environment.

The investigators' decision to conduct further in vitro experiments testing a number of the inhibitory effects of compounds closely related to pyrene in structure or in metabolism by cytochrome P-450 was logical; the same is true of their decision to test the in vivo effects of 2-aminofluorene, a potent in vitro inhibitor of 1-NP metabolism.

STATISTICAL ANALYSES

Two statistical methods were employed in this project: (1) enzyme-kinetic modeling was employed for the in vitro experiments, and (2) a repeated-measures analysis of variance (ANOVA) was used to analyze the results of the in vivo experiments.

In vitro metabolic data were analyzed using standard mathematical models. The model parameters include the apparent maximal velocity ($^{app}V_{max}$) for the metabolic rate, the apparent concentration of the substrate that produces half-maximal metabolism ($^{app}K_m$ [the Michaelis-Menten-kinetic constant]), and the apparent dissociation constant of a competitive inhibitor ($^{app}K_i$). Commercial software was used to produce estimates of these parameters using the enzyme-kinetic equations and nonlinear regression of metabolic rate on 1-NP concentration.

In vivo experimental results were based upon cumulative excretion curves obtained from mice after administrating either 1-NP or 1,6-DNP as the pollutant. Three separate experiments were conducted. The first tested the effects of pyrene as a copollutant on the metabolism of 1-NP. The second tested the effects of 2-aminofluorene as a copollutant on the metabolism of 1-NP. The third tested the effects of either pyrene or 1-NP as copollutants on the metabolism of 1,6-DNP. Each of these three experiments used nine mice, separated into three groups of three mice per cage. Thus, the level of replication was $n = 3$ per dose combination.

Compartmental analysis is the most frequently employed mathematical model used to analyze excretion data. The data from each cage is fitted to a rising exponential curve to obtain an excretory rate constant that is characteristic of the mice within that experimental group. This is done for both urinary and fecal excretion. The primary data used to compare the effects of various doses of copollutant (pyrene, 2-aminofluorine or 1-NP) are the excretory rate constants, which are compared using an ANOVA. The sample size in these comparisons is $n = 3$, the number of cages per dose combination.

The investigators chose, instead, to analyze the excretion curves by repeated-measures ANOVA. In contrast to the method described above, repeated-measures ANOVA analyzes data from within the experimental group as well as data between experimental groups in the same one-step analysis. The advantages of this approach are (1) no mathematical form (exponential or otherwise) need be assumed for the excretion data, and (2) across-time sample size and error structure are preserved and exploited statistically for each sample. The serial correlation between successive observations on a given cage is acknowledged and accounted for explicitly.

Comparisons among doses of potential inhibitor (pyrene, 2-aminofluorene, or 1-NP) were made on the basis of a whole-curve. In most instances, the investigators failed to demonstrate a statistically significant dose response. A notable exception was the metabolism of 1-NP in the presence of 2.5 μmol pyrene. As can be seen in Figure 10 of the Investigators' Report, the inhibitor pyrene was seen to significantly lower the curve for nonextractable fecal excretion.

Comparisons also were made at each individual time point. Error bars are illustrated in Figures 7 through 14 to indicate cage-to-cage variation within each time point. Asterisks mark each time point at which the dose of the inhibitor resulted in the excretion of either 1-NP or 1,6-DNP at a level that was significantly different from the control group. As a graphical device, these error bars and asterisks are somewhat misleading because they tend to obscure the serial correlation in the data. One cannot determine from the error bars whether the three cages at a particular dose produced precise, parallel excretion curves, which would provide high statistical resolving power, or alternatively, whether the curves from individual cages crossed and recrossed, which would provide lower statistical power. It would have been preferable to show a confidence band around the whole curve rather than to use multiple local error bars. A similar criticism can be applied to the use of asterisks. Because of serial correlation, the successive comparisons that are made are not statistically independent. The use of multiple asterisks give a false impression of statistical significance, whereas a single "whole curve" test would better represent the statistical strength of these comparisons.

RESULTS AND INTERPRETATIONS

The experimental results provide some evidence that copollutants, such as pyrene, may influence the genotoxic potency of the potentially carcinogenic compounds 1-NP and 1,6-DNP. The experiments performed to examine the effects of pyrene on the in vitro metabolism of 1-NP indicated that pyrene was a mixed-type inhibitor with an $^{app}K_i$ of 6.42 μM. However, the effect of pyrene on the in vitro metabolism of 1-NP was not particularly remarkable. By comparison, in vitro experiments using a number of other compounds indicated that 2-aminofluorene and 3-amino-2-methoxydibenzofuran, which had been selected on the ba-

sis of their structural similarity to 1-nitropyrene, or on the basis of their metabolism by specific cytochromes, P-450 were more effective inhibitors of in vitro metabolism than pyrene.

The effect of pyrene on in vivo metabolism of 1-NP was also not particularly remarkable. Coadministration of pyrene at either a 25- or a 250-fold molar excess did not affect the overall excretion of 1-NP, although it did alter the route of excretion when it was coadministered at the higher dose level (the amount of 1-NP metabolites excreted in the urine decreased by 20%). There was a similar increase of excretion in the feces. These changes were not, however, significant at the 95% confidence level. Coadministration of pyrene at either a 25- or 250-fold molar excess also had no effect upon the metabolism or excretion of 1,6-DNP. Finally, coadministration of 1-NP had no effect on the metabolism and excretion of metabolites of 1,6-DNP. It is difficult to interpret the significance of these results because the effects produced by 1-NP and 1,6-DNP were dependent upon the nature and concentration of the copollutant.

One interpretation of these results is that pyrene and the nitropyrenes are metabolized by different cytochrome P-450 isoforms. For example, different isoforms have been found to be involved in the detoxification pathways of 1, 3-, 1,6-, and 1,8-DNP in human liver microsomes (Shimada and Guengerich 1990). Although there is less evidence for different isoforms of P-450 in animal systems than in humans, the possibility that in the mouse different isoforms metabolize pyrene, 1-NP, and 1,6-DNP cannot be discounted.

One of the major effects of copollutants observed in this study was on the DNA adduct formation. These results also are difficult to interpret because the alterations were observed only when DNA adducts were quantified by the ^{32}P-postlabeling method, and not when DNA was examined for incorporation of [^{3}H]1-NP or [^{3}H]1,6-DNP metabolites. When male B6C3F$_1$ mice were treated with 10 nmole of 1-NP alone, no DNA adducts were detected in their livers using ^{32}P-postlabeling analysis as the means of detection. Coadministration of a 25- or 250-fold excess of pyrene did not alter this result. When mice were treated with 100 nmol of 1,6-DNP alone, a single major DNA adduct was detected in the livers. Coadministration of a 25-fold molar excess of pyrene caused a significant decrease in DNA adduct formation from 1,6-DNP when adduct formation was determined using ^{32}P-postlabeling analysis. In contrast, coadministration of 1-NP together with 1,6-DNP resulted in a significant increase in 1,6-DNP adduct formation when measured with ^{32}P-postlabeling analysis. Neither of these results was, however, confirmed when DNA adducts were measured by analyzing nonextractable ^{3}H bound to DNA isolated from the livers of treated mice under any of the treatment protocols. Although the lack of concordance of the DNA adducts experiments does not necessarily dismiss the results obtained with ^{32}P-postlabeling, it does raise concerns about the basis for the discrepancy.

Differences in the two methods of measuring DNA adducts may contribute to the lack of agreement. In the radiolabeling experiments, the animal were exposed to ^{3}H-labeled nitropyrene. The presence of DNA adducts, formed as the result of the exposure, was determined by measuring the level of nonextractable ^{3}H present in the isolated DNA. The ^{32}P-postlabeling technique, one of the more sensitive assays, does not require radiolabeled DNA and needs only 1 to 50 μg of DNA for detecting adducts. As developed by Randerath (Randerath et al. 1981), DNA samples containing adducts are digested by endonucleases and exonucleases to become 3′-monophosphates. The modified nucleotides are preferentially labeled at the 5′- position using ^{32}P–adenosine triphosphate and polynucleotide kinase. With limited amounts of ^{32}P-adenosine triphosphate, the relative labeling of some bulky adducts to unmodified nucleotides is increased. The ^{32}P-postlabeling assay is quite sensitive for detecting known DNA adducts with detection levels of approximately one adduct per 10^{9} nucleotides (Kadlubar 1992).

Where nonextractable ^{3}H levels are measured to detect DNA adducts, it is assumed that all of the radioactivity in isolated DNA is associated with covalently linked adducts, and that the levels of ^{3}H are not affected by ^{3}H exchange or by direct incorporation of ^{3}H into the DNA. Experiments using ^{32}P-postlabeling assume that the adducts formed are stable to the isolation and chromatographic conditions necessary for their analysis. When DNA adducts of polynuclear aromatic compounds or aryl amines are the adducts of concern, these assumptions have been shown not to be valid in some instances. In addition, it is possible that the additional manipulations associated with ^{32}P-postlabeling analysis may have contributed to the differences in detectable adduct levels, or that more than one DNA adduct was present. To address these possibilities and to correlate the two analytical methods, both types of analyses should focus on individual adducts. Thus, analyses quantifying nonextractable ^{3}H should not only measure undigested DNA, but should include chromatographic analysis of individual nucleosides and ^{32}P-postlabeling analysis of DNA adducts. Such an approach might provide data that could allow for a more sound interpretation of the results and their significance.

These results highlight one primary concern associated with attempting to study complex mixtures by selecting one or two components. In some experiments, the concentration of a copollutant relative to the specific carcinogen was a factor that influenced whether an effect was observed. In other cases, the effects of two individual copollutants on a selected carcinogen countered one another. In a practical

sense, therefore, it would be difficult to use such an approach to assess the effects of one or two such copollutants when isolated from the mixture and relate this behavior to effects of the pollutants within a complex mixture.

IMPLICATIONS FOR FUTURE RESEARCH

The environmental occurrence of pyrene and its close chemical relationship to both 1-NP and 1,6-DNP provide a reasonable expectation that it might exert an effect on the metabolism and genotoxic potential of 1-NP and 1,6-DNP. The data from this study, however, do not indicate that this is true. They suggest, instead, that pyrene, as a copollutant, would not exert such a profound effect on the metabolism and DNA binding of either 1-NP or 1,6-DNP as to override the effect of the many other components present in a typical complex environmental mixture. Furthermore, if the data obtained from ^{32}P-postlabeling analysis represent a true measure of effect, the coadministration of either pyrene or 1-NP together with 1,6-DNP appears to produce opposing effects with regard to the level of DNA adducts that are formed in vivo. These experimental findings, coupled with the observation that some of the effects of these copollutants are dependent on dose, suggest that further studies on the effects of individual copollutants on the genotoxic potential of environmental carcinogens may not be the most promising approach to understanding interactive effects in complex mixtures.

It is difficult, in the absence of potent synergistic or inhibitory effects, to attempt to attribute the effect of a complex mixture to one or two selected components. The difficulty in assigning definitive biological effects to the two different copollutants assessed in this study reflects the problem associated with assessing the significance of results obtained with simple synthetic mixtures. The study results suggest that the focus of future research should be on the effect of complex mixtures as opposed to one or two selected agents present in the mixture. A more useful approach may be to develop methods to determine the impact of the sum of all components within a complex mixture on the genotoxic potential of environmental carcinogenic agents identified as components of that same complex mixtures.

CONCLUSIONS

The results of this pilot study suggest that pyrene does not have a profound impact upon either the metabolism or genotoxicity of 1-NP or 1,6-DNP. Pyrene was initially selected as the copollutant for investigation in these studies because of its ubiquitous occurrence and similar chemical properties to the genotoxic agents, 1-NP and 1,6-DNP. To some extent, 1-NP did increase the formation of metabolites of 1,6-DNP in the urine and feces of mice exposed to both pollutants. Using ^{32}P-postlabeling as a means of detection, an increase in the level of 1,6-DNP–DNA adducts formed when a 25-fold molar excess of 1-NP was coadministered was apparent. This effect was dependent on dose, however, and was not confirmed by an alternative method of DNA adduct analysis.

This study suggests that further efforts to elucidate the overall effects of complex environmental mixtures by employing simple mixtures do not appear to represent a viable approach, and reaffirms the need to assess complex mixtures as a whole, and to examine the effect of a whole mixture on several select and specific environmental carcinogens known to be present in such mixtures.

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