HEI HEALTH EFFECTS INSTITUTE

Metabolism and Biological Effects of Nitropyrene and Related Compounds

Charles M. King
Michigan Cancer Foundation, Detroit, MI

Includes the Report of the Institute's Health Review Committee

Research Report Number 16

HEALTH EFFECTS INSTITUTE

HEI

The Health Effects Institute (HEI) is a nonprofit corporation founded in 1980 to assure that objective, credible, high-quality scientific studies are conducted on the potential human health effects of motor vehicle emissions. Funded equally by the U.S. Environmental Protection Agency (EPA) and 27 automotive manufacturers or marketers in the United States, HEI is independently governed. Its research projects are selected, conducted, and evaluated according to a careful public process, including a rigorous peer review process, to assure both credibility and high scientific standards. HEI makes no recommendations on regulatory and social policy. Its goal, as stated by former EPA Administrator William D. Ruckelshaus, is "simply to gain acceptance by all parties of the data that may be necessary for future regulations."

Research Agreement No. 83-14 **Designed and printed by Prime Communications, Inc.**

TABLE OF CONTENTS

Research Report No. 16

Metabolism and Biological Effects of Nitropyrene and Related Compounds

INVESTIGATOR'S REPORT C.M. King

HEALTH REVIEW COMMITTEE'S REPORT Health Effects Institute

ABBREVIATIONS

acetyl CoA	acetyl coenzyme A
HPLC	high-pressure liquid chromatography
MFH	malignant fibrous histiocytoma
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
tRNA	transfer ribonucleic acid

Metabolism and Biological Effects of Nitropyrene and Related Compounds

Charles M. King

ABSTRACT

The aim of this project was to compare the carcinogenicities of 1,3-, 1,6-, and 1,8-dinitropyrene, 1-nitropyrene, and the metabolic phenolic derivatives of 1-nitropyrene. The biochemical goal was to establish how these inert compounds are converted metabolically, by target tissues, to reactive species that can alter the DNA of the susceptible cell.

The comparative tumorigenicities were assessed by the use of female CD rats. Control groups consisted of animals that had been given only the solvent, dimethylsulfoxide, which has a low toxicity, to maximize solubility. Equimolar doses (1.7 μmole/ml; 10 μmole/kg body weight) of the compounds were administered to weanling rats by intraperitoneal injection, or by intragastric intubation, three times each week for four weeks, for a total dose of 16 μmoles. Newborn animals were treated by subcutaneous injection of the 1,3-, 1,6-, or 1,8-dinitropyrene or 1-nitropyrene within 24 hours of birth, and at seven weekly intervals, for a total dose of 6.3 μmoles. The possible carcinogenicities of phenolic metabolites of 1-nitropyrene were examined by subcutaneous treatment of newborn animals, and at seven weekly intervals, with 1-nitropyrene, 3-hydroxy-1-nitropyrene, or a mixture of 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene (70 μmoles/ml; 100 μmoles/kg body weight; total dose, 63 μmoles). The relative carcinogenicity of 1-nitropyrene in the female Fischer 344 rats was established by the concurrent treatment of appropriate animals. The smaller, inbred Fischer rats received a total dose of 40 μmoles. Subcutaneous and intraperitoneal treatments of weanling female CD rats (70 μmoles/ml; 100 μmoles/kg body weight; 5 weekly injections) with 1-nitropyrene were carried out to explore possible differences because of the different routes of administration in animals of the same age.

Dinitropyrenes are much more carcinogenic than 1-nitropyrene (1,6- > 1,8- > 1,3-dinitropyrene > 1-nitropyrene). Phenolic derivatives of 1-nitropyrene are no more carcinogenic than the parent compound. The most likely tumors to be induced in this animal model are malignant fibrous histiocytomas (MFHs), mammary gland tumors, and leukemias. Animals treated with 1,6- or 1,8-dinitropyrene by subcutaneous injection yielded MFHs at the site within 15 weeks of the first injection (i.e., at 15 weeks of age), and this did not permit them to survive more than 20 to 25 weeks. Leukemia developed in these animals within this brief period, but mammary gland tumors were observed only when survival of the animal was not limited by the formation of the more aggressive MFHs. Rats given these compounds by intraperitoneal injection developed MFHs within 16 weeks, but the 1,8-dinitropyrene-treated animals survived longer and developed mammary tumors. Oral intubation yielded no MFHs, and only a modest number of mammary tumors, with any of the compounds. Mammary tumor induction by 1-nitropyrene was detected only in CD rats after long observation periods. There appeared to be little difference in the response of the mammary gland to 1-nitropyrene administered to weanling rats either by intraperitoneal or subcutaneous injection.

Metabolism experiments employed cytosols and intact cell preparations of mammary glands from 50- to 60-day-old female CD rats. Metabolite formation was established by high performance liquid chromatography (HPLC). Nucleic acid adduct formation in cytosols was studied with transfer ribonucleic acid (tRNA) that had been added to trap activated intermediates. In intact cells, binding to DNA was examined by hydrolysis of the DNA with trifluoroacetic acid and HPLC of the adducts.

Cytosolic enzymes of the rat mammary gland have the capacity to activate the dinitropyrenes by monoreduction to the hydroxylamine, and subsequent O-acetylation. Nucleic acid adduct formation is in the order: 1,6- > 1,8- > 1,3-dinitropyrene. Only reduction and acetylation pathways were detected with these compounds in intact cells; the DNA adducts are indistinguishable from those formed on incubation of these compounds with calf thymus DNA, rat liver cytosol, and acetyl coenzyme A (acetyl CoA). Reduction of 1-nitropyrene is more limited, and the putative hydroxylamine cannot be activated by O-acetylation. In contrast to the metabolism of the dinitropyrenes, intact rat mammary cells metabolize 1-nitropyrene primarily via oxidative pathways.

These studies demonstrate that dinitropyrenes can induce tumors in rat mammary gland, an organ distant from the application site, that can only activate these compounds by reductive and acetylation mechanisms. The ability of mammary cytosols to metabolize the dinitropyrenes to nucleic acid-bound adducts parallels their carcinogenicities in this organ. The DNA adducts formed in isolated, intact mammary cells are consistent with their formation by these mechanisms.

Department of Chemical Carcinogenesis, Michigan Cancer Foundation, 110 East Warren Avenue, Detroit, Michigan 48201

INTRODUCTION

Interest in the possibility that nitroaromatics might be present as atmospheric contaminants came from the suggestion that they might be formed from the combination of polycyclic aromatic hydrocarbons and nitrogen oxides that had been produced as by-products of combustion processes (Pitts et al., 1978). Detection of these arylnitro compounds by application of a *Salmonella typhimurium* mutagenicity test system was facilitated by the presence of an endogenous bacterial metabolic activation system (Weeks et al., 1978; McCoy et al., 1983; Saito et al., 1985). The mutagenicity of extracts of atmospheric particulates, in the absence of an external metabolic activation system, supported the idea that the mutagenicity of these atmospheric mutagens was more closely related to their metabolic reduction to aromatic amine derivatives than to their conversion to oxidative metabolites of polycylic aromatic hydrocarbons present in these materials (Lofroth, 1978). Comparative testing with a *Salmonella* strain that was deficient in nitroreductase, but not in its mutagenic response to arylhydroxylamines, provided evidence that nitroreductase-dependent mutagens were present in the environment (Wang et al., 1980). Subsequent studies, employing both bacterial and chemical analyses, demonstrated that arylnitro compounds could arise from sources as diverse as photocopy toners and diesel exhaust products (Rosenkranz and Mermelstein, 1983; Schuetzle, 1983). Attention was focused on the nitropyrenes because the mononitro product of pyrene, 1-nitropyrene, is believed to be the nitroaromatic that is found in greatest quantities in environmental samples (Schuetzle, 1983), and the dinitropyrenes derived from 1-nitropyrene are among the most mutagenic compounds for *Salmonella* that have been identified (Rosenkranz and Mermelstein, 1983).

Initial studies of the carcinogenic potential of the nitropyrenes employed systems that had been useful in assays of compounds that were likely to be pertinent to the nitroaromatics (e.g., the polycyclic aromatic hydrocarbons using mouse skin [El Bayoumy et al., 1982], or aromatic amines using rat tissues [Ohgaki et al., 1985; Hirose et al., 1984]). Alternative approaches employed routes of administration that were likely to result in exposure of the respiratory tract that might more closely resemble expected human exposures (e.g., the induction of lung tumors by 1,6-dinitropyrene on intratracheal instillation into hamsters [Takayama et al., 1985]). These first efforts disclosed that nitropyrenes could induce malignant tumors at the site of subcutaneous injection into rats (Ohgaki et al., 1985; Hirose et al., 1984), and that 1-nitropyrene could induce rat mammary tumors distant from the site of injection (Hirose et al., 1984). Experiments with mice, however, yielded few tumors as compared with rats, even when dinitropyrenes were injected subcutaneously (Tokiwa et al., 1984).

The present studies were undertaken to explore the comparative carcinogenicities of 1-nitropyrene and its dinitro derivatives in the female CD rat. The mammary gland, an organ that is susceptible to the aromatic amines (King, 1985), was of particular interest, since it would be expected to reflect the chemical properties of these compounds. It would, consequently, be independent of MFH formation at the treatment site that might result from both the chemical and the physical characteristics of the agents because of deposition at the injection site. Biochemical experiments with mammary tissue also were carried out to assess whether these agents were converted to nucleic acid adducts by mechanisms analogous to those that have been implicated in tumor formation in this organ by aromatic amines.

AIMS

In Specific Aim 1, the comparative carcinogenicities of 1-nitropyrene and 1,3-, 1,6-, and 1,8-dinitropyrene were compared after intraperitoneal injection into weanling female CD rats, an animal model expected to be susceptible to tumor induction via mechanisms involving arylhydroxylamines. Treatment with equimolar doses of these compounds, as solutions in dimethylsulfoxide, ensured that the results could be compared on a molar basis. This route of administration provided for potential systemic exposure without initial contact with the microflora of the gastrointestinal tract. Thus, it was reasoned that the compounds might be delivered to target tissues without prior reduction by intestinal bacterial systems. If the nitro compounds were to be reduced to amines that could not be N-oxidized by mammalian systems, it is probable that the carcinogenic potential would be decreased.

In Specific Aim 2, the comparative carcinogenicities of the compounds employed in Specific Aim 1 were explored after administration by intragastric intubation. The experiments of Specific Aims 1 and 2 were carried out concurrently with animals that had been received in one shipment and randomized prior to treatment, to permit comparison of these two routes of administration. Exposure to agents by oral intubation differs from administration by intraperitoneal injection in that intubation results in exposure of the compounds to the gut flora. Moreover, there is a potential loss of compound via fecal excretion if the compounds are poorly absorbed. Both intubation and intraperitoneal injection are expected to result

in initial distribution of the compounds by the portal system and, consequently, metabolism by the liver prior to systemic circulation.

In Specific Aim 3, the comparative carcinogenicities of the compounds employed in Specific Aims 1 and 2 were determined after subcutaneous injection of newborn female CD rats. This animal model had originally demonstrated the ability of 1-nitropyrene to induce mammary tumors in the rat as a consequence of systemic exposure. Two features of this animal model are noteworthy. First, systemic distribution of subcutaneously administered nitropyrenes, while limited by their low solubilities, is expected to occur without first being subject to hepatic metabolism. Second, as compared with the more mature weanling animal, the developing tissues of the newborn rat may be more susceptible to the carcinogenic effects of chemicals because higher rates of DNA synthesis may tend to amplify the biological effects of DNA modification.

In Specific Aim 4, the comparative carcinogenicities of 1-nitropyrene and its major phenolic metabolites, 3-hydroxy-1-nitropyrene and a mixture of 6- and 8-hydroxy-1-nitropyrene, were determined after subcutaneous injection into newborn female CD rats. The intent was to determine whether the carcinogenic activity of 1-nitropyrene might be mediated through these metabolites.

In Specific Aim 5, the relative susceptibilities of newborn female CD and Fischer 344 rats to the subcutaneous administration of 1-nitropyrene were established. This question was of interest because of the widespread use of the inbred Fischer strain in studies supported by the Health Effects Institute and other supporting agencies. Previous studies had shown this rat strain to be less sensitive to mammary tumor induction than the outbred Sprague-Dawley-derived rats (Malejka-Giganti and Rydell, 1978).

In Specific Aim 6, the comparative effects of subcutaneous and intraperitoneal injection of 1-nitropyrene into weanling female CD rats were explored. Although mammary tumor induction had been demonstrated after subcutaneous injection of 1-nitropyrene into newborn female CD rats, a second carcinogenicity experiment, initiated prior to embarking on this Health Effects Institute project, had suggested that 1-nitropyrene might not be carcinogenic when administered by intraperitoneal injection, at least when the observation period was limited to 62 weeks. These data prompted us to attempt to determine whether the differences between the results of the first two carcinogenicity experiments with 1-nitropyrene could be accounted for by the differences in the routes of administration, and whether the limited carcinogenicity of 1-nitropyrene might require longer observation periods for expression of potential tumorigenicity.

The final specific aim, number 7, was to explore the pathways by which the rat mammary gland, a target tissue of the nitrated pyrenes, metabolizes 1-nitropyrene and 1,3-, 1,6-, and 1,8-dinitropyrene. There were two objectives. One was to explore those metabolic conversions that generate low molecular weight products, e.g., the formation of metabolites through oxidative, reductive, and acetylation mechanisms. The second objective was to attempt to identify those pathways that are responsible for DNA modification by these agents and are, therefore, likely to be crucial to the induction of tumors by these compounds.

MATERIALS AND METHODS

ANIMALS

Female CD rats from Charles River Breeding Laboratories (Wilmington, Mass.), that were either pregnant (i.e., 13 to 15 days) or weanling, were used for tumor induction studies; 50- to 60-day-old females were used for biochemical preparations. The animals were maintained, three per cage, on layers of softwood shavings in polycarbonate cages with solid bottoms. The room was air-conditioned at 22-24°C at 55% humidity, and the animals were maintained on a 12-hour light-dark cycle. Water and laboratory chow (Wayne Lab Blox, Allied Mill, Inc., Chicago, Ill.) were given ad libitum.

CHEMICALS

Throughout this report a consistent nomenclature has been used; each compound that possesses a nitro group is named as if it were a derivative of 1-nitropyrene. Although this differs from some commonly used systems, it is intended to avoid the confusion that might occur if we were to employ nomenclature conventions that require renumbering due to metabolic conversion. The specific structures of the compounds employed in tumor induction studies are shown in Figures 1 and 5.

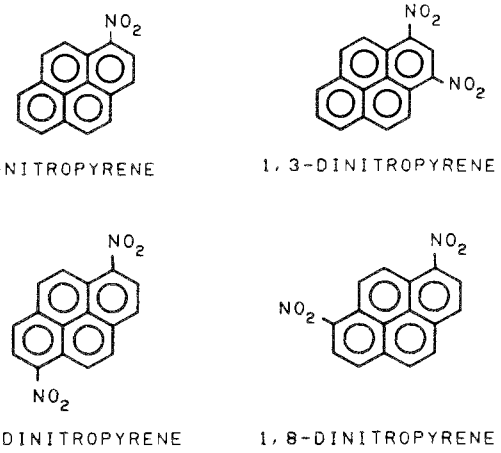

Figure 1. Nitropyrene structures.

The nitropyrenes (1-nitropyrene, 1,3-, 1,6-, and 1,8-dinitropyrene) used in this study were prepared by the Midwest Research Institute, Kansas City, Missouri, and made available to us by the Health Effects Institute, Cambridge, Mass. The 1-nitropyrene was greater than 99.9% pure, and the 1,3-, 1,6-, and 1,8-dinitropyrene were greater than 99% pure, as determined by HPLC and mass spectroscopy. The radioactive nitropyrenes contained tritium at the 4,5,9,10 positions. The labeled compounds were diluted to the required specific activities and then purified by chromatography on alumina. Radiochemical purity was established to be greater than 98% by reverse-phase HPLC by use of an on-line liquid scintillation detector (FLO-ONE model HP, Radiomatic, Tampa, Florida). 1-Aminopyrene (Kuhn, 1943) and the diaminopyrenes were prepared by reduction of the nitropyrene precursors with the use of hydrazine and Pd/C in tetrahydrofuran at 35-40°C. The corresponding aminonitropyrenes were prepared with the use of xanthine oxidase to reduce the corresponding dinitro derivatives (Bryant et al., 1984). 1-Acetylaminopyrene (Messier et al., 1981), 6-acetylamino-1-nitropyrene (Fifer et al., 1986), and 8-acetylamino-1-nitropyrene (Fifer et al., 1986) were prepared by acetylation of 1-aminopyrene, 6-amino-1-nitropyrene, and 8-amino-1-nitropyrene, respectively, by reaction with acetic anhydride at room temperature for 16 hours. Phenolic derivatives of 1-nitropyrene were synthesized by nitration of 1-acetoxypyrene and hydrolysis with sodium methoxide (El Bayoumy and Hecht, 1983). 4-Nitropyrene was prepared by nitration of hexahydropyrene and subsequent aromatization with dichlorodicyanoquinone (Bavin, 1959). The identity of these compounds was verified by infrared, mass, and nuclear magnetic spectroscopy, as required; purity was established by HPLC. 6-Nitroso-1-nitropyrene and 8-nitroso-1-nitropyrene were gifts of Dr. E. Kim Fifer of NCTR, Jefferson, AR.

METABOLISM BY RAT MAMMARY CYTOSOL

Rat mammary cytosolic fractions were freshly prepared from 50- to 60-day-old female CD rats, as previously described (King et al., 1979). Protein content was established by the Lowry method, with the use of crystalline bovine serum albumin as the standard (Lowry et al., 1951). Incubations (total volume equals 1 ml) were carried out in pyrophosphate buffer (pH 7.0; 50 mM) in the presence of dithiothreitol (1 mM), cytosolic protein (0 to 2 mg), NADPH or NADH (1 μmole) and the tritiated nitropyrene (0.035-0.05 μmole; 150 to 250 μCi/μmole) that had been added in dimethylsulfoxide (10 μl). Acetyl CoA (0.1 μmole) was included in certain experiments. Incubations were carried out at 37°C in air (aerobic) or argon (reduced oxygen tension) atmospheres. The reactions were terminated by the addition of acetone and the metabolites were extracted into ethyl acetate. The extracts were treated with sodium sulfate, evaporated to dryness under reduced pressure, and redissolved in dry tetrahydrofuran for analysis by HPLC. HPLC analysis of the ethyl acetate extracts was by reverse-phase chromatography with the use of a C-18 μBondapak column and water-methanol gradients. The tritium content of the chromatographic effluent was determined with an on-line liquid scintillation detector.

NUCLEIC ACID ADDUCT FORMATION

Determination of the abilities of tissue preparations to generate metabolites that are capable of reaction with nucleic acids was carried out with the aid of adduct formation with exogenously added tRNA. This technique permits detection of these metabolites with great sensitivity because of the possibility that higher concentrations of nucleic acid can be used. Moreover, the physical properties of tRNA, as compared to high molecular weight DNA, provide for easier manipulation of experimental material and lower values in control experiments. Reaction of the nitropyrenes with exogenously added tRNA, in the incubation systems described above, was determined in experiments that had included 0.75 mg of tRNA. Transfer RNA was recovered and purified by solvent extraction, and then assayed for tritium incorporation by liquid scintillation spectroscopy, as described previously (King, 1974).

METABOLISM BY RAT MAMMARY EPITHELIAL CELLS IN CULTURE

Rat mammary epithelial cells were obtained from 50 to 60-day-old female CD rats in the manner described by Ethier (1985), and cultured in Ham's F-12 medium supplemented with fetal bovine serum, insulin, hydrocortisone, epithelial growth factor, cholera toxin, gentamycin, and Fungizone. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The tritiated nitropyrenes (21.5 to 23 Ci/mmole) were dissolved in dimethylsulfoxide, and were added at a concentration of 1 mg/ml of medium at the time of plating. The dimethylsulfoxide concentration did not exceed 2%. Neither the nitropyrenes nor the solvent were toxic at these concentrations. After 24 hours, an aliquot of the medium was extracted twice, with equal volumes of ethyl acetate, for analysis by HPLC. DNA was isolated by solvent extraction, as described by Tay and Russo (1981). In some cases, the DNA was further purified by density gradient centrifugation in cesium chloride. The DNA was hydrolyzed with trifluoroacetic acid prior to characterization of the nitropyrene adducts by reverse-phase HPLC on C-18 μBondapak columns and water-methanol gradients (Tang and Lieberman, 1983).

TISSUE EVALUATION

Animals were necropsied when moribund, at the time of their death, or at their sacrifice at the termination of the exper-

iment. The liver, right and left kidneys, spleen, and, when detected and after measuring, tumors were weighed. All organs were examined macroscopically. The brain, pituitary gland, mammary gland, thyroid gland, esophagus, bronchus, lungs, stomach, small intestine, large intestine, liver, kidneys, spleen, ovary, preputial gland, and any pathological lesions observed macroscopically, including tumors, were fixed in phosphate-buffered formalin solution (pH 7.4). After ligation at both ends, the stomach was inflated with the same formalin solution. It was then opened by cutting the greater curvature, and was flattened on a paper surface. The tissues were numbered at the time of sacrifice to indicate the year, experiment, experimental group, and animal. These numbers were utilized throughout the evaluation process (i.e., with fixed tissues, tissue blocks, and mounted sections). All of these organs were processed for histological evaluation by trimming, embedding in paraffin, sectioning, and staining with hematoxylin and eosin. Mammary gland tumors were classified as adenocarcinoma, fibroadenoma, or adenoma, according to the criteria published by Young and Hallowes (1973). Malignant fibrous histiocytomas exhibited the typical storiform patterns, histiocytic cells, giant cells, and myxomatous patterns described by Ohgaki et al. (1982, 1984, 1985) and Hirose et al. (1984) after the administration of nitropyrenes. Other characteristics of these tumors have been described by Weiss and Enzinger (1978).

Statistical evaluation was by Chi-square analysis. The effective number of rats was the number of animals that were evaluated histologically after the detection of the first tumor-bearing animal in that particular experiment. The average tumor induction period was determined from the palpation observations of animals that were subsequently found to have histological evidence of tumor induction. It represents the length of time from the first carcinogen administration to tumor detection. The survival period was similarly determined as the time interval between the first administration of the the carcinogen and the death of the animal.

TUMOR INDUCTION PROTOCOLS

Specific Aims 1 and 2: Comparative Carcinogenicities of 1-Nitropyrene and Dinitropyrenes After Intraperitoneal Injection or Intragastric Intubation.

The nitropyrenes were dissolved in dimethlylsulfoxide (1.7 μmole/ml) for administration by intraperitoneal injection or intragastric intubation. The compounds administered were 1-nitropyrene and 1,3-, 1,6-, and 1,8-dinitropyrene. Control animals were treated only with dimethylsulfoxide in this experiment and in each of those described below. Each treatment group consisted of 36 weanling female CD rats that were administered 10 μmoles of the compound per kg, three times each week for four weeks. Preliminary experiments established that this dose, 5.9 ml dimethylsulfoxide solution per kg, was the maximum dose that could be tolerated without experiencing weight loss due to toxicity from the solvent. No nitropyrene-dependent toxicity was evident. As was the case with all of the carcinogenicity experiments, the weight of each animal was determined at each treatment and periodically throughout the remainder of the experiment. The total cumulative dose averaged 16 μmoles per rat. Animals were sacrificed when moribund, or at the end of 76 to 78 weeks. Tissues were evaluated grossly and histologically; the latter examinations were carried out after staining with hematoxylin and eosin. Statistical evaluation was by Chi-square analysis in this study and in those described below. The average tumor induction period was determined from the palpation observations of animals that were subsequently found to have histological evidence of tumor induction.

Specific Aim 3: Comparative Carcinogenicities of 1-Nitropyrene and Dinitropyrenes After Subcutaneous Injection.

Newborn female CD rats were treated eight times, at weekly intervals, with the dimethylsulfoxide solutions of the nitropyrenes described in Specific Aims 1 and 2 above, or with the solvent alone. Administration was by subcutaneous injection to the suprascapular region, starting within 24 hours of birth. The first dose was 2.5 μmoles per kg body weight, 5 μmoles per kg were used for the second and third injections, and 10 μmoles per kg were used for injections 4 through 8. This treatment schedule was also dictated by the toxicity of the dimethylsulfoxide, as established in preliminary experiments; the older animals could tolerate the larger quantities of the solvent. The animals each received an average total dose of 6.3 μmoles of compound. Each treatment group was comprised of 37 or more animals. The tumorigenic response was determined, as described above, in moribund animals, or in animals that were sacrificed at the end of 67 weeks.

Specific Aim 4: Comparative Carcinogenicities of 1-Nitropyrene and its Major Phenolic Metabolites.

The comparative carcinogenicities of 1-nitropyrene and its major phenolic metabolites, 3-hydroxy-1-nitropyrene and a mixture of 6- and 8-hydroxy-1-nitropyrene, were determined after subcutaneous injection into newborn female CD rats. Although the 3-hydroxy-1-nitropyrene was pure, as established by HPLC, the 6- and 8-hydroxy isomers were tested as a mixture because of the difficulty of obtaining sufficient quantities of pure material. 4-Nitropyrene was included for comparative purposes, since this compound has been shown

to have a greater carcinogenicity for the rat mammary gland than 1-nitropyrene on administration by intraperitoneal injection (Imaida et al., 1985). The protocol that had been employed initially with 1-nitropyrene was used (Hirose et al., 1984). Solutions of the compounds (70 μmole/ml dimethylsulfoxide; 100 μmole/kg body weight) were injected to the suprascapular region within 24 hours of birth, and at weekly intervals, for a total of eight doses. The total dose averaged 63 μmoles per animal. Animals were sacrificed when moribund or after 78 weeks of observation.

Specific Aim 5: Relative Susceptibilities of Newborn Female CD and Fischer 344 Rats to the Subcutaneous Administration of 1-Nitropyrene.

In a concurrent experiment carried out in conjunction with the tumor induction experiment described under Specific Aim 4, newborn female Fischer 344 rats were injected subcutaneously with 1-nitropyrene using the same treatment protocol. Because of the smaller size of the Fischer animals, the total dose administered averaged only 40 μmoles per animal. The animals in these two experiments were verified to be characteristic of the two strains by comparison of the differences in weight and by determination of the maternal levels of hepatic arylhydroxamic acid sulfotransferase (King and Olive, 1975).

Specific Aim 6: Comparative Effects of Subcutaneous and Intraperitoneal Injection of 1-Nitropyrene into Weanling Female CD Rats.

The relative efficiencies of tumor induction by 1-nitropyrene in weanling female CD rats was established after subcutaneous and intraperitoneal injection. The treatments consisted of five weekly injections of 1-nitropyrene (70 μmole/ml dimethylsulfoxide; 100 μmole/kg body weight), a dose rate and frequency of treatment that had yielded mammary gland tumors in a previous study (Hirose et al., 1984). The animals were sacrificed when moribund, or at the end of 88 weeks.

RESULTS

SPECIFIC AIM 1

The comparative carcinogenicities of 1-nitropyrene and 1,3-, 1,6-, and 1,8-dinitropyrene were compared after intraperitoneal injection into weanling female CD rats.

The structures of the nitropyrenes used in this study are shown in Figure 1. Treatment with these compounds did not affect the weight of the animals, compared with those injected with only the solvent, dimethylsulfoxide. Thus, each animal received, on the average, the same total molar quantity of the test compound. Intraperitoneal injection of 1,6- or 1,8-dinitropyrene resulted in the early deaths of animals, i.e., 12 to 15 weeks after the initial treatment. Characteristically, the peritoneal cavities of these animals contained bloody fluid, and the tissues showed evidence of an acute reaction without gross or histological evidence of malignancy. Tumors were first identified in a 1,6-dinitropyrene-treated animal autopsied 17 weeks after the first injection. All the animals in this treatment group that survived greater than 17 weeks had developed multiple soft tissue tumors in the peritoneal cavity that were classified histologically as MFHs (Figure 2). Residual 1,6-dinitropyrene, which co-chromatographed with the authentic compound, was observed in the peritoneal cavity on autopsy of several of these animals 16 weeks after the last injection. In the 1,8-dinitropyrene-treated group, the first intraperitoneal MFH was detected at 17 weeks; 88% of the animals had developed these tumors by 44 weeks (Table 1). Only two rats had MFHs in the 1,3-dinitropyrene treatment group. These tumors were observed only on necropsy at the end of the experiment, 78 weeks after the first injections. No MFHs were detected in animals treated with 1-nitropyrene or with dimethylsulfoxide alone.

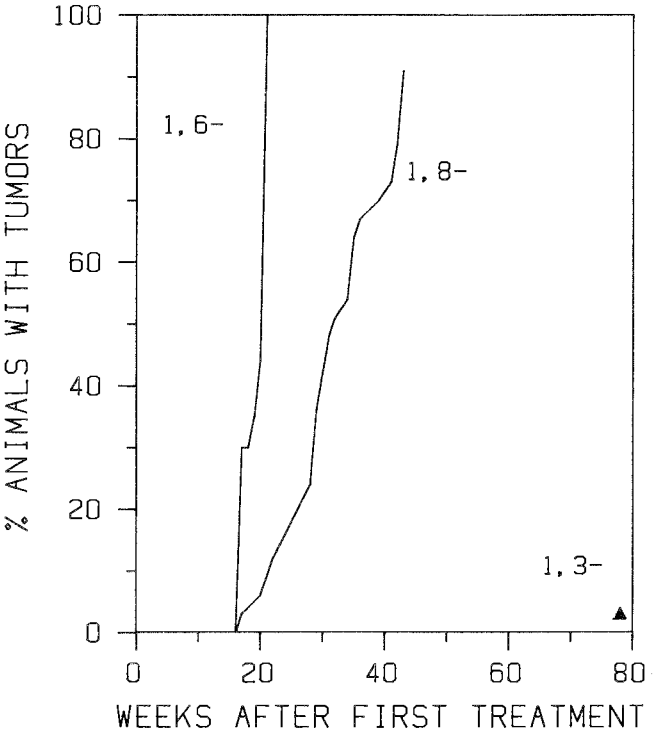

Figure 2. Malignant fibrous histiocytoma induction in weanling female CD rats after intraperitoneal injection of 1,3-, 1,6-, or 1,8-dinitropyrene, as described under Specific Aim 1 and Table 1.

Table 1. Incidences of Malignant Fibrous Histiocytoma and Leukemia in Weanling Female CD Rats Administered Nitropyrenes by Intraperitoneal Injection or Intragastric Intubation[a]

Compound Administered	Route of Administration	Effective Number of Rats	Number of Animals with Malignant Fibrous Histiocytoma	Number of Animals with Leukemia	Average Survival (days)
1-Nitropyrene	IP	36	0	1 (3%)	522
	IG	35	0	0	528
1,3-Dinitropyrene	IP	36	2 (6%)	2 (6%)	514
	IG	35	0	3 (9%)[d]	527
1,6-Dinitropyrene	IP	23	23 (100%)[b]	0	135
	IG	36	0	2 (6%)	518
1,8-Dinitropyrene	IP	33	29 (88%)[b]	7 (21%)[c]	236
	IG	36	0	1 (3%)	537
Dimethylsulfoxide	IP	31	0	0	535
	IG	36	0	0	517

[a] The animals were treated by intraperitoneal injection (IP) or intragastric intubation (IG) with the nitropyrenes (0.01 mmole/kg) three times per week for a total of 12 doses (total dose = 16 μmoles). Animals were sacrificed at 76 to 78 weeks.

[b] $p < 0.0001$, compared to solvent-treated controls administered by the same route.

[c] $p < 0.01$.

[d] $p > 0.05$.

Although the survival of the animals treated with 1,6- and 1,8-dinitropyrene was limited (Table 1), a significant incidence of myelocytic leukemia, sometimes reported as leukemia/lymphoma, developed in the 1,8-dinitropyrene group.

Mammary tumors were also detected in this experiment (Figure 3). Those treated with 1,6- or 1,8-dinitropyrene developed tumors earlier than the animals in the other groups. Although the MFHs that developed in these animals limited their survival, the incidence of palpable mammary tumors in the 1,8-dinitropyrene-treated group was approximately 50% by 44 weeks. The majority of the mammary tumors in the 1,6- and 1,8-dinitropyrene-treated groups were adenocarcinomas (Table 2). The incidence of adenocarcinoma of the mammary gland in the 1,6-dinitropyrene-treated group was not statistically significant when compared to the solvent-treated control group, but both the short induction period, and the number of tumors induced, support the conclusion that the limited survival precluded mammary tumor development. The incidences of both mammary adenocarcinoma and fibroadenoma in the 1-nitropyrene-treated group were significantly higher than in the control group, but the induction period was approximately the same in both groups, as determined by palpation of histologically confirmed, tumor-bearing animals. Although both the malignant and benign mammary tumor incidence in the 1,3-dinitropyrene-treated animals was significantly higher than in the controls, the response was more modest than with 1-nitropyrene.

At necropsy, there were few remarkable differences in organ weights, except for enlarged spleens in the 1,8-dinitropyrene-treated animals, probably due to the incidence of leukemia in this group. The livers of the 1,6- and 1,8-dinitropyrene-treated animals were 4.1 and 3.3% of their body weights, respectively. A few altered foci and hyperplastic nodules were observed in the livers, but none appeared related to treatment.

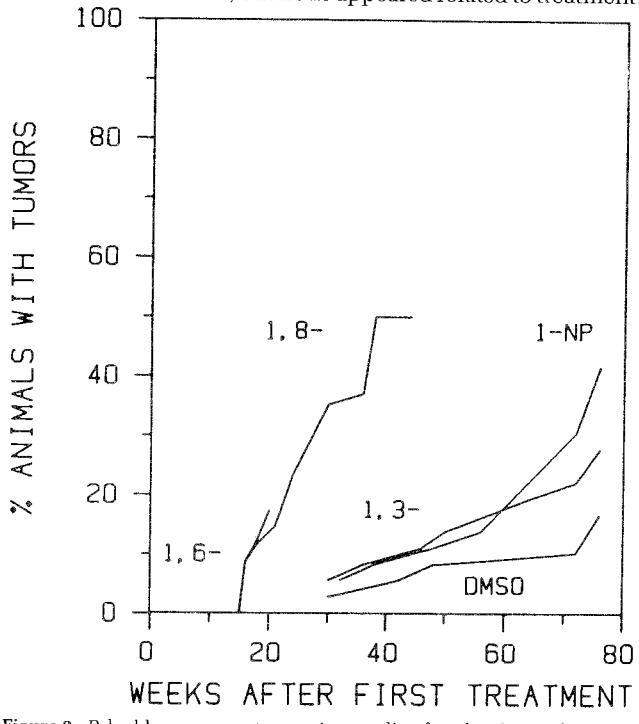

Figure 3. Palpable mammary tumors in weanling female CD rats after intraperitoneal injection of 1-nitropyrene (1-NP); 1,3-, 1,6-, or 1,8-dinitropyrene; or dimethylsulfoxide (DMSO), as described under Specific Aim 1 and Table 2.

Table 2. Comparative Induction of Mammary Tumors by Nitropyrenes After Intraperitoneal Injection into Weanling Female CD Rats[a]

Compound Administered	Effective Number of Rats	No. of Animals with Mammary Tumors				No. of Mammary Tumors			Average Induction Period (days)
		Adeno-carcinoma	Fibro-adenoma	Adenoma	Total	Adeno-carcinoma	Fibro-adenoma	Total	
1-Nitropyrene	36	14 (39%)[d]	19 (53%)[c]	0	25 (69%)[b]	19	21	40	455
1,3-Dinitropyrene	36	9 (25%)[f]	12 (33%)[f]	1 (3%)	19 (53%)[e]	16	16	33	440
1,6-Dinitropyrene	23	4 (17%)	0	1 (4%)	5 (22%)	16	0	17	130
1,8-Dinitropyrene	33	14 (42%)[c]	4 (12%)	1 (3%)	15 (45%)	28	4	33	179
Dimethylsulfoxide	31	3 (10%)	5 (16%)	0	7 (23%)	4	8	12	456

[a] Weanling female CD rats were treated by intraperitoneal injection (IP) with nitropyrenes (0.01 mmole/kg) three times per week for a total of 12 doses (total dose = 16 μmoles). Animals were sacrificed at 76 to 78 weeks.

[b] $p < 0.0001$, compared to solvent-treated controls.

[c] $p < 0.001$.

[d] $p < 0.01$.

[e] $p < 0.05$.

[f] $p > 0.05$.

As is expected in aging female CD rats, there were tumors of the adrenal and pituitary glands that were found at autopsy in treated animals, but they did not differ in incidence when compared to the control group. A papilloma was found in the forestomach of one animal treated with 1,8-dinitropyrene. Metastatic lesions, one MFH and one mammary adenocarcinoma, were found in the lungs of two animals from this group.

SPECIFIC AIM 2

The comparative carcinogenicities of 1-nitropyrene and 1,3-, 1,6-, and 1,8-dinitropyrene were explored after administration by intragastric intubation into weanling female CD rats.

There were no treatment-related differences in animal weight in this experiment, nor when compared to the previously described study in Specific Aim 1. In contrast to the results of the intraperitoneal injection of these compounds (Table 1), no MFHs were detected in any of the animals (Table 1). Neither was there any significant development of leukemia, even though the animals treated with 1,8-dinitropyrene were observed for a period more than twice as long as the intraperitoneal-treated animals survived (Table 1).

Although both 1,6- and 1,8-dinitropyrene increased the incidence of adenocarcinoma of the mammary gland, only total mammary tumor induction by 1,8-dinitropyrene was increased significantly (Table 3). Additional support for adenocarcinoma induction by these two dinitropyrenes comes from the greater number of tumors produced by these compounds, compared to solvent-treated controls.

Table 3. Comparative Induction of Tumors by Nitropyrenes After Intragastric Intubation of Weanling Female CD Rats[a]

Compound Administered	Effective Number of Rats	No. of Animals with Mammary Tumors				No. of Mammary Tumors			Average Induction Period (days)
		Adeno-carcinoma	Fibro-adenoma	Adenoma	Total	Adeno-carcinoma	Fibro-adenoma	Total	
1-Nitropyrene	35	5 (14%)	9 (26%)	2 (6%)	16 (46%)	6	11	19	464
1,3-Dinitropyrene	35	6 (17%)	7 (20%)	0	12 (34%)	11	7	18	420
1,6-Dinitropyrene	36	11 (31%)[c]	10 (28%)	1 (3%)	17 (44%)	19	11	31	372
1,8-Dinitropyrene	36	12 (33%)[c]	12 (33%)	0	22 (61%)[b]	21	16	37	421
Dimethylsulfoxide	35	5 (14%)	9 (26%)	0	12 (34%)	6	13	19	423

[a] Weanling female CD rats were treated by intragastric intubation (IG) with nitropyrenes (0.01 mmole/kg) three times per week for a total of 12 doses (total dose = 16 μmoles). Animals were sacrificed at 76 to 78 weeks.

[b] $p < 0.05$, compared to solvent-treated controls.

[c] $p > 0.05$.

No production of tumors in the gastrointestinal tract was detected, but three animals did have hyperplasia of the forestomach; two had been treated with 1,6-dinitropyrene, one with 1,8-dinitropyrene. Metastatic mammary lesions were found in the lungs of two animals; one had been treated with 1,3-dinitropyrene, the other with 1,8-dinitropyrene. Tumors of the adrenal and pituitary glands were observed in all groups of these aging animals. The incidence of carcinomas of the pituitary gland were elevated (34 to 36%) in animals receiving the dinitropyrenes, compared to an incidence of 6% in solvent-treated controls. Treatment with 1-nitropyrene yielded three animals with islet cell carcinoma of the pancreas, and two with thyroid carcinomas. One animal treated with 1,8-dinitropyrene had an islet cell carcinoma. No remarkable liver lesions were noted, nor were the organ weights abnormal at sacrifice.

SPECIFIC AIM 3

The comparative carcinogenicities of 1-nitropyrene and 1,3-, 1,6-, and 1,8-dinitropyrene were determined after subcutaneous injection into newborn female CD rats.

Treatment with 1,6- and 1,8-dinitropyrene resulted in the rapid development of MFH in 100% of the animals (Figure 4). The first tumors were observed at 15 and 16 weeks after the first injections of 1,6- and 1,8-dinitropyrene, respectively. By 18 weeks after the first injection, all animals that had been treated with 1,6-dinitropyrene had developed MFHs. The 1,8-dinitropyrene-treated animals had all developed MFHs within 20 weeks of the first injection. The aggressive nature of these tumors required that the animals be sacrificed by 24 weeks of age (Table 4). Attempts to prolong the survival of these animals by surgical removal of the tumors were unsuccessful, as new tumors appeared within seven to ten days. Evidence of metastasis to lymph nodes and lungs was obtained.

Even within this brief period, a significantly increased incidence of leukemia developed in these animals (Table 4). In contrast, only 12% of the animals injected with 1,3-dinitropyrene developed MFH, and this was after an average latent period that was almost twice that of the other dinitropyrenes. Neither MFH nor leukemia was induced by 1-nitropyrene or the solvent.

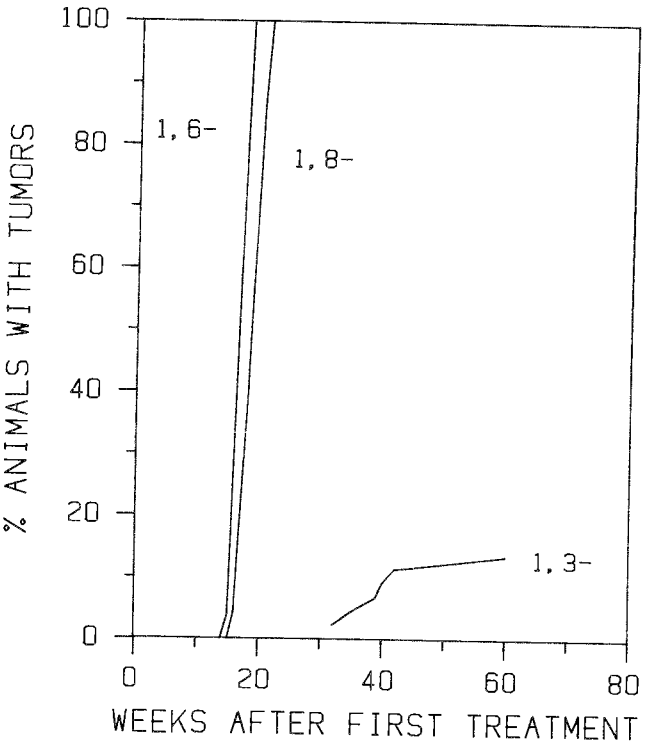

Figure 4. Malignant fibrous histiocytoma induction after subcutaneous injection of 1,3-, 1,6-, or 1,8-dinitropyrene into newborn female CD rats, as described under Specific Aim 3 and Table 4.

Table 4. Incidences of Malignant Fibrous Histiocytoma and Leukemia in Newborn Female CD Rats Administered Nitropyrenes by Subcutaneous Injection[a]

Compound Administered	Effective Number of Rats	Number of Animals with Malignant Fibrous Histiocytoma	Average MFH Induction Period (days)	Number of Animals with Leukemia	Average Survival (days)
1-Nitropyrene	49	0		0	481
1,3-Dinitropyrene	43	5 (12%)[d]	206	0	468
1,6-Dinitropyrene	46	46 (100%)[b]	115	9 (20%)[c]	149
1,8-Dinitropyrene	37	37 (100%)[b]	122	8 (22%)[c]	163
Dimethylsulfoxide	40	0		0	495

[a] The animals were treated by subcutaneous injection (SC) with nitropyrenes at eight weekly intervals (total dose = 6.3 μmoles). Animals were sacrificed at 67 weeks.

[b] $p < 0.0001$, compared to solvent-treated controls.

[c] $p < 0.005$.

[d] $p < 0.05$.

Although some mammary adenocarcinomas developed in the 1,6- and 1,8-dinitropyrene-treated animals (Table 5), the incidence was not significantly higher than in the control animals, probably due to the short survival period. A significant increase in the number of adenocarcinoma-bearing animals was observed in the 1-nitropyrene treatment group. The average latent period for tumor induction by 1-nitropyrene was greater than 65 weeks.

At sacrifice, the livers of the 1,6- and 1,8-dinitropyrene-treated animals were 4 to 4.1% of the body weight. Consistent with the leukemia in these animals, the spleens were also enlarged. The pituitary and adrenal tumors that developed in the older animals were not related to treatment.

SPECIFIC AIM 4

The comparative carcinogenicities of 1-nitropyrene and its major phenolic metabolites, 3-hydroxy-1-nitropyrene and a mixture of 6- and 8-hydroxy-1-nitropyrene, were determined after subcutaneous injection into newborn female CD rats.

The structures of the compounds employed in this experiment are shown in Figure 5. There were no substantial weight differences between the groups during the treatment period, which resulted in each animal receiving an average total dose of 63 μmoles. The average survival was greater than 523 days for each group, except for the 4-nitropyrene-treated animals that survived an average of 322 days. The 4-nitropyrene group developed mammary adenocarcinomas and fibroadenomas, as well as MFHs, leukemia, and earduct tumors (Table 6), with essentially equal induction periods of 263 and 259 days for the mammary tumors and MFHs, respectively. Each mammary tumor-bearing animal carried an average of four tumors. A significant number of 1-nitropyrene-treated rats also devel-

Table 5. Comparative Induction of Mammary Tumors by Nitropyrenes After Subcutaneous Injection into Newborn Female CD Rats[a]

Compound Administered	Effective Number of Rats	No. of Animals with Mammary Tumors				No. of Mammary Tumors			Average Induction Period (days)
		Adeno-carcinoma	Fibro-adenoma	Adenoma	Total	Adeno-carcinoma	Fibro-adenoma	Total	
1-Nitropyrene	49	10 (20%)[b]	9 (18%)	2 (4%)	16 (33%)[c]	13	10	25	441
1,3-Dinitropyrene	43	6 (14%)[c]	3 (7%)	1 (2%)	9 (21%)	7	3	11	472
1,6-Dinitropyrene	46	3 (7%)	0	2 (4%)	5 (11%)	6	0	8	150
1,8-Dinitropyrene	37	5 (14%)[c]	0	0	5 (14%)	6	0	6	143
Dimethylsulfoxide	40	1 (3%)	6 (15%)	1 (3%)	8 (20%)	1	8	10	456

[a] Newborn female CD rats were treated by subcutaneous injection (SC) with nitropyrenes at eight weekly intervals (total dose = 6.3 μmoles). Animals were sacrificed at 67 weeks.

[b] $p < 0.025$, compared to solvent-treated controls.

[c] $p > 0.05$.

Table 6. Induction of Tumors by Mononitropyrenes After Subcutaneous Injection into Newborn Female Rats[a]

Compound Administered	Rat Strain	Effective Number of Rats	Number of Animals with Mammary Tumors				Induction Period (days)	Animals with Other Tumors
			Adenocarcinoma	Fibroadenoma	Adenoma	Total		
1-Nitropyrene	CD	48	10 (21%)[d]	19 (40%)	1 (2%)	26 (54%)[e]	452	
	F344	55	0	3 (6%)	3 (6%)	5 (10%)	593	4-Leukemia[d]
4-Nitropyrene	CD	27	18 (67%)[b]	14 (52%)[e]	0	20 (74%)[c]	263	10-Malignant[b]
3-Hydroxy-1-Nitropyrene	CD	32	2 (6%)	11 (34%)	0	12 (38%)	470	
6- and 8-Hydroxy-1-Nitropyrene	CD	46	8 (17%)[e]	15 (33%)	1 (2%)	21 (46%)	457	
Dimethylsulfoxide	CD	47	3 (6%)	16 (34%)	0	17 (36%)	502	
	F344	55	0	1 (2%)	0	1 (2%)	511	

[a] Newborn female CD or F344 rats were treated by subcutaneous injection of tbe compounds (0.1 mmole/kg) and at weekly intervals, for a total of eight doses. Animals were sacrificed at 86 weeks.

[b] $p < 0.001$, compared to solvent-treated controls of the same strain.

[c] $p < 0.005$.

[d] $p < 0.05$.

[e] $p > 0.05$.

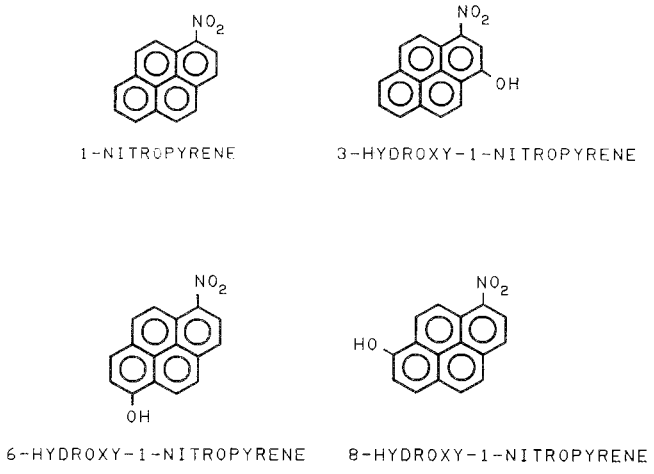

Figure 5. Phenolic metabolites of 1-nitropyrene.

oped mammary adenocarcinomas compared with solvent-treated controls. Although an increased number of animals developed mammary adenocarcinomas after treatment with the mixture of 6- and 8-hydroxy-1-nitropyrene, the increase was not significant. There were no remarkable observations regarding organ weights or their gross appearances.

SPECIFIC AIM 5

The relative susceptibilities of newborn female CD and Fischer 344 rats to the subcutaneous administration of 1-nitropyrene was established.

Newborn female Fischer 344 rats were treated concurrently with the CD rats described above in Specific Aim 4. The smaller Fischer animals received only 40 μmoles of 1-nitropyrene compared to the comparable CD group, because the inbred Fischer strain is smaller. No mammary adenocarcinomas were detected in either the 1-nitropyrene or dimethylsulfoxide groups (Table 6). Four 1-nitropyrene-treated animals developed leukemia, a significant incidence, since none were identified in the control group.

SPECIFIC AIM 6

The comparative effects of subcutaneous and intraperitoneal injection of 1-nitropyrene into weanling female CD rats were explored.

Both intraperitoneal and subcutaneous injection with 1-nitropyrene significantly increased the total mammary tumor incidence (Table 7). The modest increases were obvious by both routes of administration, although adenocarcinoma induction was significant only when 1-nitropyrene was injected intraperitoneally. The average tumor induction period was equal to or greater than 70 weeks in each of the treatment groups. Other than the liver representing a slightly greater percentage of the body weight in the subcutaneously treated 1-nitropyrene group, i.e., 3.0% compared to 2.3 to 2.5%, the organ weights were indistinguishable.

SPECIFIC AIM 7

The biochemical goal was to explore the pathways by which the rat mammary gland, a target tissue of the nitrated pyrenes, metabolizes 1-nitropyrene and 1,3-, 1,6-, and 1,8-dinitropyrene. The previous description of the carcinogenic potential of the nitrated pyrenes has clearly established three biological targets. The mammary gland, the histiocyte, and bone marrow represent the cellular targets that are the precursors of mammary tumors, MFHs, and leukemia, respectively. Biochemical efforts have been focused on the rat mammary gland,

Table 7. Comparative Induction of Tumors by 1-Nitropyrene After Subcutaneous or Intraperitoneal Injection into Weanling Female CD Rats[a]

Compound Administered	Route of Administration	Effective Number of Rats	No. of Animals with Mammary Tumors			No. of Mammary Tumors			Average Induction Period (days)
			Adeno-carcinoma	Fibro-adenoma	Total	Adeno-carcinoma	Fibro-adenoma	Total	
1-Nitropyrene	IP	29	8 (28%)[b]	14 (48%)	17 (59%)[d]	9	19	28	546
	SC	29	6 (21%)	15 (52%)[c]	17 (59%)[d]	9	19	28	474
Dimethylsulfoxide	IP	30	2 (7%)	9 (30%)	11 (37%)	2	13	15	506
	SC	30	4 (13%)	8 (27%)	11 (37%)	5	9	14	516

[a] Weanling female CD rats were treated by intraperitoneal (IP) or subcutaneous (SC) injection of 1-nitropyrene (0.1 mmole/kg) at weekly intervals for a total of five doses (total dose = 77 μmoles). Animals were sacrificed at 88 weeks.

[b] $p < 0.03$, compared to solvent-treated controls treated by the same route.

[c] $p < 0.04$.

[d] $p < 0.08$.

which is the most easily accessible for biochemical studies. Moreover, previous work has shown that this tissue responds readily to the metabolic activation of arylhydroxylamines, the putative crucial metabolites of the nitropyrenes. In contrast to other tissues, liver for example, the rat mammary gland has a very limited ability to metabolize xenobiotics (King et al., 1979; Shirai et al., 1981). Mammary tumor induction by aromatic amine derivatives is believed to result from metabolic activation within the tissue and the subsequent hormonal amplification of these early events in the carcinogenic process (King, 1985). We have explored the mammary pathways for metabolism of the nitrated pyrenes to low molecular weight products and nucleic acid adducts. Two metabolic systems have been used, mammary cytosol and isolated intact mammary epithelial cells. Only modest amounts of this tissue are available for such studies, and the method of preparation necessarily results in variations in metabolic capacity from one preparation to another. Consequently, these experiments were designed to provide for internal controls, to minimize differences in the metabolic capacities of the numerous preparations of mammary gland that must be used. In most cases, data from representative experiments are reported to provide qualitative insight into the pathways that are operative in the mammary gland.

MAMMARY CYTOSOL

1-Nitropyrene

Consistent with previous studies on the enzymatic reduction of nitropyrenes (Djuric et al., 1985), incubation of tritiated 1-nitropyrene with mammary cytosol, under reduced oxygen tension and in the presence of NADPH and tRNA, yielded nucleic acid-bound pyrene adducts (Table 8). Essentially no difference in the level of tRNA binding was observed when either NADPH or NADH was included in the incubation mixture. Adduct formation, however, was reduced by 30 to 40% in the absence of either cofactor. Incubation under reduced oxygen tension increased adduct formation.

The effect of acetyl CoA on the enzyme-catalyzed formation of adducts was examined with respect to both the time of incubation and the amount of protein concentration under reduced oxygen tension. These results, presented in Figures 6 and 7, demonstrate that adduct formation increased linearly with the time of incubation, up to 60 minutes, and with up to 2 mg protein. In agreement with the results of Djuric et al. (1985), acetyl CoA was without a consistent effect on adduct formation.

The reductive metabolism of 1-nitropyrene by rat mammary cytosol gave 1-aminopyrene as the sole product (Figure 8). The product was identified through co-chromatography with the authentic reference compound and by derivatization to 1-acetylaminopyrene, and through co-chromatography with the authentic synthetic acetamide. Figure 9 shows that the forma-

Table 8. Effect of NADPH and NADH on 1-Nitropyrene tRNA Adduct Formation After Incubation with Rat Mammary Gland Cytosol

	tRNA Adduct Formation (pmoles)	
Incubation System	Aerobic	Argon Atmosphere
Complete System[a]		
+ NADPH	87	143
+ NADH	101	143
– NADPH OR NADH	44	81
– CYTOSOL + NADH	7	8
– CYTOSOL + NADPH	5	8

[a] Complete incubation system included in a total volume of 1.0 ml: 50 mM pyrophosphate buffer (pH 7.0), 1.0 mM dithiothreitol, 1 mg cytosolic protein, 1.0 μmole NADPH or NADH, tRNA (15 A260 units) and 0.05 μmole (4,5,9,10-^{3}H)-1-nitropyrene (123.9 uCi/μmole). Incubations were carried out at 37° for 60 minutes.

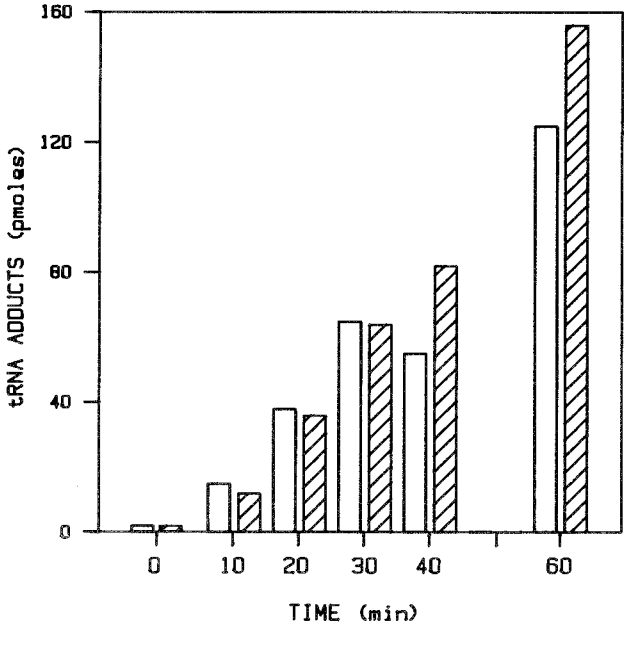

Figure 6. Transfer RNA adduct formation with 1-nitropyrene as a function of the time of incubation with rat mammary gland cytosol under reduced oxygen tension, as described under Specific Aim 7 and Table 8. The data shown were obtained in the presence (open bar) or absence (shaded bar) of exogenously added acetyl CoA.

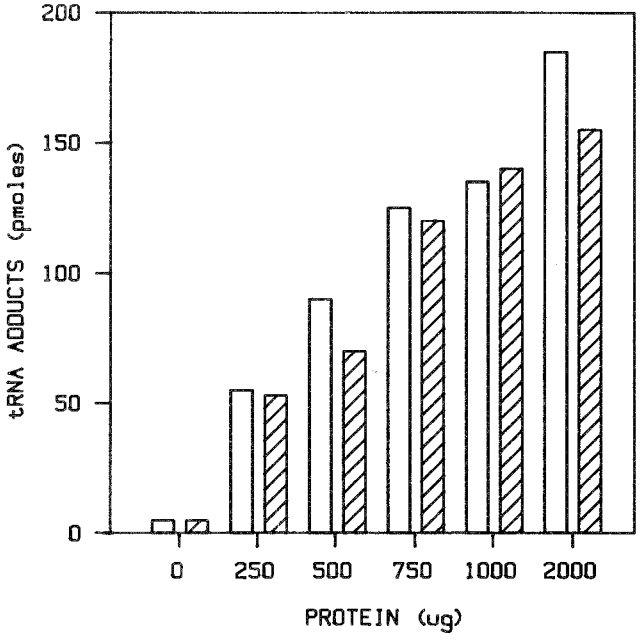

Figure 7. Transfer RNA adduct formation with 1-nitropyrene as a function of added rat mammary gland cytosol protein under argon, as described under Specific Aim 7 and Table 8. The data shown were obtained in the presence (open bar) or absence (shaded bar) of exogenously added acetyl CoA.

tion of 1-aminopyrene is linear for periods of up to 60 minutes, and that the amount of this reduction product is approximately five times the amount of adduct formation in this system.

Dinitropyrenes

Representative HPLC profiles of ethyl acetate extracts from incubations of the three dinitropyrenes, with rat mammary cytosol and NADPH under reduced oxygen tension, are shown in Figure 10. These results indicate that rat mammary cytosol is capable of reducing the three dinitropyrenes to their respective diamino- and aminonitropyrene derivatives, with the latter being the major metabolite. In addition, the peaks that were less polar than the parent 1,6- and 1,8-dinitropyrene, respectively, have been tentatively identified as their corresponding nitrosonitropyrene metabolite through co-chromatography with authentic reference samples. The radioactive peaks that are more polar than the parent dinitropyrenes have not been identified, although one might speculate that they are the aminonitrosopyrene derivatives, which have apparently not yet been synthesized. No significant reduction was observed for any of the three compounds in the absence of cytosol, either under aerobic conditions or with reduced oxygen tension (data not shown). Table 9 summarizes the amount of reduced metabolites for the three dinitropyrenes under both aerobic conditions and decreased oxygen tension. Overall, these results indicated that the extent of nitro-

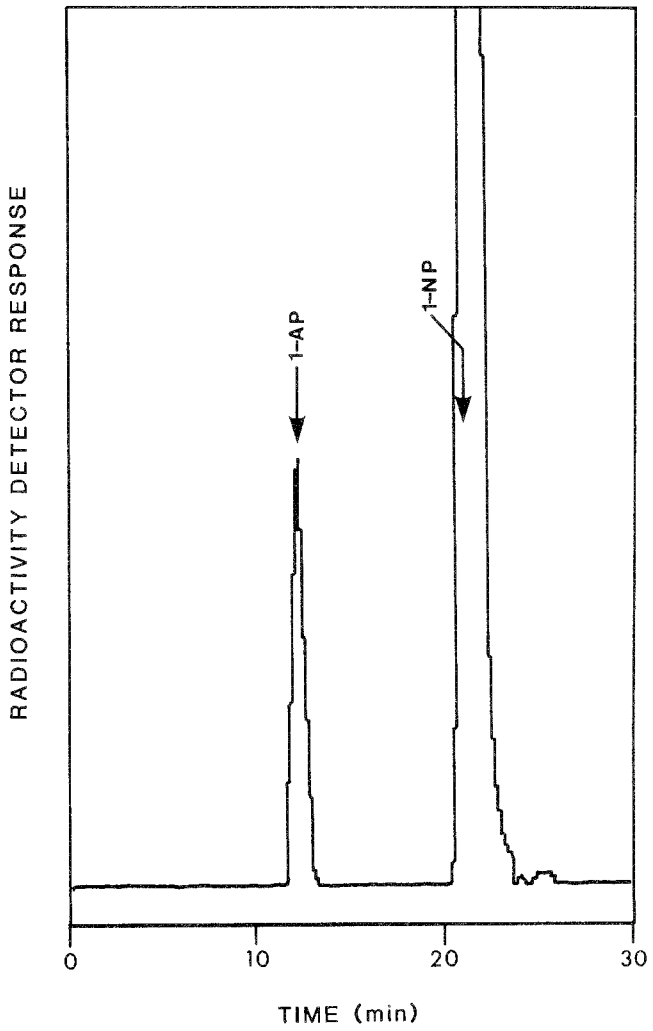

Figure 8. Formation of 1-aminopyrene (1-AP) from 1-nitropyrene (1-NP) in an argon atmosphere by rat mammary gland cytosol and NADPH, as described under Specific Aim 7, Table 8, and Table 11.

reduction, estimated as the sum of diamino, aminonitro, and nitrosonitro metabolites, was greater when oxygen availability was limited than under aerobic conditions. 1,6-Dinitropyrene was reduced more than 1,8-dinitropyrene; 1,3-dinitropyrene was reduced the least. In contrast to the 1,6- and 1,8-isomers, 1,3-dinitropyrene did not yield a nitrosonitro derivative, i.e., a metabolite that was more non-polar than the parent dinitro compound.

Transfer RNA adduct formation with the dinitropyrenes was catalyzed by rat mammary cytosol. Table 10 shows the results of adduct formation with exogenous acetyl CoA both present and absent. These data demonstrate that adduct formation was dependent on the presence of cytosol. The addition of acetyl CoA increased adduct formation by two-fold with 1,6- and

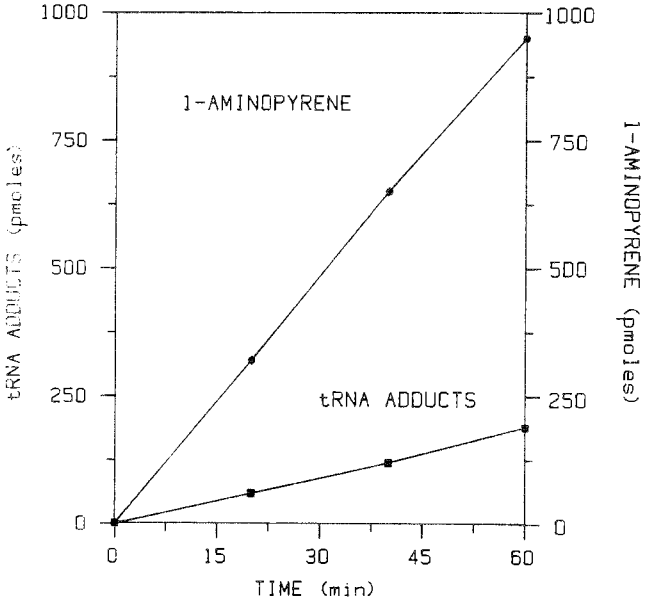

Figure 9. Metabolism of 1-nitropyrene by rat mammary gland cytosol to tRNA adducts and 1-aminopyrene under argon, as described under Specific Aim 7, Table 8, and Table 11.

1,8-dinitropyrene, but was essentially without effect in incubations with 1,3-dinitropyrene. Thus, under the conditions used, tRNA adduct formation decreased in the order 1,6-, 1,8-, and 1,3-dinitropyrene, which correlates with the extent of nitroreduction.

MAMMARY EPITHELIAL CELLS

1-Nitropyrene

Figure 11 illustrates the HPLC profile of ethyl acetate-extractable 1-nitropyrene metabolites generated after incubation with intact rat mammary epithelial cells. The amount of each metabolite, calculated from the radioactivity eluted from the HPLC column, is summarized in Table 11, together with their retention times. The major metabolites obtained when the cells were exposed to 1-nitropyrene were the ring-oxidized 3-, 6-, and 8-hydroxy-1-nitropyrene derivatives. The largest amounts of phenolic metabolites were observed in the sample taken 24 hours post-plating.

With the HPLC system used, 6- and 8-hydroxy-1-nitropyrene could not be separated; thus, these two compounds were combined for quantification (Table 11). 1-Aminopyrene was not detected at any time point. However, small amounts of 1-acetylaminopyrene were present in ethyl acetate extracts from the 24- and 48-hour incubations. The identity of the acetamide, and the mixture of 6- and 8-hydroxy-1-nitropyrene, were confirmed by chromatography on another HPLC system, which

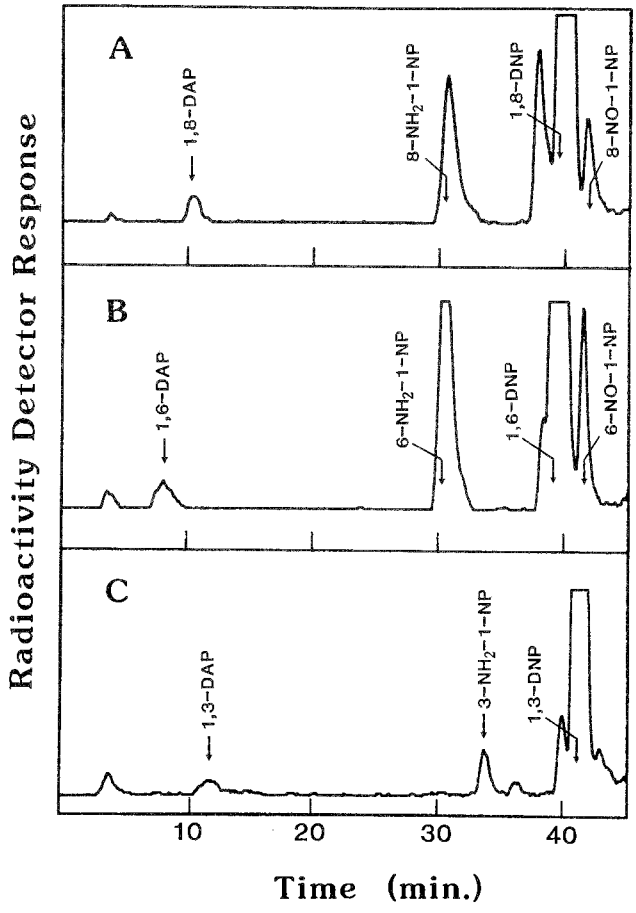

Figure 10. HPLC profiles of metabolites of 1,3-, 1,6-, and 1,8-dinitropyrene by rat mammary gland cytosol and NADPH under argon, as described under Specific Aim 7 and Table 9. The reverse-phase HPLC system employed a C-18 μBondapak column and a 45 minute concave gradient (Waters curve 8) from 60 to 100% methanol at a solvent flow rate of 1 ml/min. DAP, diaminopyrene; NH_2-1-NP, aminonitropyrene; DNP, dinitropyrene; NO-1-NP, nitrosonitropyrene.

used an isocratic solvent system of 80% methanol. In the case of the phenolic compounds, confirmation was obtained after their acetylation to yield their acetoxy derivatives, and by rechromatography on a methanol-water gradient. In control experiments, in the absence of mammary cells, the only tritiated component eluted from the HPLC was the parent 1-nitropyrene.

Dinitropyrenes

Figure 12 shows the HPLC profiles of ethyl acetate-extractable dinitropyrene metabolites generated after incubation of the compounds with rat mammary epithelial cells. The major metabolite detected from 1,6- and 1,8-dinitropyrene was the corresponding aminonitropyrene derivative. The amounts of the metabolite were 4.3% of the dose for 1,6-dinitropyrene and 1% for 1,8-nitropyrene. No evidence of any reduced metabo-

Table 9. Metabolism of Dinitropyrenes by Rat Mammary Gland Cytosol[a]

Dinitropyrene Isomer Incubated	Products Formed on Incubation with Mammary Cytosol[b]					
	Aerobic			Argon Atmosphere		
	Diamino	Aminonitro	Nitronitroso	Diamino	Aminonitro	Nitronitroso
1,3-	ND[c] (ND)	452 (20)	ND (ND)	533 (ND)	1066 (17)	ND (ND)
1,6-	272 (ND)	4406 (88)	5712 (ND)	620 (ND)	5581 (68)	2997 (ND)
1,8-	281 (ND)	2486 (ND)	3800 (ND)	658 (ND)	3909 (25)	2240 (ND)

[a] The incubation system included in a total volume of 1.0 ml: 50 μM sodium pyrophosphate buffer (pH 7.0), 1.0 mM dithiothreitol, 1.0 mg cytosolic protein, 1.0 μmole NADPH and 0.034 μmole of the corresponding (4,5,9,10-^{3}H)-dinitropyrene (150 to 250 mCi/mmole). Incubations were carried out at 37° for 60 minutes under either air (aerobic) or argon.

[b] Values for the metabolites are expressed as pmoles/mg protein/hour. Values in parentheses are from incubation in the absence of cytosol.

[c] ND = Not detected. The limit of detection was approximately 10 pmoles/mg protein/hour.

Table 10. Effect of Acetyl Coenzyme A on Dinitropyrene-tRNA Adduct Formation during Incubation with Rat Mammary Gland Cytosol

Dinitropyrene Isomer Incubated	Incubation Condition	tRNA Adduct Formation (pmoles)			
		Complete[a] System	Minus Cytosol	Minus Acetyl CoA	Minus Cytosol and Acetyl CoA
1,3-	Aerobic	17.6 ± 5.3	10.0 ± 2.0	14.5 ± 1.0	9.1 ± 4.5
	Argon	18.5 ± 4.2	8.2 ± 2.3	12.6 ± 0.2	6.2 ± 1.4
1,6-	Aerobic	37.8 ± 1.7	2.4 ± 1.5	20.9 ± 6.9	2.5 ± 1.4
	Argon	40.5 ± 3.0	2.8 ± 1.1	27.2 ± 2.3	2.5 ± 0.9
1,8-	Aerobic	19.0 ± 3.2	1.3 ± 0.3	11.7 ± 1.7	1.3 ± 0.5
	Argon	24.8 ± 3.9	1.8 ± 1.0	10.8 ± 2.0	2.4 ± 1.3

[a] The complete incubation system included in a total volume of 1.0 ml: 50 mM sodium pyrophosphate buffer (pH 7.0), 1.0 mM dithiothreitol, 1.0 mg cytosolic protein, 1.0 μmole NADPH, 0.1 μmole acetyl CoA, tRNA (15 A260 units), and 0.034 μmole of the corresponding (4,5,9,10-^{3}H)-dinitropyrene (150 to 250 mCi/mmole). Incubations were carried out at 37° for 60 minutes under either air (aerobic) or argon. All values are expressed as the mean ± standard deviation from experiments with three different rat mammary cytosol preparations.

lites was obtained after incubation of 1,3-dinitropyrene in this system. A small amount of radioactivity, 0.3% of the dose that co-chromatographed with 8-acetylamino-1-nitropyrene, was also detected. The analogous N-acetylated product was not detected in incubations with 1,6-dinitropyrene. In contrast to the reduction products observed with rat mammary cytosols, neither the diaminopyrene nor the nitrosonitropyrene derivatives were detected in experiments with intact cells. As with 1-nitropyrene, control experiments showed that incubations that contained the labeled dinitropyrenes, but not mammary cells, yielded only the parent tritiated dinitropyrenes on analysis by HPLC.

Preliminary experiments exploring the structure and quantity of DNA adducts formed in these cells are in progress. Incubation in rat liver cytosol with acetyl CoA and DNA from calf thymus has generated reference DNA adducts with the three dinitropyrenes (Djuric et al., 1985). Presumably, the adducts are C-8 guanine derivatives formed by reaction with the N-acetoxy-aminonitropyrenes that are produced in this metabolic system (Beland, 1986). Hydrolysis of the adducts generated in the liver cytosols, and in intact rat mammary epithelial cells, has been accomplished by treatment with trifluoroacetic acid (Tang and Lieberman, 1983). Reverse-phase HPLC on methanol-water gradients has disclosed that a single derivative is seen in the HPLC profile of the calf thymus DNA hydrolysate.

Mammary cell DNA adducts purified by organic extraction and digestion with pronase and ribonuclease had two components in the HPLC profiles, one at the retention time of the adduct generated in the liver system, and another, much more polar, derivative that appeared shortly after the void volume. Further purification of the mammary cell DNA on cesium, prior to hydrolysis with trifluoroacetic acid, was effective in removing the polar component, but variable amounts of a component that was only slightly more polar than the putative adduct were also detected. Calf thymus DNA adducts also

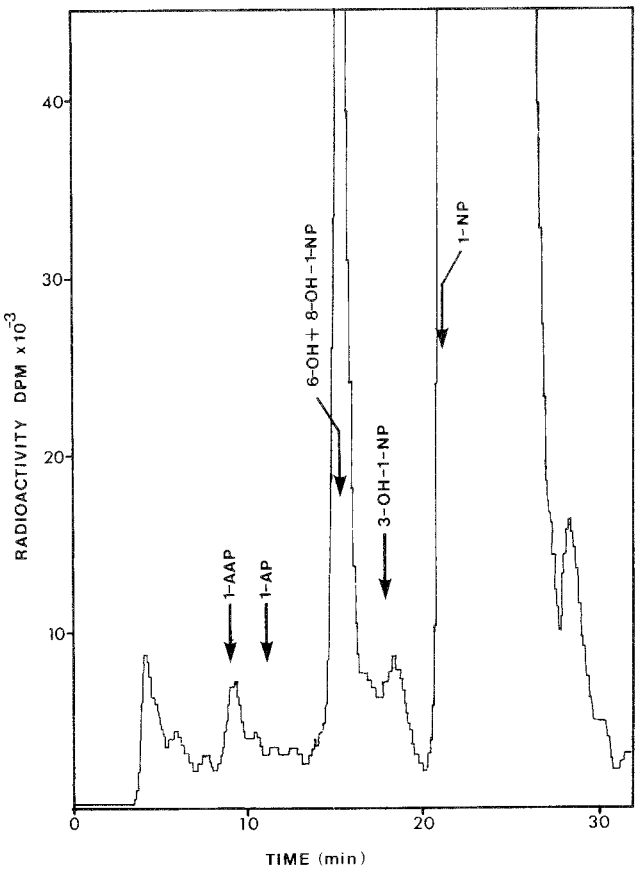

Figure 11. HPLC profile of 1-nitropyrene metabolites formed by rat mammary epithelial cells in culture, as described under Specific Aim 7 and Table 11. 1-AAP, 1-acetylaminopyrene; 1-AP, 1-aminopyrene; 6-OH + 8-OH-1-NP; mixture of 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene; 3-OH-1-NP, 3-hydroxy-1-nitropyrene; 1-NP, 1-nitropyrene. The structures of these compounds are shown in Figure 5.

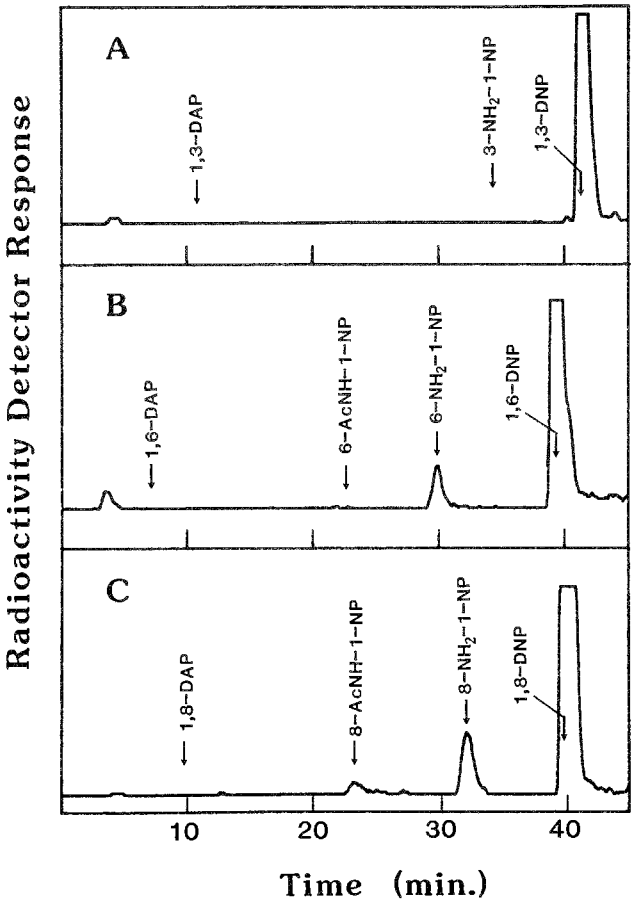

Figure 12. HPLC profile of dinitropyrene metabolites formed by rat mammary epithelial cells in culture, as described under Specific Aim 7 and Figure 10. DAP, diaminopyrene; AcNH-1-NP, acetamidonitropyrene; NH_2-1-NP, aminonitropyrene; DNP, dinitropyrene.

Table 11. Production of Ethyl Acetate-Extractable Metabolites of ^{3}H-1-Nitropyrene by Rat Mammary Epithelial Cells in Culture[a]

Metabolite Produced	Retention Time (min)	Time of Incubation (hrs) 6	24	48
		(% 1-Nitropyrene Incubated)		
Polar	3.5	ND[b]	0.17	< 0.1
1-Acetylaminopyrene	9.3	ND	0.17	0.16
1-Aminopyrene	11.4	ND	ND	ND
6-Hydroxy- and 8-Hydroxy-1-Nitropyrene	15.9	0.7	1.4	0.7
3-Hydroxy-1-Nitropyrene	18.3	< 0.1	0.2	ND
1-Nitropyrene	20.8	87.2	82.6	76.7

[a] The metabolites were subjected to HPLC on a C-18 μBondapak column (4.6 mm x 25 cm) with a 30-minute linear gradient of methanol (70 to 90%) in water. The flow rate was 1 ml per minute.
[b] ND = Not detected.

yielded two components with these same two retention times after being subjected to density gradient centrifugation on cesium chloride. Given the ease of hydrolysis of C-8 substituted arylamine adducts with guanine, the generation of the slightly more polar adduct probably results from the opening of the imidazole of the purine nucleus (Kriek and Westra, 1980). Consequently, we have elected to determine the level of DNA adduct formation in rat mammary cells by estimating the amount of adduct formation from the putative C-8 component that is chromatographically identical with the liver-generated adduct, without subjecting the DNA to cesium gradient purification.

Our preliminary results show that the level of adduct formation in rat mammary epithelial cells is a time-dependent process, and that the yield of adduct corresponds to the ease of reduction of the dinitropyrene by the rat mammary cytosol and intact mammary cells; i.e., 1,6- is greater than 1,8- is greater than 1,3-dinitropyrene.

DISCUSSION

These experiments clearly establish the ability of nitropyrenes to induce three types of tumors in the rat: MFHs, mammary tumors, and leukemia (Table 12). As judged by the induction of MFHs after intraperitoneal or subcutaneous injection, these experiments clearly demonstrate the potent carcinogenicities of 1,6- and 1,8-dinitropyrene. The 1,3-dinitropyrene isomer is much less potent in inducing these tumors. Furthermore, in the absence of additional studies with other preparations of this compound and of more extensive dose-response experiments, it cannot be excluded that the few MFHs produced by 1,3-dinitropyrene were not the consequence of small quantities of 1,6- or 1,8-dinitropyrene, or both, in the 1,3-dinitropyrene that was used in these experiments.

No MFHs were induced by 1-nitropyrene in these studies by any route of administration, in contrast to our initial observations after subcutaneous injection into newborn CD rats (Hirose et al., 1984), but consistent with our observations after intraperitoneal injection into weanling female CD rats (Imaida, et al., 1985). These findings are also consistent with those of Ohgaki et al. (1985) after subcutaneous injection into male Fischer 344 rats. Ohgaki et al. (1985) have speculated that their earlier study yielded MFHs because of small quantities of dinitropyrenes in their preparation of 1-nitropyrene. In the Hirose et al. (1984) study, MFHs were induced by 1-nitropyrene that contained no detectable dinitropyrenes, as confirmed by an independent laboratory. The production of MFHs was somewhat less than mammary gland adenocarcinoma induction. In the present study, the more potent nitropyrene isomer, 4-nitropyrene, yielded about two thirds as many MFHs as mammary adenocarcinomas when injected subcutaneously, although previous intraperitoneal injections had not induced MFHs in weanling female CD rats (Imaida et al., 1985). It cannot be excluded that the production of MFHs by the mononitropyrenes is caused by traces of dinitropyrenes. However, the more facile production of MFH, compared to mammary adenocarcinoma induction, by the dinitropyrenes suggests that mammary tumor induction by the mononitropyrenes cannot be explained by the presence of dinitropyrene contamination.

Subcutaneous injection of the dinitropyrenes is more efficient in producing MFHs than is intraperitoneal injection, as judged by incidence. However, the time from first treatment to the detection of the first MFH was approximately the same by both routes of administration. The greater response to the subcutaneous injection may come from the lesser mobility of the compounds from the site of deposition. On injection of the dimethylsulfoxide solutions, the solvent is rapidly absorbed into the surrounding tissues and the insoluble nitropyrenes are deposited in a relatively confined area. When injected intraperitoneally, the surface area to which the deposited nitropyrenes are exposed is much greater than when they are injected subcutaneously. Thus, it is expected that the absorption from the peritoneal cavity would be more rapid, with a lesser probability that local tissue reactions will develop and an increased likelihood of systemic effects. Importantly, no MFHs were induced by intragastric intubation with any of the compounds. Whether this inactivity results from inadequate absorption from the gastrointestinal tract, or from the absence of insoluble tissue deposits, is not known. Alternatively, the nitropyrenes may have been inactivated as a consequence of microbial metabolism in the gastrointestinal tract. Microbial reduction, for example, can result in the production of amines (Howard et al., 1983) that, because of an inadequate enzymatic capacity to undergo N-oxidation, may not be carcinogenic because they cannot be metabolically activated. Clearly, there was a lesser response of the mammary gland to dinitropyrenes given by intubation than when they were injected intraperitoneally. This is particularly striking when one considers that the survival of the orally treated 1,8-dinitropyrene group was twice as long as the survival of the group given the compound by intraperitoneal injection.

The relationship of structure to response is not as clear in the case of mammary tumor induction. Malignant mammary tumor development by 1,8-dinitropyrene was demonstrated conclusively by intraperitoneal administration. The survival period was so short in the group of animals that had been given intraperitoneal injections of 1,6-dinitropyrene, or subcutaneous injections of either the 1,6- or 1,8-dinitropyene isomers, that the incidence of mammary tumors was not significantly higher than in the solvent-treated controls. However, the

Table 12. Summary of Tumor Induction by Nitropyrenes in Female Rats

Specific Aim	Data Presentation	Treatment			Animal		Observation Period (weeks)	Tumor Induction		
		Compound[a]	Dose (μmole)	Route[b]	Age	Strain[c]		MFH[d]	Mammary Adeno-carcinoma	Leukemia
1	Tables 1 and 2	1-NP	16	IP	Weanling	CD	77		Increase**	
		1,3-DNP	16	IP	Weanling	CD	77			
		1,6-DNP	16	IP	Weanling	CD	77[e]	Increase***		
		1,8-DNP	16	IP	Weanling	CD	77[e]	Increase***	Increase***	Increase**
2	Tables 1 and 3	1-NP	16	IG	Weanling	CD	77			
		1,3-DNP	16	IG	Weanling	CD	77			
		1,6-DNP	16	IG	Weanling	CD	77		Increase^{NS}	
		1,8-DNP	16	IG	Weanling	CD	77		Increase^{NS}	
3	Tables 4 and 5	1-NP	6.3	SC	Newborn	CD	67		Increase*	
		1,3-DNP	6.3	SC	Newborn	CD	67	Increase*		
		1,6-DNP	6.3	SC	Newborn	CD	67[e]	Increase***		Increase**
		1,8-DNP	6.3	SC	Newborn	CD	67[e]	Increase***		Increase**
4 and 5	Table 6	1-NP	63	SC	Newborn	CD	86		Increase*	
		1-NP	40[f]	SC	Newborn	F344	86			
		4-NP	63	SC	Newborn	CD	86		Increase***	
		3-OH-1-NP	63	SC	Newborn	CD	86			
		6-OH-1-NP and 8-OH-1-NP	63	SC	Newborn	CD	86			
6	Table 7	1-NP	77	IP	Weanling	CD	88		Increase*	
		1-NP	77	SC	Weanling	CD	88		Increase^{NS}	

[a] 1-NP, 1-nitropyrene; 1,3-DNP, 1,3-dinitropyrene; 1,6-DNP, 1,6-dinitropyrene; 1,8-DNP, 1,8-dinitropyrene; 4-NP, 4-nitropyrene; 3-OH-1-NP, 3-hydroxy-1-nitropyrene; 6-OH-1-NP and 8-OH-1-NP, mixture of 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene.

[b] IG, intragastric; IP, intraperitoneal; SC, subcutaneous.

[c] CD, non-inbred Sprague-Dawley-derived rat; F344, inbred Fischer 344 rat.

[d] MFH, malignant fibrous histiocytoma.

[e] Survival was severely limited in these groups due to MFH formation.

[f] The smaller total dose administered to the Fischer rats reflects the lower body weights of this strain as compared to the CD rat. The dose rate administered to the two strains was equivalent, as expressed by body weight (μmole/kg body weight).

[NS] Non-significant increase compared with the control group of the same strain that had been given only the solvent by the same route of administration.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

appearance of mammary adenocarcinomas in these animals during this brief survival period (less than 22 weeks) is consistent with a carcinogenic potential of both of these compounds for this organ. Similarly, after intragastric intubation, mammary adenocarcinoma induction was increased by both 1,6- and 1,8-dinitropyrene, as judged both by incidence and by the total number of tumors. This increased tumorigenic response was, however, more modest than with the intraperitoneal administration, and a much longer survival period permitted a greater opportunity for the expression of carcinogenic activity. Clearly, the intraperitoneal route of administration is a more effective means of producing mammary tumors, but the results of these studies cannot rule out the possibility that partially reduced derivatives of the dinitropyrenes absorbed from the intestinal tract, or produced metabolically in the liver, are responsible for their induction. The biochemical data support the conclusion that the mammary gland is capable of carrying out the metabolic activa-

tion of the parent dinitropyrenes. Structural studies of the adducts formed in this tissue after systemic administration of the carcinogens, and in vitro malignant transformation experiments, will be required to determine more precisely the actual biochemical events that are responsible for tumor induction.

The weak carcinogenic potential of 1-nitropyrene for the mammary gland in these experiments (i.e., the appearance of modest numbers of malignant tumors only after a long observation period) is in qualitative agreement with the results of the initial carcinogenicity experiments with this compound (Hirose et al., 1984), and establishes that both 1,6- and 1,8-dinitropyrene are more potent carcinogens than 1-nitropyrene or 1,3-dinitropyrene for this organ. The failure of the 3-, 6-, or 8-phenols of 1-nitropyrene to substantially increase mammary tumor formation after subcutaneous injection into newborn CD rats suggests that the weak carcinogenic activity of the parent nitro compound is not mediated through these oxidized metabolites. Previous studies had revealed little evidence for the carcinogenicity of 1-acetylaminopyrene (Miller et al., 1955; Imaida et al., 1985) or N-hydroxy-1-acetylaminopyrene (Imaida et al., 1985). Thus, the carcinogenicity of 1-nitropyrene is most closely associated with the parent nitro compound that serves as an immediate precursor of the hydroxylamine that is believed to be crucial for tumor induction by this parent compound (Figure 1).

Comparison of the CD and Fischer 344 strains demonstrated that the Fischer 344 rat is unlikely to exhibit a carcinogenic response of the mammary gland to these agents and is, consequently, not a valid animal model to explore the carcinogenic potential of these compounds. These observations are consistent with previous mammary tumor induction studies with aromatic amine derivatives (Malejka-Giganti et al., 1977). Direct comparison of subcutaneous and intraperitoneal injection into weanling rats suggested that the response of the mammary gland was essentially independent of these routes of administration in the weanling animal.

Leukemia induction by 1,6- and 1,8-dinitropyrene was observed in the brief survival period after subcutaneous injection, as well as by 1,8-dinitropyrene after intraperitoneal injection. Given the previous observation that hamsters developed leukemia after intratracheal instillation of 1,6-dinitropyrene (Takayama et al., 1985), these data suggest that similar mechanisms of tumor induction by these compounds may be operative in these two species. Furthermore, given the wide diversity of tumors induced by related N-substituted aryl compounds in the rat (Garner et al., 1984), it is likely that other organ systems of the rat may well be susceptible to these agents, if the treatment protocol ensures exposure of the appropriate organ and sufficient survival for tumor expression. In this regard, one unexpected result of the present studies was the failure to induce tumors of the forestomach. Previous studies have clearly demonstrated the ability of N-hydroxylated carcinogenic aromatic amines (Miller et al., 1961) and the nitro compound, 4-nitroquinoline-N-oxide, to induce tumors of the rat forestomach after oral administration (Endo, 1971).

The present metabolic studies support the conclusion that the mechanisms of tumor induction by nitropyrenes are analogous to those of the carcinogenic amines (King, 1985). N-Substituted aryl compounds are believed to induce tumors and mutations as a consequence of their sequential 1) conversion to N-hydroxylated derivatives, 2) esterification of the N-hydroxylated metabolite, and 3) reaction with nucleic acid. The metabolic pathways thought to be involved in the induction of tumors by the nitropyrenes is outlined in Figure 13. The nitro group is believed to be reduced via the nitroso derivative to the arylhydroxylamine. This N-hydroxylated compound can yield reactive nitrenium ions directly. Alternatively, the levels of nitrenium ions can be increased as a consequence of acetylation of the oxygen of the hydroxylamine; this gives an unstable N-acetoxyarylamine that more readily yields the key reactive nitrenium derivative. The nitrenium ion can react with both protein and nucleic acid, but nucleic acid adduct formation is believed to be more closely associated with the carcinogenic process. Alteration of nucleic acids by nitrenium ions has been shown to involve arylamine substitution of C-8 of guanine most frequently. Phenols, in view of our carcinogenicity studies, may not be intermediates in the carcinogenic process, and are not generally regarded as intermediates in metabolic activation pathways. Epoxides, on the other hand, are regarded as potentially reactive per se (Djuric et al., 1986a,b; El Bayoumy et al., 1986), although carcinogenicity tests of these metabolites have yet to be carried out.

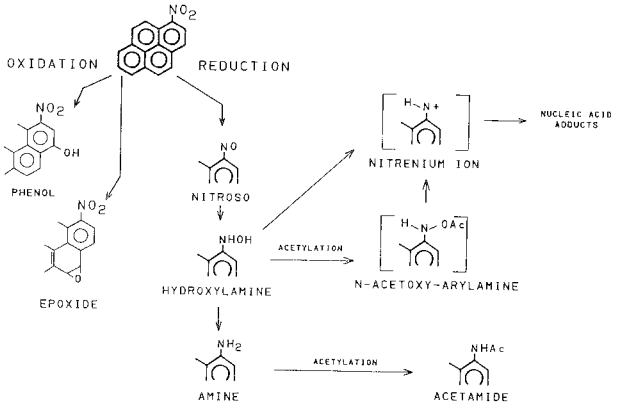

Figure 13. Metabolism of nitropyrenes.

Of the three susceptible organ systems of the rat, the mammary gland is the most readily accessible for biochemical studies. Mammary tumor formation in the rat has been related to the ability of this organ to activate carcinogenic aromatic amines, by the enzymatic synthesis of N-acetoxy-arylamines, that can react with the nucleic acid of the target organ (King, 1985). Several lines of evidence support this relationship. Direct administration to the mammary gland of precursors of this reactive metabolite results in tumor formation in this organ (Malejka-Giganti et al., 1977). Tumor induction in the mammary gland after the systemic or direct administration of arylhydroxamic acids that can be activated by N,O-acyltransferase by this organ is related to the ability of the rat mammary gland to activate these compounds (Shirai et al., 1981; Allaben et al., 1982). The structures of nucleic acid adducts that are isolated from mammary glands treated in vivo with these carcinogens are consistent with this metabolic activation pathway (King et al., 1979; Allaben et al., 1983).

Reduction of the nitropyrenes to amines provides indirect evidence of the production of N-hydroxy-aminopyrenes as intermediates. The instability of these key putative metabolites has generally precluded their synthesis and isolation from biochemical systems. In the present study, the ease of reduction of the nitropyrenes by mammary gland preparations generally parallels the abilities of these compounds to induce tumors in this organ. Remarkably, the nitropyrenes that are most able to produce mammary tumors, 1,8- and 1,6-dinitropyrene, undergo reduction on incubation with intact mammary cells; no metabolites suggestive of ring oxidation were observed. Furthermore, these nitropyrenes are activated to the greatest extent by the addition of acetyl CoA to incubations of mammary cytosols. Earlier studies by Beland and his coworkers (Djuric et al., 1985) had shown the activation of these two isomers to be greatly enhanced by the inclusion of acetyl CoA in rat liver cytosols, preparations that have greater concentrations of enzymes capable of activation of N-hydroxyarylamines by acetylation (King, 1985). Since arylhydroxamic acids that occupy peri positions (King, unpublished observations) or carry ortho methyl groups (Flammang et al., 1985) cannot serve as acetyl donors for O-acetylation, it is likely that the activation of the dinitropyrenes by acetyl CoA results from direct O-acetylation of their hydroxylaminonitro derivatives. Direct evidence of the ability of the rat mammary gland to acetylate N-substituted pyrene derivatives was demonstrated in the present study by the detection of small quantities of 8-acetylamino-1-nitropyrene after incubation of 1,8-dinitropyrene with intact mammary cells.

The results of the present study support the conclusion that the greater ease of reduction of 1,6- and 1,8-dinitropyrene by mammalian enzymes to reactive hydroxylamine derivatives, and the further activation of these putative hydroxylamine metabolites by acetyl CoA, is responsible for their potent carcinogenicity. These characteristics of tumorigenesis are analogous to those believed to be responsible for the carcinogenic activity of other N-substituted aryl compounds, a class of carcinogens known to be capable of inducing urinary bladder tumors in humans.

ACKNOWLEDGMENTS

These studies were carried out in the A. Alfred Taubman Facility for Environmental Carcinogenesis. Katsumi Imaida, M.D. and Ching Y. Wang, D.V.M., Ph.D. were primarily responsible for the tumor induction studies. Dr. Imaida evaluated the tissue specimens histologically. Lee Tay, Ph.D. and Thomas M. Reid, M.A. carried out the majority of the metabolism experiments. Mei-Sie Lee, Ph.D., with Dr. Tay, prepared the necessary compounds and characterized them by chromatographic and spectroscopic methods. Nuclear magnetic resonance and mass spectroscopy data were obtained by Dr. Robin Hood and his staff at the Instrumentation Center of the Comprehensive Cancer Center of Metropolitan Detroit.

REFERENCES

Allaben WT, Weeks CE, Weis CC, Burger GT, King CM, 1982. Mammary tumor induction in the rat by *N*-acyl-*N*-2-fluorenylhydroxylamines: Structure-activity relationship. Carcinogenesis; 3:233-240.

Allaben WT, Weis CC, Fullerton NF, Beland FA, 1983. Formation and persistence of DNA adducts from the carcinogen N-hydroxy-2-acetylaminofluorene in rat mammary gland in vivo. Carcinogenesis; 4:1067-1070.

Bavin PMG, 1959. 4-Nitropyrene. Can J Chem; 37:1614-1615.

Beland FA, 1986. The metabolic activation and DNA adducts of dinitropyrenes. Health Effects Institute Research Report No. 4, pp. 3-30.

Bryant, DW, McCalla DR, Lultschik P, Quilliam MA, McCarry BE, 1984. Metabolism of 1,8-dinitropyrene by *Salmonella typhimurium* is deficient in an acetyl CoA acetyltransferase. Chem-Biol Interact; 49:351-368.

Djuric Z, Fifer EK, Beland FA, 1985. Acetyl coenzyme A-dependent binding of carcinogenic and mutagenic dinitropyrenes to DNA. Carcinogenesis; 6:941-944.

Djuric Z, Fifer EK, Howard PC, Beland FA, 1986a. Oxidative microsomal metabolism of 1-nitropyrene and DNA-binding of oxidized metabolites following nitroreduction. Carcinogenesis; 7:1073-1079.

Djuric Z, Potter DW, Heflich RH, Beland FA, 1986b. Aerobic and anaerobic reduction of nitrated pyrenes. Chem-Biol Interact; 59:309-324.

El Bayoumy K, Hecht SS, 1983. Identification and mutagenicity of metabolites of 1-nitropyrene formed by rat liver. Cancer Res; 43:2052-2058.

El Bayoumy K, Hecht SS, Hoffmann D, 1982. Comparative tumor initiating activity on mouse skin of 6-nitrobenzo[a]pyrene, 6-nitrochrysene, 3-nitroperylene, 1-nitropyrene and their parent hydrocarbons. Cancer Letters; 15:1-7.

El Bayoumy K, Villucci P, Roy AK, Hecht SS, 1986. Synthesis of K-region derivatives of the carcinogen 1-nitropyrene. Carcinogenesis; 7:1577-1580.

Endo H., 1971. Carcinogenicity. In: Endo H, Ono T, Sugimura T, eds. Recent Results in Cancer Research. Chemistry and Biological Actions of 4-Nitroquinoline-1-oxide. Berlin: Springer-Verlag, pp. 32-52.

Ethier SP, 1985. Primary culture and serial passage of normal and carcinogen treated rat mammary epithelial cells *in vitro*. J Natl Cancer Inst; 74:1307-1318.

Fifer EK, Heflich RH, Djuric Z, Howard PC, Beland FA, 1986. Synthesis and mutagenicity of 1-nitro-6-nitrosopyrene and 1-nitro-8-nitrosopyrene, potential intermediates in the metabolic activation of 1,6- and 1,8-dinitropyrene. Carcinogenesis; 7:65-70.

Flammang, TJ, Westra JG, Kadlubar FF, Beland FA, 1985. DNA adducts formed from the probable proximate carcinogen, N-hydroxy-3,2'-dimethyl-4-aminobiphenyl by acid catalysis or S-acetyl coenzyme A-dependent enzymatic esterification. Carcinogenesis; 6:251-258.

Garner RC, Martin CN, Clayson DB, 1984. Carcinogenic aromatic amines and related compounds. In: Searle CE, ed. Chemical Carcinogens. Washington, DC: American Chemical Society, pp. 175-276.

Hirose M, Lee MS, Wang CY, King CM, 1984. Induction of rat mammary gland tumors by 1-nitropyrene, a recently recognized environmental mutagen. Cancer Res; 44:1158-1162.

Howard PC, Beland FA, Cerniglia CE, 1983. Reduction of the carcinogen 1-nitropyrene to 1-aminopyrene by rat intestinal bacteria. Carcinogenesis; 4:985-990.

Imaida K, Hirose M, Lee M-S, Wang CY, King CM, 1985. Comparative carcinogenicities of 1-, 2- and 4-nitropyrenes (NP) and structurally related compounds for female CD rats following intraperitoneal injection. Proc Amer Assn Cancer Res; 26:93.

King CM, 1974. Mechanism of reaction, tissue distribution and inhibition of arylhydroxamic acid acyltransferase. Cancer Res; 34:1503-1516.

King CM, 1985. Metabolism and the "initiation" of tumors by chemicals. In: Marnett LJ, ed. Arachidonic Acid Metabolism and Tumor Initiation. Boston: Martinus Nijhoff Publishing, pp. 1-37.

King CM, Olive CW, 1975. Comparative effects of strain, species and sex and the acyltransferase- and sulfotransferase-catalyzed activations of *N*-hydroxy-*N*-2-fluorenylacetamide. Cancer Res; 35:906-912.

King CM, Traub NR, Lortz ZM, Thissen MR, 1979. Metabolic activation of arylhydroxamic acid *N,O*-acyltransferase of rat mammary gland. Cancer Res; 39:3369-3372.

Kriek E, Westra JG, 1980. Structural identification of the pyrimidine derivatives formed from N-(deoxyguanosin-8-yl)-2-aminofluorene in aqueous solutions at alkaline pH. Carcinogenesis; 1:459-468.

Kuhn WE, 1943. 2-Nitrofluorene and 2-aminofluorene. Org Syn Coll; 2:447-448.

Lofroth G, 1978. Mutagenicity assays of combustion emissions. Chemosphere; 17:791-798.

Lowry OH, Rosebrough HJ, Farr AL, Randall RF, 1951. Protein determination with the folin phenol reagent. J Biol Chem; 193:265-275.

Malejka-Giganti D, Rydell RE, 1978. Lack of susceptibility of F344 rats to mammary tumor induction by topical application of fluorenylhydroxamic acids. J Nat Cancer Inst; 60:433-435.

Malejka-Giganti D, Rydell RE, Gutmann HR, 1977. Mammary carcinogenesis in the rat by topical application of fluorenylhydroxamic acids and their acetates. Cancer Res; 37:111-117.

McCoy EC, Anders M, Rosenkranz HS, 1983. The basis of the insensitivity of *Salmonella typhimurium* strain TA98/1,8-DNP_6 to the mutagenic action of nitroarenes. Mutation Res; 121:17-23.

Messier F, Lu C, Andrews P, McCarry BE, Quilliam MA, McCalla DR, 1981. Metabolism of 1-nitropyrene and formation of DNA adducts in *Salmonella typhimurium*. Carcinogenesis; 2:1007-1011.

Miller EC, Miller JA, Hartman HA, 1961. N-Hydroxy-2-acetylaminofluorene: A metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. Cancer Res; 31:815-824.

Miller JA, Sandin RB, Miller EC, Rusch HP, 1955. The carcinogenicity of compounds related to 2-acetylaminofluorene, II. Variations in the bridges and the 2-substituent. Cancer Res; 15:188-199.

Ohgaki H, Hasagawa H, Kato T, Negishi C, Sato S, Sugimura T, 1985. Absence of carcinogenicity of 1-nitropyrene, correction of previous results, and new demonstration of carcinogenicity of 1,6-dinitropyrene in rats. Cancer Letters; 25:239-245.

Ohgaki H, Matsukura N, Morino K, Kawachi T, Sugimura T, Morita K, Tokiwa H, Hirota T, 1982. Carcinogenicity in rats of the mutagenic compounds 1-nitropyrene and 3-nitrofluoranthene. Cancer Letters; 15:1-7.

Ohgaki H, Negishi K, Wakabayashi K, Kusama K, Sato S, Sugimura T, 1984. Induction of sarcomas in rats by subcutaneous injection of dinitropyrenes. Carcinogenesis; 5:583-585.

Pitts JN, van Cauwenberghe KA, Grosjean D, Schmid JP, Fitz DR, Belser WL Jr, Knudson GB, Hynds PM, 1978. Atmospheric reactions of polycyclic aromatic hydrocarbons: Facile formation of mutagenic nitro derivatives. Science; 202:515-519.

Rosenkranz HS, Mermelstein R, 1983. Mutagenicity and genotoxicity of nitroarenes; all nitro-containing chemicals were not created equal. Mutation Res; 114:217-267.

Saito K, Shinohara A, Kamataki T, Kato R, 1985. Metabolic activation of mutagenic N-hydroxylamines by O-acetyltransferase in *Salmonella typhimurium* TA98. Arch Biochem Biophys; 239:286-295.

Schuetzle D, 1983. Sampling of vehicle emissions for chemical analysis and biological testing. Environmental Health Perspectives; 47:65-80.

Shirai T, Fysh JM, Lee MS, Vaught JB, King CM, 1981. Relationship of metabolic activation of N-hydroxy-N-acylarylamines to biological response in the liver and mammary gland of the female CD rat. Cancer Res; 41:4346-4353.

Takayama S, Ishikawa T, Nakajime N, Sato S, 1985. Lung cancer induction in Syrian golden hamsters by intratracheal instillation of 1,6-dinitropyrene. Japanese Journal of Cancer Research; 76:457-461.

Tang MS, Lieberman MW, 1983. Quantification of adducts formed in DNA treated with N-acetoxy-2-acetylaminofluorene or N-hydroxy-2-aminofluorene: Comparison of trifluoroacetic acid and enzymatic degradation. Carcinogenesis; 4:1001-1006.

Tay LK, Russo J, 1981. 7,12-Dimethylbenz(a)anthracene (DMBA)-induced DNA binding and repair synthesis in susceptible and non-susceptible mammary epithelial cells in culture. J Natl Cancer Inst; 67:155-161.

Tokiwa H, Otofuji T, Horikawa K, Kitamori S, Otsuka H, Manabe Y, Kirouchi T, Ohnishi Y, 1984. 1,6-Dinitropyrene: Mutagenicity in *Salmonella* and carcinogenicity in BALB/c mice. J Natl Cancer Inst; 73:1359-1363.

Wang CY, Lee M-S, King CM, Warner PO, 1980. Evidence for nitroaromatics as direct-acting mutagens of airborne particulates. Chemosphere; 9:83-87.

Weeks CE, Allaben WT, Louie SC, Lazear EJ, King CM, 1978. Role of hydroxamic acid acyltransferase in the mutagenicity of N-hydroxy-N-2-fluorenylacetamide in *Salmonella typhimurium*. Cancer Res; 38:613-618.

Weiss SW, Enzinger FM, 1978. Malignant fibrous histiocytoma: An analysis of 200 cases. Cancer; 41:2250-2277.

Young S, Hallowes RC, 1973. Tumors of the mammary gland. In: Tursuov VS, ed. Pathology of Tumors in Laboratory Animals. Lyons, France: International Agency for Research on Cancer, Vol. 1, pp. 31-74.

INTRODUCTION

A Request for Applications (RFA 82-1), which solicited proposals for "Studies on the Metabolism and Biologic Effects of Nitropyrene and Related Nitro-Polycyclic Aromatic Compounds," was issued by the HEI in the summer of 1982. Dr. Charles M. King of the Michigan Cancer Foundation in Detroit, Michigan, submitted a proposal entitled, "Metabolism and Biological Effects of Nitropyrene and Related Compounds." The HEI approved the three-and-a-half year project and authorized a total expenditure of $502,925. The project began in August, 1983, and the final report was accepted by the Health Review Committee in July, 1987.

The Health Review Committee report is intended to place the investigator's final report in perspective as an aid to the sponsors of the HEI and to the public.

CLEAN AIR ACT

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

Under these provisions, the EPA has taken regulatory actions with respect to diesel engines. In 1980, the Agency set light-duty diesel particulate standards, and, in 1984, granted a two-year delay in their effective date. The Agency established emissions averaging in 1983, and it set nitrogen oxides standards in 1985. For heavy-duty diesel engines, the Agency set hydrocarbon and carbon monoxide standards in 1983, and nitrogen oxides and particulate standards in 1985. In addition, under Section 109 of the Act, EPA has established national ambient air quality standards for particulate matter. Those standards were most recently revised in July, 1987.

Research on the metabolism and carcinogenesis of 1-nitropyrene and several of its metabolic derivatives can contribute to increased understanding of the risks to humans from exposure to diesel particulates, and can thereby contribute to informed decision-making with respect to standards under the Clean Air Act.

BACKGROUND

An important environmental issue of the past decade has been the potential health effects from the use of diesel fuel in vehicles (McClellan, 1986). Such vehicles emit relatively large amounts of particulate matter, 30 to 100 times as much as gasoline-fueled vehicles. Diesel particles are submicronic in size and contain a carbon core. The particles have a large surface-to-volume ratio, which allows for the adsorption of organic combustion products to the particle surface. Diesel exhaust thus consists of respirable particles with adsorbed organic materials that are potentially bioavailable after the particles have been deposited in the lung.

Early findings, which showed that the polar organic fraction of diesel particle extracts was tumorigenic in mouse skin initiation assays (Kotin et al., 1955; Nesnow et al., 1984), and was genotoxic in a variety of bacterial and mammalian cell short-term assays (reviewed in Lewtas, 1983), aroused concern about the potential carcinogenic risk from exposure to diesel exhaust. To address this concern, a few chronic studies using different laboratory species (rats, mice, and hamsters) were launched to study the tumorigenicity of inhaled diesel exhaust. The results of these early efforts were negative or inconclusive (White et al., 1983; Shefner et al., 1985). Recent studies, however, have demonstrated that if rats are exposed to high levels of diesel exhaust (2 to 7 mg/m^3) over their lifetimes, lung tumors, as well as tumors at distant sites, are induced (Ishinishi et al., 1986; Mauderly et al., 1986; Heinrich et al., 1986; Brightwell et al., 1986; Iwai et al., 1986).

Another important development in the research on diesel-related carcinogenicity during the last decade was the determination that nitropyrene compounds adsorbed to diesel particles contribute significantly to the total mutagenicity of particle extracts, as measured by the Ames *Salmonella typhimurium* test (Huisingh et al., 1978; Lewtas, 1978; Scheutzle et al., 1982; Nishioka et al., 1982; Pederson and Siak, 1981; Mermelstein et al., 1981). In short-term tests on mammalian cells, the nitropyrene compounds have been shown to produce mutations (Li and Dutcher, 1983), DNA damage (Saito et al., 1984), chromosomal aberrations (Danford et al., 1982), and neoplastic transformation (DiPaolo et al., 1983; Howard et al., 1983). Particular attention has centered on 1-nitropyrene, which is the most abundant nitropyrene on the particles (approximately 100 μgram per gram particle),

as well as on three isomers of dinitropyrene (1,3-, 1,6-, and 1,8-dinitropyrene). The dinitropyrenes are less abundant than 1-nitropyrene (approximately 1 μg per gram), but are more than two orders of magnitude more potent as mutagens than is 1-nitropyrene in the Ames assay. The relative potencies of these compounds have not been systematically compared in intact mammals.

Nitropyrenes exhibit genotoxicity after nitroreduction and, in the case of dinitropyrenes, after subsequent acetylation (Beland, 1986). The structural similarities between nitropyrenes and aromatic amines (some of which are known human carcinogens), as well as certain similarities between the metabolism of the two classes of compounds (in particular, the role of acetylation), raise the possibility that the mechanism of tumor induction by nitropyrenes is analogous to that of aromatic amines. Several acetylation reactions (viz., N-, O-, and N,O-acetylation) appear to be involved in the metabolism of aromatic amines (Weber et al., 1987). In the case of dinitropyrenes, O-acetylation is believed to be the major pathway in the production of genotoxic intermediates; however, detailed information to assess the role of other acetylation pathways is not yet available.

Metabolic pathways leading to activated intermediates have been investigated in cells in which the metabolic enzymes are endogenous, or where the enzymes are added from an external source, such as the supernatant from liver homogenate. When the "appropriate" metabolic conditions are present in these systems, 1-nitropyrene and dinitropyrene are converted to electrophilic intermediates that form adducts with DNA at the C-8 position of deoxyguanosine (Beland, 1986). In animals, adduct formation is believed to be linked with carcinogenicity, and it has been considered as a marker for carcinogenesis in humans (Perera and Weinstein, 1982; Harris et al., 1985; Wogan and Gorelick, 1985).

Although much attention in the short-term bioassays has focused on the metabolic pathways responsible for reducing nitropyrenes to DNA-binding products, the more complex metabolic systems present in mammalian tissues, such as liver and lung, engage in oxidative, as well as reductive, metabolism of nitropyrenes (El-Bayoumy and Hecht, 1983; King et al., 1984). After 1-nitropyrene is administered, these tissues yield a complex array of products, which include several hydroxylated derivatives formed as a result of the oxidation of the parent compound. Many of these derivatives are themselves mutagenic, and some are capable of forming adducts with DNA, which include the C-8 deoxyguanosine adduct, as well as other adducts not observed in the single cell systems (Jackson et al., 1985).

In recent years, a number of studies have been conducted to determine whether nitropyrenes are tumorigenic in laboratory animals. The doses used in these experiments are many orders of magnitude higher than the nitropyrene body burdens that would result from humans' inhalation of diesel exhaust, even under the worst-case situations. These experiments serve as useful screens for carcinogens, explore mechanisms of action within in vivo systems, and provide information on the metabolic pathways by which these compounds are activated or deactivated.

For example, bacteria (also referred to as microflora) in the gastrointestinal tract are capable of converting orally administered nitropyrenes to a number of products, some of which are genotoxic. These products are transported by way of the portal circulation to the liver, in which they are further metabolized. Alternatively, nitropyrenes injected intraperitoneally bypass intestinal conversion and also reach the liver through portal blood. In the liver, 1-nitropyrene is oxidized or reduced, and often is conjugated to products destined for excretion in either urine or feces. On the other hand, upon subcutaneous injection, nitropyrenes have direct access to various organs before they are metabolized. The role of the metabolic pathways, and the production of genotoxic products and ultimate carcinogenicity in vivo, can be tested experimentally using different routes of administration.

Several studies in whole animals have demonstrated that dinitropyrenes are carcinogenic and, depending on the route of administration, induce skin tumors, lung tumors, and leukemia (for review, see Rosenkranz, 1987). These studies also have shown that 1,6- and 1,8-dinitropyrene are much more potent as carcinogens than is 1,3-dinitropyrene. On the other hand, the results of animal experiments with 1-nitropyrene have been inconsistent. Although some investigators have reported tumors in animals given 1-nitropyrene, others have failed to observe significant tumorigenic activity. Furthermore, it has not been possible to explain these observations in terms of the route of administration of 1-nitropyrene. (It should be noted that at the time that Dr. King proposed his studies, much of this information was not available.)

JUSTIFICATION FOR THE STUDY

Nitropyrenes and related nitroaromatics are of interest for several reasons: their ubiquity in diesel emissions; their well-established direct mutagenicity in the Ames assay; their reported carcinogenicity in rodents; and the relative paucity of information on this class of compounds in terms of metabolism, mutagenicity, carcinogenicity, or other toxic effects in mammalian systems. The HEI solicited proposals that would provide novel and fundamental information on one or more aspects of the metabolism and biologic effects of 1-nitropyrene and related nitroaromatics. More specifically, research areas of interest to the Institute included the elucidation of biotrans-

formation, the formation of adducts, and the comparison of activity and relative potencies of these compounds.

Dr. King proposed to test the hypothesis that the nitro derivatives of noncarcinogenic polycyclic aromatic hydrocarbons, such as nitropyrenes, would be carcinogenic by virtue of metabolic conversion to products that are closely related to the aromatic amines known to be human and animal carcinogens. Extensive carcinogenicity studies were planned to compare systematically the potency of several nitropyrenes given by various routes of administration to intact animals. In addition, metabolic studies were proposed that would elucidate the metabolic pathways by which nitropyrenes are activated by mammalian tissues. The strength of Dr. King's proposal centered on his approach of relating the metabolism of these compounds to their carcinogenic potential.

OBJECTIVES OF THE STUDY

Dr. King's study had two overall goals: first, to investigate in rats the carcinogenicities of 1-nitropyrene, the metabolic derivatives of 1-nitropyrene, and 1,3-, 1,6-, and 1,8-dinitropyrene; and, second, to determine how these compounds are metabolically activated in target tissues.

The specific aims of the study were:

1. To investigate the carcinogenicities of 1-nitropyrene, 1,3-, 1,6-, and 1,8-dinitropyrene administered by intraperitoneal injection into weanling female Sprague Dawley-derived CD rats;

2. To investigate the carcinogenicities of the same compounds administered by intragastric intubation to rats of the same strain, age, and sex in order to ascertain whether initial contact of the compounds with the gastrointestinal microflora would alter their activities;

3. To investigate the carcinogenicities of the same compounds administered subcutaneously to newborn rats of the same strain and sex, in order to explore the influence of age on susceptibility to the action of these agents, as well as to examine their effects under conditions where the compounds become distributed systematically before undergoing hepatic metabolism;

4. To investigate the carcinogenicities of 1-nitropyrene and its major phenolic metabolites, 3-hydroxy-1-nitropyrene and a mixture of 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene administered subcutaneously to newborn female rats in order to determine whether the carcinogenic activity of 1-nitropyrene might be mediated through these metabolites;

5. To determine the relative susceptibility of newborn female Fischer 344 (F344) rats to 1-nitropyrene administered subcutaneously, as compared with newborn female CD rats, in view of the widespread use of the Fischer rat in toxicological studies;

6. To investigate the carcinogenic effects of subcutaneous, as opposed to intraperitoneal, administration of 1-nitropyrene in weanling female CD rats, in order to confirm previous observations suggesting that 1-nitropyrene might not be carcinogenic when administered by the intraperitoneal route; and

7. To explore the capacity of the rat mammary gland, a target tissue for the carcinogenic effects of the aromatic amines, to metabolize 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene, and 1,8-dinitropyrene.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

This is a well-designed and well-conducted study. All the specific aims were accomplished in the experiments described in the report. The results provide important information about the biological action of nitropyrenes.

METHODS AND STUDY DESIGN

Well-conceived protocols and appropriate methods were used in these studies. The purity of the compounds used was confirmed during the course of this study; this is an important issue because, in other studies, the use of impure materials may have given misleading results. Because sufficient quantities of the 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene metabolites could not be readily obtained, these compounds were tested together as a mixture.

The bioassay systems for the evaluation of the carcinogenicity of the nitropyrenes were well-chosen. Because most diesel and nitropyrene tumor studies are conducted using the F344 strain, the decision to compare the results in this strain was wise.

The biochemical experiments, which used rat mammary cytosol and rat mammary epithelial cells in culture, also were conducted appropriately. It would have been ideal, however, to have measured the binding to DNA in all portions of the work, instead of only the gross binding to tRNA, which was measured in most instances in these studies. Nevertheless, this approach is a justifiable cost- and time-saving compromise, as explained in the report.

STATISTICAL ANALYSIS

The statistical analysis of the data is straightforward. However, within the different protocols, groups of animals are compared only to their solvent controls; different experimental conditions were not formally compared. It would have been appropriate to have analyzed the results of the tumorigenicity experiments by life-table methods, such as the Kaplan-Meier

technique, which take into account both the tumor incidence rate and the tumor induction time for the entire experimental group. Hence, a later attempt was made to analyze the data of this study by the Kaplan-Meier technique, which provided results that were of limited utility and that did not change the overall conclusions of the study. However, because the duration of the experiments reported in Tables 2 and 3 (76 to 78 weeks) was insufficient for tumors to develop in all animals, this analysis suggested that the average time-to-tumor in the experimental population may be greater than that reported in Tables 2 and 3.

RESULTS AND INTERPRETATION

Depending upon the conditions of exposure, three principal types of neoplasms were observed to be induced by the agents tested: malignant fibrous histiocytomas (MFHs) at the site of subcutaneous or intraperitoneal injection, mammary gland tumors (adenocarcinomas and fibroadenomas), and myelocytic leukemias.

For the induction of MFHs, 1,6-dinitropyrene, 1,8-dinitropyrene, and 4-nitropyrene appeared to be more effective than 1,3-dinitropyrene; 1-nitropyrene, 3-hydroxy-1-nitropyrene and the mixture of 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene appeared to be ineffective. In animals treated with 1,6- or 1,8-dinitropyrene, the MFHs were induced rapidly and with great frequency; such animals did not survive more than 20 to 25 weeks. In this study, 1-nitropyrene did not cause MFHs; this is in contrast with earlier experiments reported by the same investigator and should be noted (Hirose et al., 1984). Other investigators also have reported negative results for the induction of MFHs at the site of 1-nitropyrene injection (Ohgaki et al., 1985; Tokiwa et al., 1984). In studies that tested dinitropyrenes, however, MFHs at the site of injection have been reported (Tokiwa et al., 1984, 1986; Ohgaki et al., 1984, 1985). The significance of MFHs at the site of injection has been questioned because of the insolubility, and the consequent precipitation, of nitropyrenes at the site of injection. The precipitates are believed to produce high local concentrations of the compound, and to irritate the tissue; both of these factors are thought to be involved in the induction of MFHs. The biological significance of the induction of MFHs at the injection sites, therefore, remains equivocal.

With respect to the induction of leukemia, 1,6- and 1,8-dinitropyrene were more effective than 1,3-dinitropyrene, and 1-nitropyrene appeared to be ineffective. These results are consistent with a report by Takayama et al. (1985), who exposed Syrian golden hamsters to 1,6-dinitropyrene by intratracheal administration, and observed leukemia (and lung tumors) in all the animals in his experiment.

For the induction of mammary gland tumors, 1,8-dinitropyrene, 1,6-dinitropyrene, and 4-nitropyrene appeared to be more effective than 1,3-dinitropyrene; 1-nitropyrene appeared to be weakly effective; and 3-hydroxy-1-nitropyrene and the mixture of 6-hydroxy-1-nitropyrene and 8-hydroxy-1-nitropyrene appeared to be ineffective. Three kinds of mammary tumors were observed: fibroadenoma, adenoma, and adenocarcinoma. The incidence of total mammary tumors was high in the solvent-exposed CD rats; however, the background tumor rate in the solvent-exposed animals was relatively modest for the malignant adenocarcinoma. In terms of biological significance, the authors gave greater emphasis to adenocarcinomas than to the other tumors, and performed appropriate statistical tests to confirm their conclusions. The results on mammary gland tumor induction by 1-nitropyrene are in agreement with earlier results reported from the same laboratory (Hirose et al., 1984). This is the first report in the literature regarding the induction of mammary gland tumors by the dinitropyrenes.

Carcinogenicity by the intragastric route of administration appeared to be substantially lower than by the subcutaneous or intraperitoneal routes. Although there was a suggestion that the incidence of mammary gland tumors was increased by 1,6- and 1,8-dinitropyrenes administered intragastrically, the observation that MFHs were not found after the intragastric administration of 1-nitropyrene suggests that intestinal bacteria may detoxify the nitropyrenes. Whether a reduced metabolite was activated by the mammary gland, or the tumors were induced by the parent compound, cannot be determined from the results of this study. The appearance of tumors in tissues distant from the site of administration confirms that specific tissues, in this case the mammary gland, are capable of generating carcinogenic metabolites. This finding was supported in the metabolic studies.

Although the differences among compounds in carcinogenic potency appear to be definitive, it is difficult to quantify them precisely in the absence of appropriate dose-response curves. Thus, on the basis of the results reported, the intended comparison of carcinogenic potencies can be no more than semi-quantitative. However, this study was designed primarily to explore the potential target organs and the influence of the route of administration of nitropyrenes; therefore, detailed dose-response experiments were considered beyond the scope of this study.

Two other significant findings of these studies should be noted. First, rats of the Fischer strain appeared to be much less susceptible to the induction of neoplasms than were rats of the CD strain. The absence of malignant mammary tumors in the F344 strain suggests that this strain may be inappropriate for mammary studies, and caution should be exercised in the interpretation of results from previous or future studies which use this strain. Second, with respect to age, neonatal CD rats appeared to be more susceptible to the induction of MFHs than were weanling animals. However, it should be noted that

this observation may be related to the differences in the routes of exposure (subcutaneous versus intragastric, respectively).

Studies on the metabolism of nitropyrenes were of a more limited nature and were performed with rat mammary gland cytosols and isolated mammary epithelial cells. The objective of these experiments was to obtain information regarding the metabolic pathways by which the nitropyrenes are activated. The author reports that, in both cytosols and intact cells, dinitropyrenes are activated by monoreduction and subsequent O-acetylation. In cytosols, the dinitropyrenes formed adducts with exogenously added tRNA; the order of potency was 1,6-, 1,8-, 1,3-dinitropyrene. On the basis of the results of preliminary experiments, the investigator states that the DNA adducts formed in intact cells of the mammary gland were indistinguishable from those formed on incubation of these compounds with calf thymus DNA in rat liver cytosol and acetyl CoA. The results described here support the investigator's hypothesis that, in mammary tissue, dinitropyrenes are metabolized in a manner analogous to carcinogenic aromatic amines.

The major product of 1-nitropyrene metabolism in mammary cytosols was 1-aminopyrene. This suggested that mammary cells are capable of reductive metabolism of 1-nitropyrene. 1-Nitropyrene also formed adducts with tRNA added to the cytosols. However, when 1-nitropyrene was added to intact mammary epithelial cells, the products of metabolism were phenolic derivatives of 1-nitropyrene; little or no 1-aminopyrene was detected. This finding suggested that, in contrast to cytosols, the major path of 1-nitropyrene metabolism in intact cells was oxidative. As discussed above, the investigator also found a moderate elevation in the incidence of mammary tumors in animals exposed to 1-nitropyrene. The studies reported here do not clarify whether 1-nitropyrene-induced tumorigenesis is related to metabolism of 1-nitropyrene in mammary tissues (and if so, what is the relative importance of reductive versus oxidative pathways of metabolism) or whether products of 1-nitropyrene metabolism are transported to mammary tissue from other organs.

REMAINING UNCERTAINTIES

Additional tumorigenicity experiments in F344 rats, especially with the dinitropyrenes, would be of interest. These would provide further information on the mammary gland carcinogenicity of nitropyrenes. Studies on DNA adducts formed in intact cells derived from the target tissue, including studies on the structure of such adducts, would further enhance our understanding of the mechanism of action of nitropyrenes in the target tissue.

It also would be useful to extend the studies described here by using inhalation as the route of exposure. Because such experiments (especially with the dinitropyrenes) present numerous difficulties, it would be desirable to approach extrapolating the results of the current study to respiratory tissues by performing the appropriate biochemical and metabolic experiments.

CONCLUSIONS

Several important conclusions can be derived from this study. First, nitropyrenes administered by a variety of routes induce mammary tumors in CD rats. This observation, together with the metabolic studies reported here, suggests that nitropyrenes behave like aromatic amines in the mammary tissue. The use of different routes of administration also allowed an indirect estimation of the importance of intestinal microflora in detoxifying orally ingested nitropyrenes.

Second, it is noteworthy that Fischer 344 rats that were administered 1-nitropyrene did not develop mammary tumors. Because most studies on the carcinogenicity of nitropyrenes, as well as on whole diesel exhaust, use the Fischer rat, it may not be an appropriate model for mammary gland carcinogenesis. Dr. King has provided evidence for the mammary gland carcinogenesis of 1-nitropyrene and 1,8-dinitropyrene; the question of mammary gland carcinogenesis of other nitropyrenes, as well as of whole diesel exhaust, remains unresolved.

Third, the metabolism of dinitropyrenes appeared to proceed via the O-acetylation pathway in mammary cytosol. Thus, dinitropyrenes appeared to behave as aromatic amines; this observation may be significant in view of the suggestion that the susceptibility to arylamine-induced tumors in humans may be a function of the individual acetylator phenotype (Cartwright, 1984; Weber et al., 1987). However, the acetylation of aromatic amines appears to occur by one or more acetyl transfer reactions (N-, O-, or N,O-acetylation), which may differ in their potential to produce genotoxic products. The relative contributions of these pathways in the overall acetylation reaction depend upon the substrate, the tissue, and the species. The roles and the significance of these alternative pathways in the metabolism of dinitropyrenes have not been systematically investigated. Therefore, the analogy between the metabolism of aromatic amines and nitropyrenes should be interpreted cautiously.

Certain implications of these studies also deserve comment. The metabolic data, coupled with the carcinogenicity results, imply that the tumorigenic potential of these compounds results from their resemblance to aromatic amines, which are recognized to be human urinary bladder carcinogens. This suggests that these compounds also may have the potential to cause human bladder cancer. The extent of such a risk from these compounds is beyond the scope of the present study, but deserves further exploration. In addition, the studies presented here provide evidence for mammary gland carcinogenicity of nitropyrenes in the rat. Carcinogenic risk assessments for diesel exhaust have relied on studies in Fischer rats, which appear resistant to mammary gland carcinogenesis; therefore, we should be mindful that the potential of diesel exhaust for mammary gland carcinogenesis in animals has not been fully explored.

REFERENCES

Beland FA, 1986. The metabolic activation and DNA adducts of dinitropyrenes. Research Report No. 4, Health Effects Institute, Cambridge, MA.

Brightwell J, Fouillet X, Cassano-Zoppi AL, Gatz R, Duchosal F, 1986. Neoplastic and functional changes in rodents after chronic inhalation of engine exhaust emissions. Dev Toxicol Environ Sci; 13:471-485.

Cartwright RA, 1984. Epidemiological studies in N-acetylation and C-center ring oxidation in neoplasia. In: Omen GS, Gelboin HV, eds. Genetic Variability in Responses to Chemical Exposure, Banbury Report 16. Cold Spring Harbor, New York: Cold Spring Harbor Laboratories, pp. 359-365.

Danford N, Wilcox P, Parry JM, 1982. The clastogenic activity of dinitropyrenes in rat-liver epithelial cell line. Mutation Res; 105:349-355.

DiPaolo JA, DeMarinis AJ, Chow FL, Garner RC, Martin CN, Doniger J, 1983. Nitration of carcinogenic and non-carcinogenic polycyclic aromatic hydrocarbons results in products able to induce transformation of Syrian hamster cells. Carcinogenesis; 4:357-359.

El-Bayoumy K, Hecht SS, 1983. Identification and mutagenicity of metabolites of 1-nitropyrene formed by rat liver. Cancer Res; 43:3132-3137.

Harris CC, Vahakangas K, Autrup H, Trivers GE, Shamsuddin AKM, Trump BF, Bowman BM, Mann DL, 1985. Biochemical and molecular epidemiology of human cancer risk. In: The Pathologist and the Environment. International Academy of Pathology Monograph No. 26, pp. 140-167.

Heinrich U, Muhle H, Takenaka S, Ernst H, Fuhst R, Mohr U, Pott F, Stober W, 1986. Chronic effects on the respiratory tract of hamsters, mice and rats after long-term inhalation of high concentrations of filtered and unfiltered diesel engine emissions. J Appl Toxicol; 6(6):383-395.

Hirose M, Lee MS, Wang CY, King CM, 1984. Induction of rat mammary gland tumors by 1-nitropyrene, a recently recognized environmental mutagen. Cancer Res; 44:1158-1162.

Howard PC, Gerrard JA, Milo GE, Fu PP, Beland FA, Kadlubar FF, 1983. Transformation of normal human skin fibroblasts by 1-nitropyrene and 6-nitrobenzo(a)pyrene. Carcinogenesis; 4:353-355.

Huisingh J, Bradow R, Jungers R, Claxton L, Zweidinger R, Tejada S, Bumgarner J, Duffield F, Waters M, Simmon VF, Hare C, Rodriguiz C, Snow L, 1978. Application of bioassay to the characterization of diesel particle emissions. In: Waters MD, Nesnow S, Huisingh JL, Sandhu SS, Claxton L, eds. Application of Short-Term Bioassay in the Fractionation and Analysis of Complex Environmental Mixtures. New York: Plenum Press, pp. 381-418.

Ishinishi N, Kuwabara N, Nagase S, Suzuki T, Ishiwata S, Kohno T, 1986. Long-term inhalation studies on effects of exhaust from heavy and light duty diesel engines on F344 rats. Dev Toxicol Environ Sci; 13:329-348.

Iwai K, Udagawa T, Yamagishi M, Yamada H, 1986. Long-term inhalation studies of diesel exhaust on F344 SPF rats. Incidence of lung cancer and lymphoma. In: Ishinishi N, Koizumi A, McClellan RO, Stober W, eds. Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust. Amsterdam: Elsevier, pp. 349-360.

Jackson MA, King LC, Ball LM, Ghayourmanesh S, Jeffrey AM, Lewtas J, 1985. Nitropyrene: DNA binding and adduct formation in respiratory tissues. Environ Health Perspect; 62:203-207.

King LC, Kohan MJ, Ball LM, Lewtas J, 1984. Mutagenicity of 1-nitropyrene metabolites from lung S9. Cancer Lett; 22:255-262.

Kotin P, Falk HL, Thomas M, 1955. Aromatic hydrocarbons. III. Presence in the particulate phase of diesel engine exhausts and the carcinogenicity of exhaust extracts. Arch Ind Health; 11:113-120.

Lewtas J, 1978. Mutagenic activity of diesel emissions. In: Lewtas J, ed. Toxicological Effects of Emissions from Diesel Engines. New York: Plenum Press, pp. 69-82.

Lewtas J, 1983. Evaluation of the mutagenicity and the carcinogenicity of motor vehicle emissions in short-term bioassays. Environ Health Perspect; 47:141-152.

Li AP, Dutcher JS, 1983. Mutagenicity of mono-, di-, and trinitropyrenes in Chinese hamster ovary cells. Mutat Res Lett; 119:387-392.

Mauderly JL, Jones RK, McClellan RO, Henderson RF, Griffith WC, 1986. Carcinogenicity of diesel exhaust inhaled chronically by rats. Dev Toxicol Environ Sci; 13:397-409.

McClellan RO, 1986. Health effects of diesel exhaust: A case study in risk assessment. Am Ind Hyg Assoc J; 47:1-13.

Mermelstein R, Kiriazides DK, Butler M, McCoy EC, Rosenkranz HS, 1981. The extraordinary mutagenicity of nitropyrenes in bacteria. Mutation Res; 89:187-196.

Nesnow S, Triplett LL, Slaga TJ, 1984. Tumor initiating activities of 1-nitropyrene and its nitrated products in Sencar mice. Cancer Lett; 23:1-8.

Nishioka MG, Peterson BA, Lewtas J, 1982. Comparison of nitroaromatic content and direct-acting mutagenicity of diesel emissions. In: Cooke M, Dennis AJ, Fisher GL, eds. Polynuclear Aromatic Hydrocarbons: Physical and Biological Chemistry. Columbus, OH: Battelle Columbus Press, pp. 603-613.

Ohgaki H, Hasegawa H, Kato T, Negishi C, Sato S, Sugimura T, 1985. Absence of carcinogenicity of 1-nitropyrene, correction of previous results, and new demonstration of carcinogenicity of 1,6-dinitropyrene in rats. Cancer Lett; 25:239-245.

Ohgaki H, Negishi C, Wakabayashi K, Kusama K, Sato S, Sugimura T, 1984. Induction of sarcomas in rats by subcutaneous injection of dinitropyrenes. Carcinogenesis; 5:583-585.

Pederson TC, Siak JS, 1981. The role of nitroaromatic compounds in the direct-acting mutagenicity of diesel particulate extracts. J Appl Toxicol; 1:54-60.

Perera FP, Weinstein IB, 1982. Molecular epidemiology and carcinogen-DNA adduct detection: New approaches to studies of human cancer causation. J Chron Dis; 35:581-600.

Rosenkranz HS, 1987. Predicting carcinogenic potencies of environmental nitropyrenes. Environ Mutagenesis; 10:149-156.

Saito K, Mita S, Kamataki T, Kato R, 1984. DNA single-strand breaks by nitropyrenes and related compounds in Chinese hamster V79 cells. Cancer Lett; 24:121-127.

Scheutzle D, Lee FSC, Prater TJ, Tejada SB, 1981. The identification of polynuclear aromatic (PAH) derivatives in mutagenic fractions of diesel particulate extracts. Int J Environ Anal Chem; 9:93-144.

Scheutzle D, Riley TL, Prater TJ, Harey TM, Hunt DF, 1982. Analysis of nitrated polycyclic aromatic hydrocarbons in diesel particulates. Anal Chem; 54:265-271.

Shefner AM, Collins BR, Fisks A, Graff JL, Thompson CA, 1985. Project Summary: Respiratory Carcinogenesis of Diesel Fuel Emissions. Health Effects Research Laboratory, Environmental Protection Agency, NC.

Takayama S, Ishikawa T, Nakajima H, Sato S, 1985. Lung carcinoma induction in Syrian golden hamsters by intratracheal instillation of 1,6-dinitropyrene. Jpn J Cancer Res; 76:457-461.

Tokiwa H, Otofuji T, Horikawa K, Kitamori S, Otsuka H, Manabe Y, Kinouchi T, Ohnishi Y, 1984. 1,6-Dinitropyrene: Mutagenicity in *Salmonella* and carcinogenicity in BALB/c mice. JNCI; 73:1359-1363.

Tokiwa H, Otofuji T, Nakagawa R, Horikawa K, Maeda T, Sano N, Izumi K, Otsuka H, 1986. Dinitro derivatives of pyrene and fluoranthene in diesel emission particulates and their tumorigenicity in mice and rats. In: Ishinishi N, Koizumi A, McClellan RO, Stober W, eds. Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust. Amsterdam: Elsevier, pp. 253-270.

Vostal JJ, 1986. Factors limiting the evidence for chemical carcinogenicity of diesel emissions in long-term inhalation experiments. In: Ishinishi N, Koizumi A, McClellan RO, Stober W, eds. Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust. Amsterdam: Elsevier, pp. 381-396.

Weber WW, Mattano SS, Levy GN, 1987. Acetylation pharmacogenetics and aromatic-induced cancer. In: King CM, Romano LJ, Scheutzle D, eds. Carcinogenicity and Mutagenicity of Aromatic Amines and Nitroarenes. New York: Elsevier, pp. 115-124.

White H, Vostal JJ, Kaplan HL, MacKenzie WF, 1983. A long-term inhalation study evaluates the pulmonary effects of diesel emissions. J Appl Toxicol; 3:332.

Wogan GN, Gorelick NJ, 1985. Chemical and biochemical dosimetry of exposure to genotoxic chemicals. Environ Health Perspec; 62:5-18.

ABOUT THE AUTHOR

Charles M. King was awarded a Ph.D. in Biochemistry from the University of Minnesota in 1962. His thesis studies were carried out under Dr. Helmut R. Gutmann. He was an American Cancer Society Postdoctoral Fellow at the Antoni van Leeuwenhoek-Huis (Netherlands Cancer Institute) in Amsterdam, in the laboratory of Dr. Pieter Emmelot. After his return, he was in the Division of Cancer Research, Michael Reese Hospital and Medical Center in Chicago. From 1975 until 1977, he was an Expert of the National Cancer Institute at the National Center for Toxicological Research in Jefferson, Arkansas. Since 1977, he has been Chairman of the Department of Chemical Carcinogenesis, Michigan Cancer Foundation, Detroit. He is also an Adjunct Professor in the Department of Chemistry, Wayne State University. Dr. King's research interests are focused on the mechanisms of chemical carcinogenesis and mutagenesis.

LIST OF HEI PUBLICATIONS

Special Reports

Title	Publication Date
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	Supplement January 1988

Research Reports

Report No.	Title	Principal Investigator	Publication Date
1	Estimation of Risk of Glucose 6-Phosphate Dehydrogenase-Deficient Red Cells to Ozone and Nitrogen Dioxide	M. Amoruso	January 1985
2	Disposition and Metabolism of Free and Particle-Associated Nitropyrenes after Inhalation	J. Bond	January 1985
3	Transport of Macromolecules and Particles at Target Sites for Deposition of Air Pollutants	T. Crocker	January 1985
4	The Metabolic Activation and DNA Adducts of Dinitropyrenes	F.A. Beland	August 1986
5	An Investigation into the Effect of a Ceramic Particle Trap on the Chemical Mutagens in Diesel Exhaust	S.T. Bagley	January 1987
6	Effect of Nitrogen Dioxide, Ozone, and Peroxyacetyl Nitrate on Metabolic and Pulmonary Function	D.M. Drechsler-Parks	April 1987
7	DNA Adducts of Nitropyrene Detected by Specific Antibodies	J.D. Groopman	April 1987
8	Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung	J.L. Mauderly	May 1987
9	Biochemical and Metabolic Response to Nitrogen Dioxide-Induced Endothelial Injury	J.M. Patel	June 1987
10	Predictive Models for Deposition of Inhaled Diesel Exhaust Particles in Humans and Laboratory Species	C.P. Yu	July 1987
11	Effects of Ozone and Nitrogen Dioxide on Human Lung Proteinase Inhibitors	D.A. Johnson	August 1987
12	Neurotoxicity of Prenatal Carbon Monoxide Exposure	L.D. Fechter	September 1987
13	Effects of Nitrogen Dioxide on Alveolar Epithelial Barrier Properties	E.D. Crandall	October 1987
14	The Effects of Ozone and Nitrogen Dioxide on Lung Function in Healthy and Asthmatic Adolescents	J.Q. Koenig	January 1988
15	Susceptibility to Virus Infection with Exposure to Nitrogen Dioxide	T.J. Kulle	January 1988

Copies of these reports can be obtained by writing to the Health Effects Institute, 215 First Street, Cambridge, MA 02142

The Health Effects Institute (HEI) is an independent nonprofit corporation which, according to its charter, is "organized and operated... specifically to conduct or support the conduct of, and to evaluate, research and testing relating to, the health effects of emissions from motor vehicles." It is organized in the following ways to pursue this purpose:

INDEPENDENCE IN GOVERNANCE

HEI is governed by a four-member Board of Directors whose members are William O. Baker, Chairman Emeritus of Bell Laboratories and Chairman of the Board of Rockefeller University; Archibald Cox, Carl M. Loeb University Professor (Emeritus) at Harvard University; Donald Kennedy, President of Stanford University; and Charles Powers, Partner, Resources for Responsible Management. Archibald Cox chairs the Board. These individuals, who select their own successors, were chosen initially, after consultation with industry and other individuals, by then Environmental Protection Agency Administrator Douglas M. Costle.

TWO-SECTOR FINANCIAL SUPPORT

The Institute receives half of its funds from the United States government through the Environmental Protection Agency and half from the automotive industry. Twenty-seven leading manufacturers of vehicles or engines that are certified for use on U.S. highways contribute to the Institute's budget, in shares proportionate to the number of vehicles or engines that they sell.

RESEARCH PLANNING AND PROJECT EVALUATION

HEI is structured to define, select, support, and review research that is aimed at investigating the possible health effects of mobile source emissions. Its research program is developed by the Health Research Committee, a multi-disciplinary group of scientists knowledgeable about the complex problems involved in determining the health effects of mobile source emissions. The Committee seeks advice from HEI's sponsors and from other sources prior to independently determining the research priorities of the Institute.

After the Health Research Committee has defined an area of inquiry, the Institute announces to the scientific community that research proposals are being solicited on a specific topic. Applications are reviewed first for scientific quality by an appropriate expert panel. Then they are reviewed by the Health Research Committee both for quality and for relevance to the mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, which also reviews the procedures, independence, and quality of the selection process.

When a study is completed, a draft final report is reviewed by a separate HEI committee, the Health Review Committee. Members are also expert scientists representing a broad range of experience in environmental health sciences. The Health Review Committee has no role either in the review of applications or in the selection of projects and investigators for funding. This Committee assesses the scientific quality of each study and evaluates its contribution to unresolved scientific questions.

Each funded proposal is assigned in advance of completion to a member of the Health Review Committee, who acts as "primary reviewer." When the draft report is received, the primary reviewer directs a peer review by outside scientists and, when appropriate, a biostatistician. After the investigator has had a chance to comment on the technical evaluations, the primary reviewer drafts a final report review. This document is sent to the investigator for comment. It is subsequently examined by the full Health Review Committee and revised as necessary. The investigator's final report, as well as the Review Committee's report, are then made available to the sponsors and to the public after evaluation by the HEI Board of Directors.

All HEI investigators are urged to publish the results of their work in the peer-reviewed literature. The timing and nature of HEI report releases are tailored to ensure that the Health Review Committee's report does not interfere with the journal publication process. The reports of the Health Review Committee are as thorough as necessary to evaluate any individual report.

HEI HEALTH EFFECTS INSTITUTE 215 First Street, Cambridge, Massachusetts 02142 (617) 491-2926

Research Report Number 16 February, 1988