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Evaluation of the Potential Health Effects of the Atmospheric Reaction Products of Polycyclic Aromatic Hydrocarbons

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

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HEI Statement

Synopsis of Research Report Number 84

Genotoxicity of Polycyclic Aromatic Hydrocarbons and Their Atmospheric Reaction Products

BACKGROUND

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants that are formed from the incomplete combustion of fossil fuels. These compounds can react with other chemicals in the atmosphere to produce oxy- and nitro-PAHs. Many of the atmospheric PAHs, as well as their chemical derivatives, are genotoxic—that is, they can damage DNA. Therefore, the presence of these atmospheric transformation products may pose a human health risk.

Because of the need to understand better the potential for PAHs and their atmospheric reaction products to damage DNA, HEI funded this study to evaluate the genotoxicity of select PAHs found in combustion emissions.

APPROACH

Dr. Arey and colleagues of the University of California, Riverside, examined the genotoxic potential of two PAHs (naphthalene and phenanthrene) that are common air pollutants, and a subset of their atmospheric transformation products. The investigators evaluated the genotoxicity of these compounds using a variety of human cell lines with a range of metabolic capabilities. They examined the ability of these compounds to produce small-scale (damage to genes) and large-scale (damage to chromosomes) genetic damage.

RESULTS AND IMPLICATIONS

The investigators' results indicate that some transformation products of common atmospheric PAHs are more genotoxic in mammalian cell lines than their parent compounds. In addition, some compounds produced chromosomal damage without damaging individual genes. The results also indicated that enzymatic transformation of some compounds was necessary to induce genotoxicity in the mammalian cell lines tested. The genotoxicity of these atmospheric transformation products in human cells, together with their high concentrations in ambient air, validates the underlying premise of this work: that atmospheric reaction products of PAHs should be considered in genotoxicity assessments of air pollutants. Because the findings of this study are based on in vitro testing only, the ability to extrapolate the results to human exposures and the subsequent risk to human health is limited. The results are, however, very useful in identifying the most significant PAHs to study in future in vivo studies.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1994 to 1997. This study was conducted by Drs. Andrew J. Grosovsky, Janet Arey, David A. Eastmond, Karyn K. Parks, and Roger Atkinson at the University of California, Riverside, and Jennifer C. Sasaki at Lawrence Livermore National Laboratory. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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ABSTRACT

The genotoxic risks from exposure to polycylic aromatic hydrocarbons (PAHs)* have long been recognized. Less well understood are the potential genotoxic risks of the atmospheric reaction products of this class of compounds. In this investigation, we have utilized several human cell assays to evaluate the genotoxicity of naphthalene, phenanthrene, and their atmospheric reaction products 1-nitronaphthalene, 2-nitronaphthalene (2NN), 1-hydroxy-2NN, 2-hydroxy-1-nitronaphthalene, 1,4-naphthoquinone, and 2-nitrodibenzopyranone (2NDBP). In addition, simulated atmospheric reaction products of naphthalene were generated in a 6,700 liter (L) Teflon environmental chamber, collected on a solid adsorbent, extracted, and fractionated by normal-phase high-performance liquid chromatography (HPLC). Individual fractions were then analyzed using gas chromatography/mass spectrometry (GC/MS), and tested for genotoxic effects. Genotoxicity was primarily determined using the human B-lymphoblastoid cell line, MCL-5, which expresses several transfected P450 and epoxide hydrolase genes. Mutagenicity was evaluated at both the heterozygous thymidine kinase (*tk*) locus and the hemizygous hypoxanthine phosphoribosyl transferase (*hprt*) locus, permitting detection of both intragenic and chromosomal scale mutational events. Test compounds were also screened using the CREST modified micronucleus assay. The results indicate that 2NN and 2NDBP possess greater mutagenic potency than their parent compounds, and, interestingly, both compounds induced significant increases in mutation frequency at the *tk* but not the *hprt* locus. These findings suggest a mechanistic difference in human cell response to 2NN and 2NDBP as compared to bacteria, where both compounds were previously shown to induce point mutations in the *Salmonella typhimurium* reversion assay. The genotoxicity of 2NN and 2NDBP in human cells, together with their high concentrations in ambient air relative to nitro-PAHs directly emitted from combustion sources, emphasizes the need to consider atmospheric reaction products of PAHs in assessments of the genotoxicity of air pollutants.

We also investigated whether transfected cytochrome P450 monooxygenase activities were required to activate 2NN and 2NDBP to genotoxic species, and whether a single enzyme could be sufficient for metabolic activation. Three directly related cell lines with multiple (MCL-5), single (AHH-1 1A1), or no (L3) transfected cytochrome P450 genes were used. AHH-1 is additionally distinguished by elevated mutagenic response at the *tk* locus, a heterozygous mutation in p53, and apoptosis capacity. The effect of these metabolic and genetic differences on genotoxicity of 2NN, 2NDBP, and β-naphthylamine (βNA) was also investigated. The results indicated that 2NN and 2NDBP were not activated to genotoxic species through nitroreduction pathways. Mutagenicity induced at the *tk* locus was dependent on oxidative metabolism, provided by transfected cytochrome P450 enzymes in MCL-5 and AHH-1 1A1. Mutagenicity was not observed in the L3 cell line, which does not carry transfected cytochrome P450 activities. The negative response of βNA in all cell lines indicates that, contrary to previous hypotheses, 2NN and βNA are not activated by similar metabolic pathways in these human cell lines. Taken as a whole, these results suggest that the genotoxicity of nitro-PAHs in human cells requires oxidative metabolism.

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

This Investigators' Report is one part of Health Effects Institute Research Report Number 84, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project for which Dr. Janet Arey was the principal investigator. Correspondence concerning the Investigators' Report may be addressed to Dr. Andrew Grosovsky, University of California, Riverside, Environmental Toxicology, 5419 Boyce Hall, Riverside, CA 92521.

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INTRODUCTION

Polycyclic aromatic hydrocarbons are atmospheric pollutants formed as a result of natural and anthropogenic combustion processes (Nickolaou et al. 1984). Polycylic aromatic hydrocarbons with two to four rings are found at least partially in the vapor phase under ambient atmospheric

Table 1. Products Observed and Their Formation Yields from Gas-Phase OH Radical– and NO_3 Radical–Initiated Reactions of Naphthalene[a]

	% Yield from Reaction with	
Product	OH Radical	NO_3 Radical
2-Formylbenzaldehyde[b]	~ 2.7	
Phthalic anhydride[b]	~ 3	
1,4-Naphthoquinone	1.0	1.9
Compound of molecular weight 160	1.4	
2-Formylcinnamaldehyde (*cis* + *trans*)	35	
Compound of molecular weight 160	1.5	
1-Naphthol	2.9	
2-Naphthol	3.8	Observed
1-Nitronaphthalene	1.2	24.4
2-Nitronaphthalene	1.3	11.0
1-Hydroxy-2-nitronaphthalene	1.1	1.5
2-Hydroxy-1-nitronaphthalene	Observed	1.2
Compound of molecular weight 174[c]	~ 5	
Compound of molecular weight 176	13	
Total	~ 67	≥ 40[d]

[a] From Sasaki et al. 1997.

[b] Expected to be secondary product from reaction of 2-formylcinnamaldehyde with the OH radical; therefore not included in total yield.

[c] Tentatively identified as 2,3-epoxy-1,4-naphthoquinone.

[d] Because of the expected high reactivity of 2-naphthol toward NO_3 radicals, significant amounts of the reaction products could have proceeded via the initial formation of 2-naphthol.

conditions, and can undergo relatively rapid gas-phase atmospheric transformation reactions. For example, the daytime reaction of these PAHs with hydroxyl (OH) radical results in PAH lifetimes of generally less than one day (Atkinson and Arey 1994), and both daytime reaction with OH radicals and nighttime reaction with nitrate (NO_3) radicals lead to nitro-PAH products (Arey et al. 1986, 1989a,b, 1990; Zielinska et al. 1989a,b; Atkinson et al. 1990; Atkinson and Arey 1994; Gupta et al. 1996; Ciccioli et al. 1995, 1996). Because these atmospherically formed nitro-PAHs are often found at higher concentrations than the nitro-PAH isomers directly emitted from combustion sources (Arey et al. 1989b; Ciccioli et al. 1995, 1996; Gupta et al. 1996), it is apparent that the toxic effects of atmospheric transformation products derived from the parent PAH compounds need to be an integral component of any comprehensive evaluation of the health effects of atmospheric PAHs.

Bioassay-directed fractionation and chemical analysis has been used successfully to identify mutagenic components in complex mixtures (Schuetzle et al. 1981; Schuetzle and Lewtas 1986) and mutagenic atmospheric reaction products produced in simulated atmospheric reactions in the laboratory (Arey et al. 1992; Sasaki et al. 1995). High-performance liquid chromatography (HPLC) fractionation of gas- and particle-phase ambient air extracts followed by bioassay with a modified Ames assay (Kado et al. 1983), together with GC/MS analyses of mutagenic fractions, has identified two distinct compound classes—the nitro-PAHs and the nitro-PAH lactones—as major bacterial mutagens formed from atmospheric transformation of PAHs (Arey et al. 1992; Helmig et al. 1992a,b; Sasaki et al. 1995).

Nitro-PAHs appear to be dominant contributors to gas-phase ambient mutagenicity (Gupta et al. 1996), whereas the nitro-PAH lactones, due to their lower vapor pressure and their predominance in the particle phase, are major contributors to ambient air particle phase mutagenicity (Helmig et al. 1992a,b; Sasaki et al. 1995). For example, in vapor-phase ambient samples collected in Redlands, CA, nitronaphthalenes and methylnitronaphthalenes—products of the atmospheric photooxidations of naphthalene and the methylnaphthalenes—accounted for 18% of the total daytime and 32% of the total nighttime vapor-phase mutagenicities (Gupta et al. 1996) using the preincubation microsuspension modified Ames assay (Kado et al. 1983). Furthermore, in one ambient Riverside, CA, sample, the nitro-PAH lactone, 2NDBP, an atmospheric transformation

product of phenanthrene, accounted for approximately 20% of the total activity of the ambient particle extract in the microsuspension assay (Helmig et al. 1992a,b).

Naphthalene and phenanthrene were selected for evaluation of genotoxicity because they are two of the most abundant PAHs observed in ambient atmospheres (Arey et al. 1989b), and product studies of their OH radical– and NO_3 radical–initiated reactions have previously been carried out (Atkinson et al. 1987; Arey et al. 1989a; Atkinson et al. 1990; Helmig et al. 1992a,b; Bunce and Zhu 1994; Kwok et al. 1994; Lane and Tang 1994; Sasaki et al. 1995). In particular, two atmospheric transformation products of naphthalene and phenanthrene, 2NN and 2NDBP, have been shown to be highly mutagenic in the microsuspension preincubation modified Ames assay (Arey et al. 1992; Helmig et al. 1992a; Watanabe et al. 1996).

Ongoing efforts to characterize fully the atmospheric reaction products of naphthalene have now resulted in the identification of ~70% of the reaction products of the OH radical–initiated reaction of naphthalene and ~40% of the NO_3 radical–initiated reaction products (Sasaki et al. 1997). The products identified and their yields are given in Table 1. Proposed reactions leading to these products are shown in Figures 1 and 2 for the OH radical– and NO_3 radical–initiated reactions, respectively. Because naphthalene is the most abundant PAH in ambient atmospheres (Arey et al. 1989b; Atkinson et al. 1988), nitronaphthalenes are expected to be the most abundant nitro-PAH, especially under conditions when NO_3 radical–initiated chemistry has occurred (Gupta et al. 1996). For example, in one nighttime sample collected in Glendora, CA, in August 1986, the ambient concentrations of nitro-PAH formed from atmospheric processes were 1-nitronaphthalene, 5.7 ng/m^3; 2NN, 3.1 ng/m^3; and 2-nitrofluoranthene, 2.0 ng/m^3. 1-Nitropyrene, the nitro-PAH generally most abundant in diesel exhaust emissions, was 0.015 ng/m^3; benzo[*a*]pyrene was 0.32 ng/m^3 (Atkinson et al. 1988).

The majority of the atmospheric reaction products of phenanthrene are presently unknown. Although 2NDBP has been identified as a relatively minor product of phenanthrene's atmospheric reaction, ambient concentrations of 2NDBP comparable with those of 2-nitrofluoranthene, and thus greater than that of 1-nitropyrene, have been reported (Helmig et al. 1992b).

The expected pathway for metabolic activation of 2NN involves nitroreduction to form the corresponding N-hydroxyamino metabolite and derived species that can bind to DNA, forming covalent adducts (Fu 1990). Although detection of this reactive intermediate has not been reported, βNA has been detected in in vivo mammalian studies (Johnson and Cornish 1978). Anaerobic incubation of 2NN with liver postmitochondrial supernatants and cy-

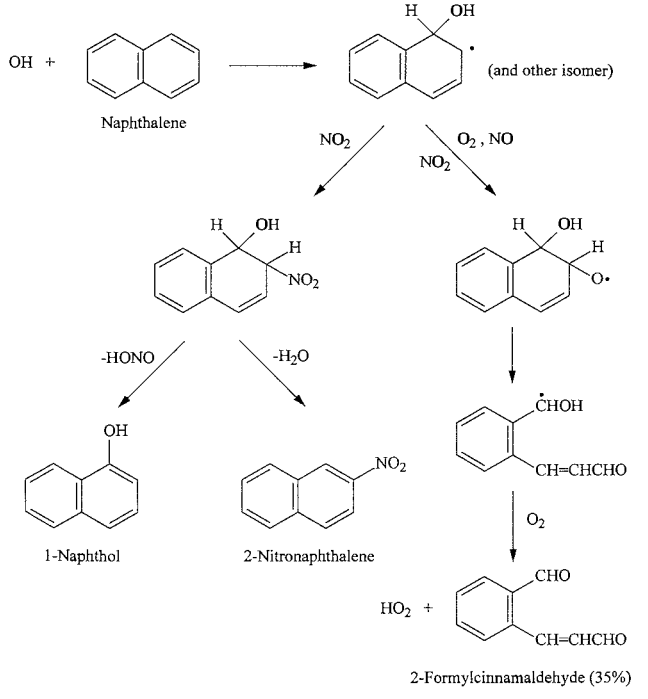

Figure 1. Postulated reaction mechanism for gas-phase hydroxyl (OH) radical–initiated reaction of naphthalene.

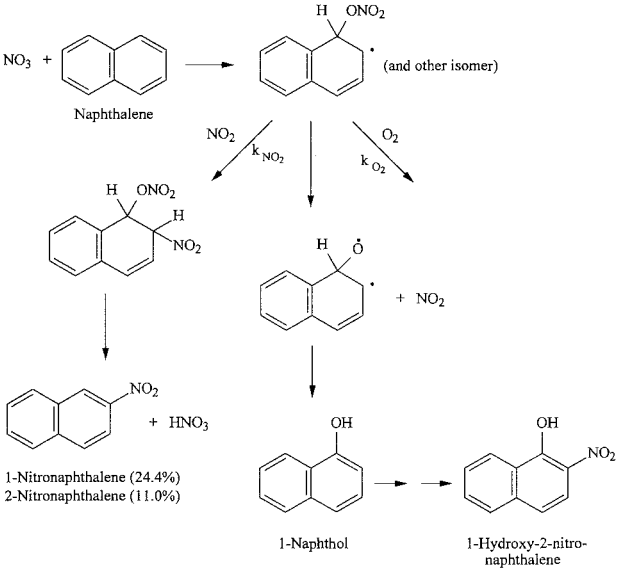

Figure 2. Postulated reaction mechanism for gas-phase nitrate (NO_3) radical–initiated reaction of naphthalene.

tosol also resulted in the conversion of 2NN to βNA (Poirier and Weisburger 1974). β-Naphthylamine has been shown to cause bladder cancer in monkeys and dogs (Conzelman and Moulton 1972; Conzelman et al. 1970), and has been clearly associated with an increased risk for bladder cancer in humans (Garner et al. 1984). Because both βNA and 2NN may be converted to the N-hydroxyamino species, 2NN has also been regarded as a potential mutagenic and carcinogenic agent (Hecht and El-Bayoumy 1990). It is unknown whether the nitro-group of 2NDBP would also be metabolized.

The human B-lymphoblastoid cell line MCL-5 has facilitated the investigation of the mutagenicity of PAHs (Busby et al. 1995) and nitro-PAH compounds (Busby et al. 1994, 1997) since the cell line has been transfected with two plasmids encoding multiple P450 and epoxide hydrolase genes. The cell line also constitutively expresses endogenous cytochrome P450 1A1 (CYP 1A1) (Crespi et al. 1991). Therefore, exogenous metabolic activation of PAHs is not required. Related cell lines, derived from AHH-1, are also available. These would be expected to have comparable nitroreductase activities, but provide only one transfected cytochrome P450 enzyme. One of the AHH-1 derivative cell lines, L3, the parent cell line of MCL-5, was selected by exposure to benzo[*ghi*]perylene for elevated levels of CYP 1A1 activity, but contains no transfected enzymatic activities (Crespi et al. 1991, 1993). These cell lines provide a variety of metabolic environments for investigating the genotoxicity of nitroarene compounds.

There are inherent limitations in utilizing a bacterial reversion assay, such as the Ames assay, in the evaluation of human mutagenic and/or carcinogenic potential. The Ames assay detects only agents that induce specific point mutations, such as frameshifts or base substitutions; larger scale chromosomal events are not detectable. Because many fundamental differences exist between prokaryotic and eukaryotic systems, the use of a human cell line rather than bacteria may better predict the genotoxic activity of a particular compound in humans. Two selectable markers, *hprt* and *tk*, are available in MCL-5 and AHH-1 cells, permitting a wide range of mutational events to be detected. The *hprt* and *tk* loci are distinguished in a number of important characteristics including size, zygosity, chromosomal context, and G:C content, all of which could potentially influence the recovered spectrum of mutations (Giver et al. 1995).

Assay of mutagenicity using *hprt* as a marker gene is simpler and more widely available than using *tk*, since *hprt* is X-linked and physically or functionally hemizygous. Many pathways for mutagenesis are unavailable at hemizygous loci, however, and the heterozygous *tk* locus in MCL-5 provides the opportunity to assay these other mechanisms of mutagenesis. In particular, large deletions of many megabases in length are recoverable at *tk* (Dobo et al. 1995; Grosovsky et al. 1995), and homologous interactions may result in a recombinationally mediated loss of heterozygosity (LOH) of the marker locus and surrounding sequences. Mutational spectra at *tk* (Dobo et al. 1995; Giver et al. 1995) often include a large component of mutants attributable to multi-locus LOH. In addition to quantification of mutation frequency at the *hprt* and *tk* loci, we also performed the CREST micronucleus assay to evaluate cytogenetic effects (Eastmond and Tucker 1989). Thus, in conjunction with the mutational studies at *hprt* and *tk*, a wide range of genotoxic events ranging from point mutations to chromosome loss can be rapidly identified and quantified in human cells.

Besides enzymatic differences, MCL-5 and AHH-1 cells also differ in mutagenic response at the *tk* locus, p53 status, and apoptotic capacity. AHH-1 carries a heterozygous mutation in p53 that is associated with diminished and/or reduced cellular apoptotic response to DNA damage (Morris et al. 1996; Dobo et al. 1997). Alteration in cellular apoptotic capacity is associated with elevation in tk^- mutants, which is specifically attributable to increased recovery of multi-locus LOH events but not with an increased frequency of $hprt^-$ mutants (Xia et al. 1995; Dobo et al. 1997). To provide additional insights into the mechanisms underlying the mutagenesis induced by 2NN and 2NDBP, we investigated the role of oxidative metabolism in the bioactivation of the two compounds by comparing their genotoxic effects in three cell lines of various metabolic capacities.

SPECIFIC AIMS

The overall objective was to address an important aspect of the health effects of motor vehicle emissions that had not been previously evaluated, specifically the genotoxic effects of the selected atmospheric reaction products of selected PAHs emitted in vehicle exhaust. This evaluation was made using techniques that assayed chromosomal as well as point mutational mechanisms of genotoxicity.

Specific objectives were:

1. To screen naphthalene and phenanthrene, two PAHs important in vehicle exhaust, and an extract of their gas-phase atmospheric reaction products, as produced in and collected from our environmental chambers. Genotoxic assessment included determining mutagenicity at two gene loci and chromosomal alterations in human cells.
2. To fractionate the simulated reaction products of naphthalene, characterize the activity of the fractions, and attempt to identify chemically the compounds respon-

sible for any mutagenic activity observed in the human cell line.

3. To compare the mutagenic activity of selected fractions or identified products at the *hprt* and *tk* loci of two human lymphoblastoid cell lines, AHH-1 and the derivative line MCL-5, both possessing endogenous means of metabolic activation.

METHODS AND STUDY DESIGN

TEST CHEMICALS

In addition to naphthalene and phenanthrene, six previously identified and commercially available atmospheric transformation products of these two PAHs were included in our compound survey: 1-nitronaphthalene (99% stated purity), 2NN (98% stated purity), 1-hydroxy-2NN (95% stated purity), 2-hydroxy-1-nitronaphthalene (purity not stated), 1,4-naphthoquinone (97% stated purity), and 2NDBP (98% stated purity). All chemicals were purchased from Aldrich Chemical Company (Milwaukee, WI) with the exception of 1-hydroxy-2NN, which was purchased from TCI America (Portland, OR) and used without further purification.

For experiments in the AHH-1 and L3 cell lines, 2NN was purified by open column silica chromatography to remove dinitronaphthalene impurities. Separate experiments were conducted in which MCL-5 cells were exposed to low concentrations of dinitronaphthalene in order to evaluate the potential contribution of dinitronaphthalene impurities in the genotoxicity of 2NN. These experiments demonstrated that the genotoxicity of 2NN could not be accounted for by contaminating impurities.

GENERATION, COLLECTION, EXTRACTION, AND HPLC FRACTIONATION OF ATMOSPHERIC CHAMBER REACTION PRODUCTS OF NAPHTHALENE

The techniques to generate, collect, extract, and HPLC fractionate photooxidation products of PAHs have been described in detail elsewhere (Sasaki et al. 1995). Briefly, photooxidation products of naphthalene were produced in a 6,700 L Teflon chamber equipped with two parallel banks of blacklamps for irradiation, a Teflon-coated fan for rapid mixing of reactants, and Pyrex sampling ports for introducing reactants and collecting gas samples for analysis. Naphthalene was introduced into the chamber at parts-per-million by volume concentrations by passing a stream of nitrogen gas through a Pyrex tube packed with solid naphthalene. The photooxidation involved generating OH radicals, NO3 radicals, and ozone (O_3) in the chamber. The OH radicals were produced by photolysis of methyl nitrite in the presence of NO, with the concentrations of reactants and irradiation times allowing for subsequent production of O_3 and NO_3 (Arey et al. 1992; Sasaki et al. 1995). After the 10-minute photolysis time, chosen to be long enough to generate O_3 and hence NO_3 radicals, large volume samples (~6,500 L) were collected on polyurethane foam plugs, with subsequent Soxhlet extraction with dichloromethane (Fisher, Pittsburgh, PA). The extract was then fractionated by normal-phase HPLC (on a semi-preparative Regis Spherisorb SSW silica column) into nine 9-minute fractions of increasing polarity. Each fraction was diluted with a known amount of dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) until total solubility of the fraction was obtained. Serial dilutions of the fractions were made to obtain a range of fraction doses for mutagenicity testing.

DERIVATION OF CELL LINES

Three human B-lymphoblastoid cell lines—AHH-1 variant h1A1v2 (referred to as AHH-1 1A1), L3, and MCL-5—were obtained from GENTEST Corporation (Woburn, MA). AHH-1 is a clonal isolate derived from RPMI 1788 cells, selected for sensitivity to benzo[*a*]pyrene (B[*a*]P)(Crespi and Thilly 1984); and the 1A1 derivative has been further transfected to express constitutive CYP 1A1 activity (Penman et al. 1994). The L3 variant (Davies et al. 1989) of the AHH-1 cell line was selected in benzo[*ghi*]perylene for elevated levels of endogenous cytochrome P450 1A1 activity and has a lower spontaneous mutant fraction at the *tk* locus. MCL-5 is a metabolically competent human lymphoblastoid cell line derived from L3, which expresses epoxide hydrolase and four cytochrome P450 genes. Expression of microsomal epoxide hydrolase, CYP 1A2, and CYP 2A6 were obtained by incorporating the cDNA for these enzymes into the pME23 vector (Crespi et al. 1991). Likewise, the cDNA for CYP 2E1 and CYP 3A4 have been transfected via incorporation into the pH441 vector. Both vectors remain episomal, and MCL-5 cultures are routinely grown in media containing 200 μg/mL hygromycin B and 2 mM 1-histidinol to ensure the retention of the plasmids in cell populations.

MAINTENANCE AND GROWTH OF CULTURES

Human B-lymphoblastoid cell lines were maintained as recently described in Dobo and colleagues (1995). Cultures were maintained in GENTEST RPMI 1640 media (GENTEST, Woburn, MA, without histidine and with 2 mM histidinol, henceforth referred to as GENTEST media) supplemented with 9% horse serum (PAA Laboratories, Newport Beach, CA) that was not heat-inactivated. L-Glutamine, 100 μg/mL penicillin G, and 100 μg/mL streptomycin sulfate were also added to the media. Cells grew to a density of

approximately 10^6 cells/mL and were subcultured to 2×10^5 cells/mL every other day and supplemented with 200 μg/mL of hygromycin B. The following were used in cell culture: RPMI 1640 (Cellgro, Mediatech, Herndon, VA); RPMI 1640 powdered media without histidine and supplemented with 2 mM histidinol (GENTEST), prepared as recommended by the manufacturer; horse serum (PAA Laboratories); penicillin-streptomycin and L-glutamine (Cellgro, Mediatech); and hygromycin B (Calbiochem, San Diego, CA).

Prior to dosing the cells with the chemicals of interest, the cells were grown for 3 days in GENTEST media containing all three of the following compounds: 2×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin, and 3.5×10^{-5} M thymidine (HAT) to reduce the number of pre-existing mutants within the cell population. The cells were centrifuged out of the HAT media after day 3 and resuspended in media containing hypoxanthine and thymidine. Exposure of cells to test compounds was carried out after an additional 3 or 4 days.

SELECTION OF DOSES FOR TESTING

Cytotoxicity was estimated for each chemical selected for testing by analysis of cell growth after chemical exposure. Cell density was determined each day using a Coulter Counter (Coulter Electronics, Hialeah, FL), in order to maintain density in the range (4 to 10) $\times 10^5$ cells/mL and to determine the number of daily population doublings. The number of population doublings for each day was summed to obtain a cumulative population doubling (CPD). After one week of measurements, the CPD for each flask of dosed cells was divided by the CPD of a corresponding negative control culture to obtain an estimate of toxicity. Ideally, we intended to dose with each chemical at the 50% to 80% toxicity level. However, the maximum dose achievable was limited by the compound solubility, and for most of the compounds examined the maximum dose resulted in < 50% toxicity.

CHEMICAL EXPOSURE AND CLONOGENIC DETERMINATION OF CYTOTOXICITY

Dosing solutions of each compound were made up in DMSO (Sigma) at 1,000 × the final desired concentration of the compound in the cell culture. Approximately 2×10^7 cells were centrifuged out of the maintenance medium and into 40 mL of RPMI 1640 medium (Cellgro, Mediatech) containing histidine without serum. Compound exposure was performed in sealed glass vials with minimal (< 2 cc) headspace. A 120 μL aliquot of DMSO was added to each cell culture prior to addition of 40 μL aliquots containing the desired concentration of compound. Benzo[*a*]pyrene (2×10^{-4} μmol/mL) was used as a positive control, and DMSO was used as a negative control. The cultures were exposed for 10 hours at 37°C. Exposed cells were centrifuged at the completion of the exposure period and re-suspended in fresh serum-containing medium. Generally the treated cells were split into separate cultures for the mutation, cell survival, and micronucleus assays. Immediately after the dosing period, survival plates were prepared at cell densities of 10 to 50 cells/mL and scored for clonogenic survival after 14 days.

NAPHTHALENE PHOTOOXIDATION PRODUCT DOSING AND CLONOGENIC DETERMINATION OF CYTOTOXICITY

The procedure chosen for dose selection of naphthalene photooxidation products ensured that each HPLC fraction could be tested up to the solubility limit; maximum DMSO concentration in cell cultures was 1%. The total residue from each fraction was thus dissolved in a minimal volume of DMSO and serial dilutions were made of this original stock solution. However, for some HPLC fractions, toxicity rather than solubility limited the maximum dose tested. Exposed cells were centrifuged at the completion of the exposure period and re-suspended in fresh serum-containing medium. Generally the treated cells were split into separate cultures for the mutation, cell survival, and micronucleus assays. Immediately after the dosing period, survival plates were prepared at cell densities of 10 to 50 cells/mL and scored for clonogenic survival after 14 days.

SELECTION OF *tk*$^-$ AND *hprt*$^-$ MUTANTS

The cells were allowed a phenotypic expression period of five days prior to selection of *tk*$^-$ mutants. Selection was performed by distributing cells at a density of 2×10^4 cells/well in 96 well plates (4 plates/culture) in media containing 4 μg/mL trifluorothymidine and 9% heat-inactivated horse serum. Cloning efficiency (2 cells/well) was determined in nonselective medium at the same time that mutant selection was performed. Plates were scored for colonies 14 days after seeding. The remainder of the cells from each culture were carried to day 7 of the expression period and plated for mutation frequency at the *hprt* locus using identical techniques and media containing 0.6 μg/mL 6-thioguanine and 9% horse serum (not heat-inactivated). Cloning efficiency plates were also prepared in parallel with the selection of *hprt*$^-$ mutants. The mean cloning efficiency in untreated MCL-5 cultures was 0.26, and typical cloning efficiencies at the time of mutant selection in cultures treated with test compounds were similar, ranging from 0.16 to 0.36. Plates were scored by identifying and counting wells with colonies under a low-power microscope.

For each culture, the number of positive wells was pooled and mutation frequencies were calculated using the P_0 method (Luria and Delbruck 1943).

PREPARATION OF CELLS FOR THE CREST MICRONUCLEUS ASSAY

In the CREST micronucleus assay, chromosome loss and breakage events are distinguishable by staining with an antibody that recognizes proteins at the centromere-associated kinetochores, thereby detecting chromosomal breakage and chromosomal loss that may not otherwise be observed at either mutational marker (Eastmond and Tucker 1989). Shortly following treatment with the test compound or HPLC fraction, cytochalasin B (3 μg/mL) was added to the media of the cultures reserved for micronucleus assay. The MCL-5 cells were then cultured for an additional 36 hours and harvested by cytocentrifugation. After fixation in methanol, slides were stored in a nitrogen atmosphere at –20°C until use. The CREST staining, microscopy, and scoring procedures were performed as previously described (Eastmond and Tucker 1989; Krishna et al. 1992).

STATISTICAL METHODS AND DATA ANALYSIS

Dose-related increases in the induction of total, kinetochore-positive, and kinetochore-negative micronuclei were tested for significance using the Cochran-Armitage test for trend in binomial proportions (Margolin and Risko 1988). Following the observation of a significant trend in the Cochran-Armitage test, a one-tailed Fisher's exact test or chi-squared test was used as a post hoc test to determine the significance of each test concentration.

The analysis of the mutagenesis data was performed using a variety of statistical tests. Comparisons between control and a single test concentration were performed using a one-tailed Student's *t*-test. Dose-related increases in mutation frequency were performed using linear regression. Following the observation of a significant dose-related increase in mutation frequencies, a one-tailed *t*-test was used as a post hoc test to determine the significance at each concentration. For comparison of 3 or more groups, analysis of variance was used followed by the Fisher's least significant difference test to identify individual differences. Critical values for all tests were determined using a 0.05 probability of Type I error.

RESULTS

EVALUATION OF EXPOSURE CONDITIONS AND PHENOTYPIC EXPRESSION PERIOD FOR GENOTOXICITY ASSAYS

Protocols for exposure of MCL-5 cells to test chemicals have been developed for nonvolatile PAHs such as B[*a*]P. These have been used for genotoxicity evaluation of B[*a*]P and dibenzopyrenes (Crespi et al. 1991, 1993; Busby et al. 1995). Because of the anticipated volatility of the 2- and 3-ring PAHs, which were studied here, an exposure protocol was developed that would minimize sample volatilization but not diminish cellular response to mutagenic agents. Benzo[*a*]pyrene was used as a positive control to evaluate the influence of modified treatment parameters on genotoxic response. Published protocols for exposure of MCL-5 cells to test chemicals (Table 2, Row 3) involved a 28-hour

Table 2. Parameters Influencing Determination of Induced Mutation Frequency[a] per 10^6 Viable Cells

Treatment[b]	Conditions	*tk*⁻ Mutants 3-Day Expression Period	*tk*⁻ Mutants 5-Day Expression Period	*hprt*⁻ Mutants 7-Day Expression Period	Clonogenic Survival
Negative control	10-hr treatment Serum-free medium Minimal headspace	6.70 ± 2.04	1.27	7.34	1.00
Benzo[*a*]pyrene	10-hr treatment Serum-free medium Minimal headspace	73.4 ± 43.2	408 ± 26	260 ± 33	0.94 ± 0.13
Benzo[*a*]pyrene	28-hr treatment Serum-containing medium Large-volume headspace	35[c]	Not determined	80[d]	~ 0.90[c,d]

[a] Induced mutation frequency is defined here as the overall mutation frequency minus the background frequency.

[b] The concentration of benzo[*a*]pyrene used in these experiments was 50 ng/mL.

[c] Data from Busby et al. 1995.

[d] Data from Crespi et al. 1991.

Table 3. Mutation Frequency Induced in MCL-5 Cells Treated with PAHs and Selected Atmospheric Reaction Products

Compound	Concentration[a] (μmol/mL)	n[b]	Clonogenic Survival (± SEM)	tk^- Mutation Frequency (per 10^6 viable cells)[c]	$hprt^-$ Mutation Frequency (per 10^6 viable cells)[d]
Negative control	—	5	1.00	5.23 ± 1.32	4.91 ± 0.74
Benzo[*a*]pyrene	2.0×10^{-4}	5	1.00 ± 0.26	411 ± 67*	304 ± 30*
Naphthalene	0.31	2	0.38 ± 0.02	6.28 ± 3.50	8.71 ± 3.10
1-Nitronaphthalene	0.23	2	1.17	4.36 ± 0.56	3.20 ± 1.76
2-Nitronaphthalene[e]	0.46	3	0.13	88.7 ± 18.3*	4.53 ± 1.61
1-Hydroxy-2-nitronaphthalene	0.53	3	0.74 ± 0.02	3.97 ± 0.90	3.76 ± 1.73
2-Hydroxy-1-nitronaphthalene	0.53	3	0.86 ± 0.12	2.38 ± 0.35	4.88 ± 1.99
1,4-Naphthoquinone	6.3×10^{-4}	2	0.66 ± 0.20	4.99 ± 0.60	10.3 ± 4.1
Phenanthrene	0.06	2	0.08 ± 0.01	5.78 ± 1.73	1.52 ± 0.01
2-Nitrodibenzopyranone	4.1×10^{-3}	4	0.85 ± 0.14	11.3 ± 3.2*	2.95 ± 1.13

[a] The reported value is the highest concentration of compound tested. These concentrations were determined by either solubility or survival limitations.

[b] Number of observations.

[c] Compounds that induced a significant increase in mutation frequency at *tk* were benzo[*a*]pyrene ($p < 0.0001$) and 2-nitronaphthalene ($p < 0.001$). Induction of tk^- mutants by 2-nitrodibenzopyranone ($p = 0.048$) was weakly significant. Significance was calculated by Student's *t*-test.

[d] A significant increase in mutation frequency at *hprt* was induced by benzo[*a*]pyrene ($p < 0.0001$). A weak increase in mutation frequency at *hprt* was also induced by 1,4-naphthoquinone ($p = 0.04$).

[e] Significant inductions in mutation frequency at *tk* were also observed at 0.23 ($p = 0.001$) and 0.35 ($p < 0.001$) μmol/mL (see Figure 7).

* Significant increase in mutation frequency.

exposure period of cells growing in medium containing 9% horse serum in standard tissue culture flasks. Exposure conditions (Table 2, Rows 1 and 2) were developed to minimize the air headspace in the treatment vessel, and sealed 40 mL glass sample vials with minimal headspace were employed in place of standard tissue culture flasks. The cultures were treated in serum-free medium to prevent the possible interaction of test compound with serum proteins. Exposure times were restricted to 10 hours to avoid any effect of growth in serum-free conditions on cell viability. To test these modified exposure conditions mutant fractions at *hprt* and *tk* induced by 50 ng/mL B[*a*]P were compared with previously reported data (Table 2) (Crespi et al. 1991; Busby et al. 1995). We routinely use five-day expression periods for the selection of normal growth tk^- mutants from TK6 human lymphoblasts (Giver et al. 1995), and the influence of phenotypic expression period on the recovery of tk^- mutants from MCL-5 cells was, therefore, also examined. The results indicate that these conditions, particularly an extension of the phenotypic expression period to five days, resulted in a significant increase in recovery of tk^- mutants. These parameters were, therefore, routinely utilized in subsequent determinations of mutagenicity in test compounds. Benzo[*a*]pyrene was used as a positive control to demonstrate that the cells were metabolically competent, and that the mutational markers were responsive to mutagenic treatment.

Table 4. Mutagenicity at *tk* in MCL-5 Cells and Ambient Concentrations for Atmospheric Nitro-PAHs

Compound	tk^- Mutation Frequency[a] (μmol/mL compound)	Ambient Concentration (pg/m^3)[b]
2-Nitronaphthalene	190	2,300
2-Nitrodibenzopyranone	2,830	230[c]
1-Nitropyrene	460[d]	19

[a] Mutation frequency is defined here as the number of mutants per 10^6 viable cells.

[b] Ambient data from Atkinson et al. 1988.

[c] Ambient data from Helmig et al. 1992a,b.

[d] Mutation frequency data from Busby et al. 1994.

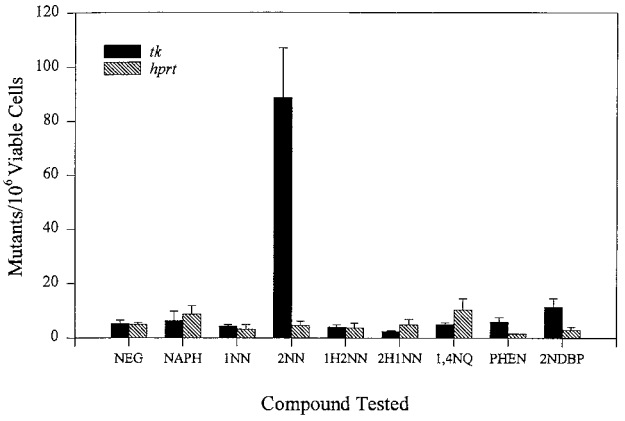

Figure 3. Mutation frequencies at the *tk* (black bars) and *hprt* (hatched bars) loci following exposure to various chemicals. Chemical concentrations are as noted in Table 3. Negative and positive control mutation frequencies were calculated with data from 11 independent cultures and 8 independent experiments. Test compound mutation frequencies are the result of averaged data from 3 to 5 independent cultures and up to 4 independent experiments. Standard error of the mean is shown by error bars. Compounds are abbreviated as follows: NEG = negative control using 0.4% DMSO, NAPH = naphthalene, 1NN = 1-nitronaphthalene, 2NN = 2-nitronaphthalene, 1H2NN = 1-hydroxy-2-nitronaphthalene, 2H1NN = 2-hydroxy-1-nitronaphthalene, 14NQ= 1,4-naphthoquinone, PHEN = phenanthrene, 2NDBP = 2-nitrodibenzopyranone.

MUTAGENICITY OF NAPHTHALENE, PHENANTHRENE, AND THEIR ATMOSPHERIC TRANSFORMATION PRODUCTS

An overall summary of mutation frequency induced at the *tk* and *hprt* loci, and corresponding clonogenic survival data, is presented in Table 3. The mutagenesis results are also graphically depicted in Figure 3. The data presented are for the highest soluble dose for each compound, and surviving fractions ranged from 0.08 to no detectable cytotoxicity (Table 3). A significant increase in the tk^- mutation frequency was induced by 2NN (Table 3, Figure 3; $p < 0.0001$). A modest statistically significant increase in the tk^- mutation frequency was also induced by 2NDBP ($p = 0.048$). The evaluation of mutagenicity for 2NDBP was constrained by the low solubility of the compound. However, on the basis of induced tk^- mutants/μmole of compound, 2NDBP is an order of magnitude more potent than 2NN (Table 4). Neither compound induced a significant increase in mutation frequency at the *hprt* locus (Table 3, Figure 3), although the sensitivity of the mutagenesis assay at *hprt* was demonstrated by response to B[*a*]P. The six remaining test compounds did not induce a significant increase in mutation frequency at *tk* (Table 3, Figure 3). Although naphthalene ($p = 0.062$) and 1,4-naphthoquinone ($p = 0.04$) cause small increases in mutation frequency at the *hprt* locus, these were not significantly different from the tk^- mutation frequency induced by the same compounds. Since the types of mutations recoverable at *hprt* are expected to be a subset of those recoverable as tk^- mutants (Giver et al. 1995), these data suggest that the small increases in $hprt^-$ mutation frequency observed following exposure to naphthalene and 1,4-naphthoquinone may not be biologically meaningful.

MICRONUCLEI INDUCED BY NAPHTHALENE, PHENANTHRENE, AND THEIR ATMOSPHERIC TRANSFORMATION PRODUCTS

Compounds were assayed for cytogenetic effects using the CREST modified micronucleus technique, which distinguishes chromosomal loss from chromosomal breakage. The same compounds that produced the greatest increases in mutation frequency at the *tk* locus, 2NN and 2NDBP, also induced a statistically significant increase in micronuclei (Table 5, Figure 4). 2-Nitrodibenzopyranone appears to be more potent than 2NN, since a twofold higher induction of micronuclei was induced by an over 50-fold lower concentration of 2NDBP (Table 5). 1,4-Naphthoquinone and naphthalene also induced statistically significant increases in micronuclei (Table 5, Figure 4), although exposure to 1-nitronaphthalene, 1-hydroxy-2NN, 2-hydroxy-1-nitronaphthalene, and phenanthrene did not produce a significant elevation in the frequency of micronuclei.

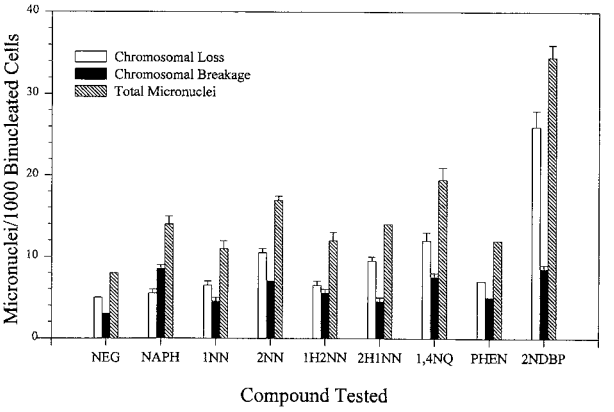

Figure 4. Number of micronuclei per 1,000 binucleated cells induced by exposure to various chemicals. Chemical concentrations are as noted in Table 5. CREST$^+$ micronuclei (white bars) denote chromosomal loss events and CREST$^-$ micronuclei (black bars) denote chromosomal breakage events. The total number of micronuclei are shown by the hatched bars. Results shown are from two independent experiments. Standard error of the mean is shown by error bars. Compounds are abbreviated as described in the legend to Figure 3.

Table 5. Micronuclei Analysis of Cells Treated with PAHs and Selected Atmospheric Reaction Products

Compound	Concentration (μmol/mL)	n[a]	Clonogenic Survival (± SEM)	CREST$^+$ (± SEM)[b,c,d]	CREST$^-$ (± SEM)[b,c,e]	Total Number of Micronuclei (± SEM)[b,f]
Negative control (DMSO)	—	2	1.00 ± 0.00	5.0 ± 0.0	3.0 ± 0.0	8.0 ± 0.0
Benzo[*a*]pyrene	2.0×10^{-4}	2	1.00 ± 0.18	5.5 ± 0.5	3.5 ± 0.5	9.0 ± 0.0
Naphthalene	0.23	2	0.70 ± 0.30	5.5 ± 0.5	8.5 ± 0.5*	14.0 ± 1.0
1-Nitronaphthalene	0.23	2	0.43 ± 0.05	6.5 ± 0.5	4.5 ± 0.5	11.0 ± 1.0
2-Nitronaphthalene	0.35	2	0.76 ± 0.04	10.5 ± 0.5	7.0 ± 0.0	17.5 ± 0.5*
1-Hydroxy-2-nitronaphthalene	0.53	2	0.66 ± 0.14	6.5 ± 0.5	5.5 ± 0.5	12.0 ± 1.0
2-Hydroxy-1-nitronaphthalene	0.53	2	0.43 ± 0.04	9.5 ± 0.5	4.5 ± 0.5	14.0 ± 0.0
1,4-Naphthoquinone	6.3×10^{-4}	2	1.05 ± 0.16	12.0 ± 1.0*	7.5 ± 0.5	19.5 ± 1.5*
Phenanthrene	0.042	2	0.58 ± 0.05	7.0 ± 0.0	5.0 ± 0.0	12.0 ± 0.0
2-Nitrodibenzopyranone	0.0062	2	1.34 ± 0.16	26.0 ± 2.0*	8.5 ± 0.5*	34.5 ± 1.5*

[a] Number of observations.

[b] Total number of micronuclei per 1000 scored binucleated cells.

[c] CREST$^+$ = centromere was present, indicating micronucleus formed from chromosome loss;
CREST$^-$ = centromere was absent, indicating micronucleus formed from chromosome fragment.

[d] There was a statistically significant increase in CREST$^+$ micronuclei for 1,4-naphthoquinone ($p = 0.025$) and 2-nitrodibenzopyranone ($p < 0.0001$) using the chi-squared approximation of the Fisher's exact test.

[e] There was a statistically significant increase in CREST$^-$ micronuclei for naphthalene ($p = 0.031$) and 2-nitrodibenzopyranone ($p = 0.037$).

[f] There was a statistically significant increase in total micronuclei for 1,4-naphthoquinone ($p = 0.003$), 2-nitronaphthalene ($p = 0.11$), and 2-nitrodibenzopyranone ($p < 0.0001$).

* Significant increase in micronuclei.

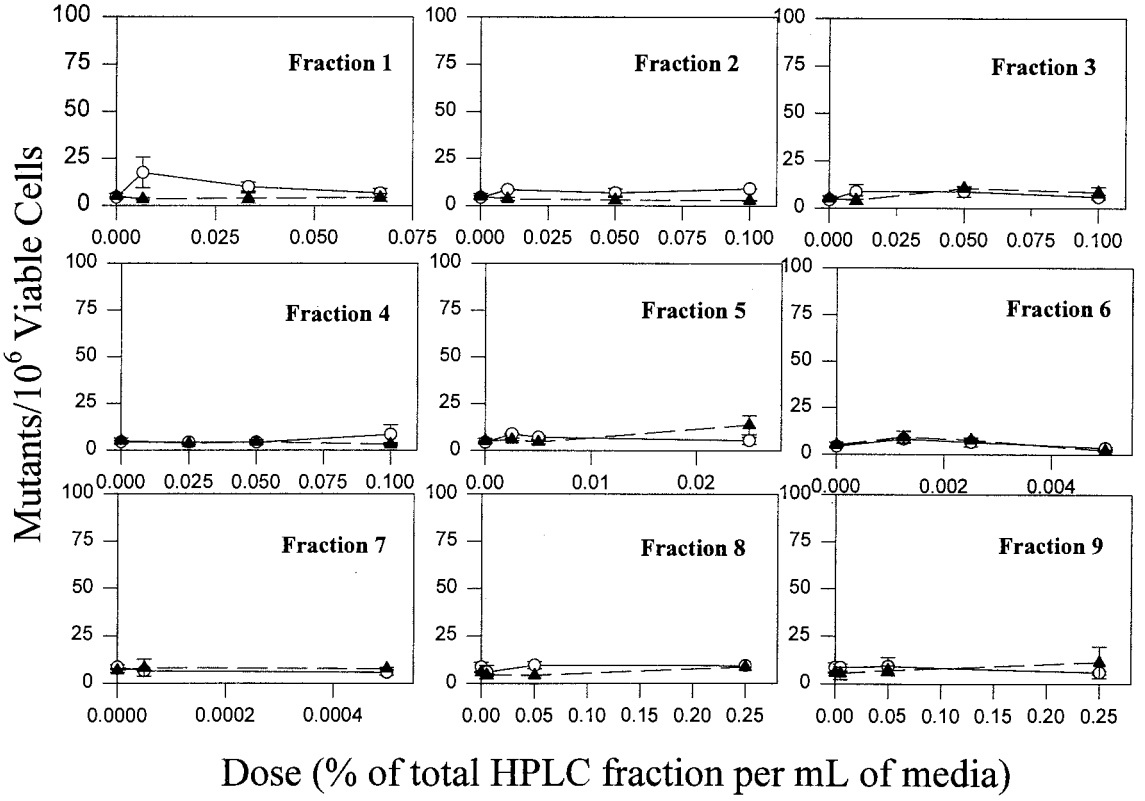

Figure 5. Mutation frequencies at *tk* (circles) and *hprt* (triangles) loci followijng exposure to HPLC fractionated atmospheric reaction products of naphthalene. Results are from duplicate cultures. Standard error of the mean is shown by error bars.

MUTAGENICITY OF FRACTIONATED MIXTURES OF ATMOSPHERIC REACTION PRODUCTS OF NAPHTHALENE

Simulated atmospheric reaction products were collected and assayed in order to test additional reaction products of naphthalene that are as yet unidentified, or unavailable in purified form. A determination of the dose-dependence of mutation frequency at *hprt* and *tk* was obtained for the nine HPLC serial fractions of naphthalene's atmospheric reaction products (Figure 5). The total residue from each of the nine serial fractions was dissolved in a minimal volume of DMSO, which was used as the stock dosing solution. All doses are expressed as the percentage of the total HPLC fraction residue per mL of media (Figure 5).

Since the HPLC fractions were collected based on polarity, the dose to the test cells for the first four HPLC fractions (which contained less polar atmospheric reaction products) was significantly affected by solubility limitations. No substantial cell killing (survival fraction $[S/S_0] \geq 0.92$) was detected for fractions 1 through 3, even at the highest doses tested. No increase was observed in the frequency of tk^- mutants for any HPLC fraction (Figure 5), including fraction 4, which contained 2NN. Chemical analysis of this fraction indicated that the concentration of 2NN in the culture medium during mutagenicity testing was approximately 0.012 μmol/mL, approximately 30-fold lower than the concentration required to induce significant elevation in the mutant fraction at the *tk* locus (Figure 3, Table 3). Although increases in *hprt* mutants were not seen for most of the fractions, modest dose-related increases in the frequency of *hprt* mutants were seen for fractions 5 and 8. However, the associations were due to increases at only one or two points at the highest concentrations tested. This, combined with the lack of effect at *tk*, suggests that the observed increases are not likely to be biologically significant. These results indicate that there was no unidentified naphthalene reaction product, or combination of products, that induced an unequivocal increase in mutagenicity at the tested concentrations.

SURVEY OF MICRONUCLEI INDUCED BY A FRACTIONATED MIXTURE OF ATMOSPHERIC REACTION PRODUCTS OF NAPHTHALENE

Micronucleus experiments were performed for fractions 3 and 4, which were shown by GC/MS analysis to contain the genotoxic compound 2NN and its isomer 1-nitronaphthalene. A small dose-related increase ($p \leq 0.005$; Cochran-Armitage trend test) was observed in the induction of micronuclei by cellular exposure to fraction 3 (Figure 6). The increase in total micronuclei was primarily due to an increase in $CREST^+$ micronuclei, indicating that a significant increase in chromosomal loss was induced by this fraction. Based on the induction of micronuclei by pure compounds (Table 5, Figure 4), the concentrations of 2NN and 1-nitronaphthalene dissolved in fraction 3 (< 0.001 μmol/mL and 0.006 mmol/mL, respectively, as determined by GC/MS), were not sufficient to account for the observed induction of micronuclei. Furthermore, fraction 4, which contained the highest concentrations of 2NN and 1-nitronaphthalene (0.012 μmol/mL and 0.023 μmol/mL, respectively), did not induce a significant increase in micronuclei ($p \geq 0.11$; Cochran-Armitage trend test, Figure 6). Therefore, the induction of micronuclei in fraction 3 is likely to be attributable to unidentified chemical components in the fractions, or synergistic effects induced by the mixture of compounds in the fraction.

Analysis of fraction 7 by GC/MS showed it to be dominated by isomeric compounds of molecular weight 160.

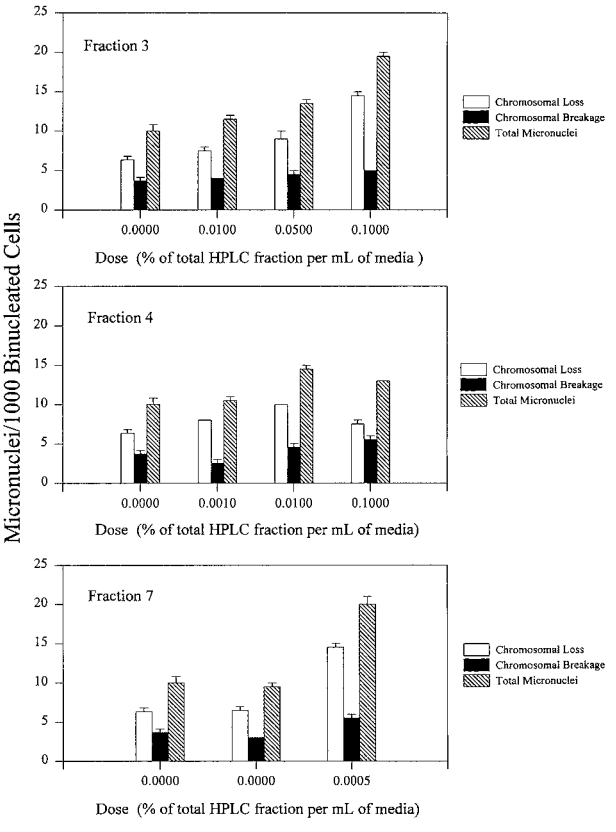

Figure 6. Number of micronuclei per 1,000 binucleated cells induced by exposure to HPLC fractions 3, 4, and 7. $CREST^+$ micronuclei (white bars) denote chromosomal loss events and $CREST^-$ micronuclei (black bars) denote chromosomal breakage events. The total number of micronuclei is shown by the hatched bars.

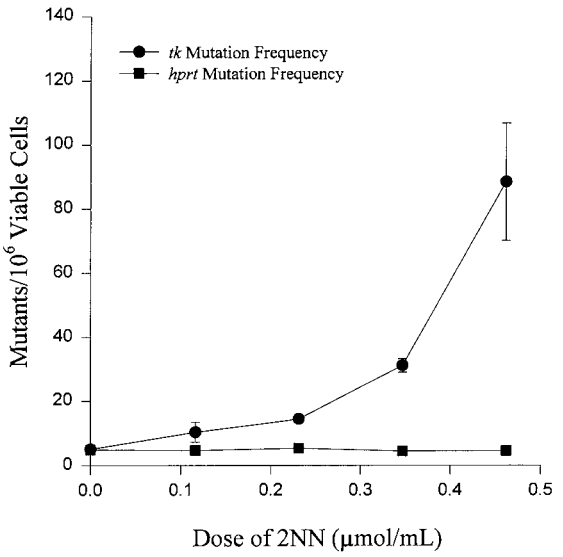

Figure 7. Induction of mutations at *tk* and *hprt* by 2NN in MCL-5 human lymphoblasts.

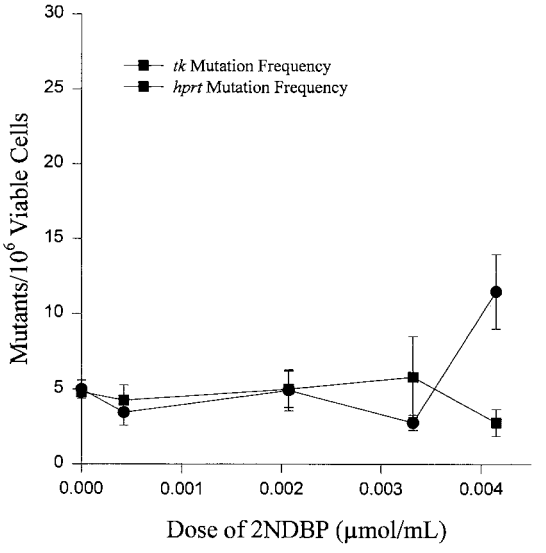

Figure 8. Induction of mutations at *tk* and *hprt* by 2NDBP in MCL-5 human lymphoblasts.

Two ring-breakage products have recently been identified as the major products from the OH radical–initiated photooxidation of naphthalene (Sasaki et al. 1997). Because one of these products, *trans*-2-formyl-cinnamaldehyde, was the most abundant component in fraction 7, induction of micronuclei by fraction 7 was also tested. Although fractions 1 through 4 contained nonpolar compounds of limited solubility, and, in most cases, compound treatment did not result in a significant amount of cell killing, fractions 5 through 9 contained compounds of greater polarity, which also tended to be more toxic. For example, testing of fraction 7 was significantly limited by cytotoxicity, so that the concentrations used for mutagenicity and micronucleus assays were 1 to 2 orders of magnitude lower than those used in testing of fractions 3 and 4 (Figure 6). Nevertheless, a significant dose-related induction of micronuclei ($p \leq 0.001$; Cochran-Armitage trend test) was observed in cells exposed to fraction 7. Again, this increase was primarily due to chromosomal loss ($CREST^+$ micronuclei). Further evaluation of the genotoxicity of 2-formyl-cinnamaldehyde may be warranted.

DOSE-DEPENDENT INDUCTION OF GENOTOXICITY BY 2NN AND 2NDBP IN MCL-5

The dose-response relationship for mutagenicity induced by exposure of MCL-5 cells to 2NN and 2NDBP across a series of concentrations is shown Figures 7 and 8. No increase in mutation frequency was induced by either compound at the *hprt* locus, suggesting that point mutations do not contribute significantly to the mutagenicity induced by these compounds in these cells. However, a dose-dependent increase in the frequency of tk^- mutants was observed for 2NN. A reproducible increase ($p = 0.048$) was also observed at a substantially lower molar concentration for 2NDBP (Figure 9). This increase was not observed at higher concentrations. However, testing at the highest concentrations was problematic and considered unreliable due to solubility limitations. 2-Nitronaphthalene also induced a significant dose-dependent increase in micronuclei (Figure 10). Most of the induced micronuclei were $CREST^+$, indicating that they were formed from chromosomal loss. Low molar concentrations of 2NDBP also induced a substantial increase in micronuclei, and there was a significant dose-related increase in both the $CREST^+$ and $CREST^-$ micronuclei ($p < 0.05$, Figure 11). The dose-response curve flattened for doses over 0.0047 μmol/mL, probably reflecting the solubility limit of the compound in cell culture medium.

DOSE-DEPENDENT INDUCTION OF GENOTOXICITY BY 2NN AND 2NDBP IN AHH-1 1A1

MCL-5 cells express several enzymes involved in the oxidative metabolism of organic compounds (Crespi et al. 1991). To determine whether these enzymatic activities were required for the genotoxicity of 2NN and 2NDBP, and to determine whether a single cytochrome P450 activity would be sufficient for metabolic activation, related cell lines with multiple, single, or no transfected cytochrome P450 genes were utilized. AHH-1 1A1 was used in these experiments because CYP 1A1 has been shown to be important for metabolic activation of PAHs (Crespi et al. 1991).

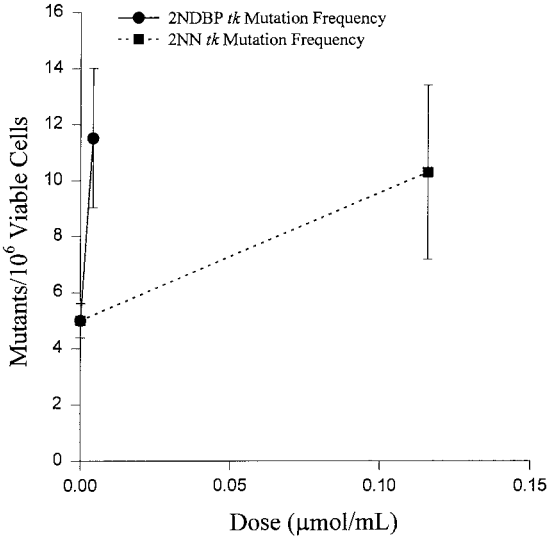

Figure 9. Comparative induction of mutations at *tk* and *hprt* by 2NN and 2NDBP in MCL-5 human lymphoblasts.

AHH-1 cells have also been shown to have increased sensitivity for the detection of multilocus mutational events, such as those detected at the *tk* locus (Dobo et al. 1997), probably due to reduced cellular apoptotic response (Morris 1996; Dobo et al. 1997).

Significant increases in the tk^- mutant fraction were observed following exposure of AHH-1 1A1 cells to 2NN (p = 0.0003) or 2NDBP (p = 0.043) (Figures 12 and 13). The spontaneous background of tk^- mutants was approximately fivefold higher than it was for MCL-5 cells, as previously reported (Dobo et al. 1995). However, 2NN-induced mutant fractions (corrected for background frequency) were comparable to those observed in MCL-5 cells (Table 6). The effect induced by 2NN was much less at *hprt*, although

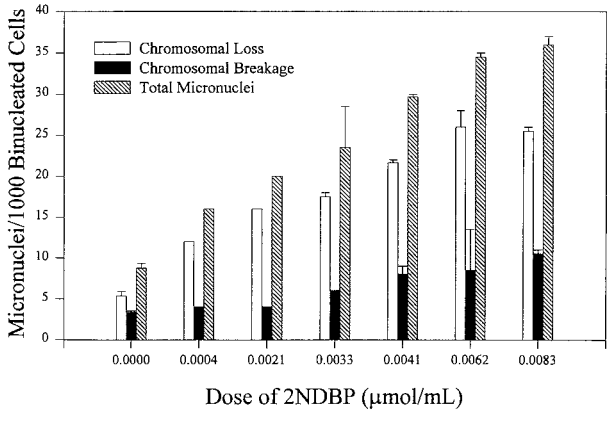

Figure 11. Micronuclei per 1,000 binucleated cells induced by exposure of MCL-5 human lymphoblasts to indicated doses of 2NDBP.

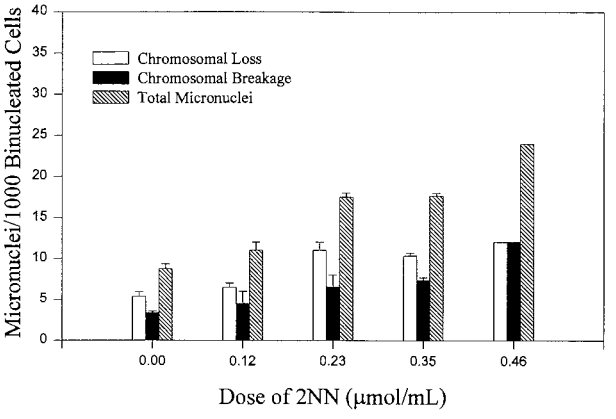

Figure 10. Micronuclei per 1,000 binucleated cells induced by exposure of MCL-5 human lymphoblasts to indicated doses of 2NN.

there was a possible increase in $hprt^-$ mutant fraction (p = 0.09, Figure 12), primarily because of a slightly elevated increase at the highest dose (0.46 μmol/mL). Similarly, 2NDBP produced tk^- mutants but not $hprt^-$ mutants in AHH-1 1A1 cells (Figure 13). Although the lower 0.0042 and 0.0062 μmol/mL concentrations of 2NDBP exhibited clear increases above the controls ($p \leq 0.005$), the tk^- mutant fraction exhibited a lesser and more variable increase at the 0.0083 μmol/mL concentration ($p = 0.059$). As indicated previously, the results at the highest concentrations were considered less reliable due to solubility limitations. The induced tk^- mutant fraction for 2NDBP was

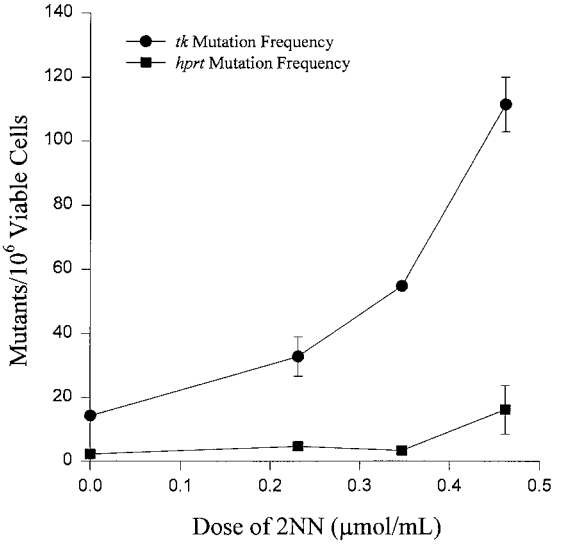

Figure 12. Induction of mutations at *tk* and *hprt* by 2NN in AHH-1 1A1 human lymphoblasts.

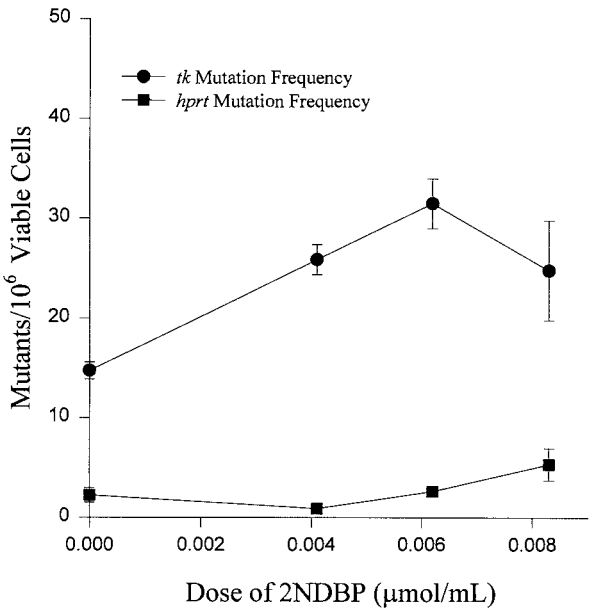

Figure 13. Induction of mutations at *tk* and *hprt* by 2NDBP in AHH-1 1A1 human lymphoblasts.

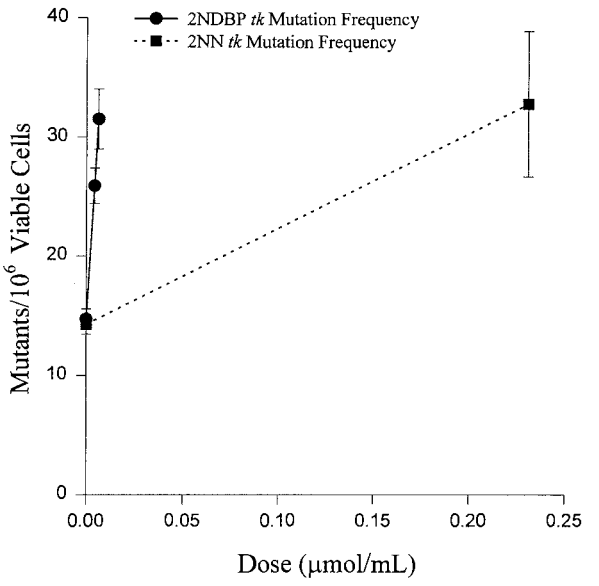

Figure 14. Comparative induction of mutations at *tk* and *hprt* by 2NN and 2NDBP in AHH-1 1A1 human lymphoblasts.

slightly higher than the effect observed in MCL-5 cells, but not to a statistically significant level ($p = 0.08$).

These results, for both compounds, did not correspond with expectations of a strong induction of tk^- mutants in AHH-1 1A1 following exposure to 2NN and 2NDBP. The tk^- mutant fractions induced by 2NN and 2NDBP are compared on an equimolar basis in Figure 14 using the linear lower portions of the dose response curves. Comparable tk^- mutations were induced by 2NDBP concentrations 30- to 40-fold lower than the concentrations that were used for 2NN. 2-Nitronaphthalene exhibited a curvilinear dose response (Figure 12), which may diminish the magnitude of this difference at higher concentrations. Due to the solubility limitations of 2NDBP the shape of its dose response curve at higher concentrations remains undetermined.

Micronucleus results for 2NN and 2NDBP in AHH-1 1A1 cells are shown in Figure 15. For 2NN, the highest dose (0.46 μmol/mL) produced excessive cellular debris and the slides obtained were not scorable. Therefore, only the two lower doses have been plotted (Figure 15). A dose-dependent induction of micronuclei similar to the increase induced by 2NN in MCL-5 cells (Figure 10) was observed. 2-Nitrodibenzopyranone also induced an increase in micronuclei in AHH-1 1A1 cells (Figure 16). The dose-dependent increase in micronuclei flattens at concentrations higher than 0.005 μmol/mL (Figure 16), which resembles the mutagenicity data and which may reflect the poor solubility of 2NDBP at higher doses. The induction of micronuclei in AHH-1 1A1 cells following exposure to 2NDBP was comparable to the effect observed in MCL-5 cells (Figure 11).

Table 6. Mutation Frequencies at *tk* in MCL-5 and AHH-1 1A1 Cells

Compound	MCL-5–Induced Mutation Frequency[a]	AHH-1–Induced Mutation Frequency	Statistical Difference
2-Nitronaphthalene	83.6	96.7	$p = 0.55$
2-Nitrodibenzopyranone	7.2	16.7	$p = 0.08$
N-Nitrosodimethylamine[b]	18.7	66	$p < 0.0001$

[a] Induced mutation frequency is defined as the number of mutants per 10^6 viable cells minus the background mutants per 10^6 viable cells.

[b] Data from Dobo et al. 1997.

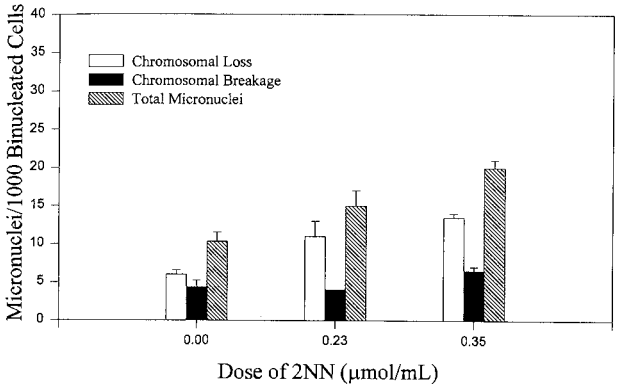

Figure 15. Micronuclei per 1,000 binucleated cells induced by exposure of AHH-1 1A1 human lymphoblasts to indicated doses of 2NN.

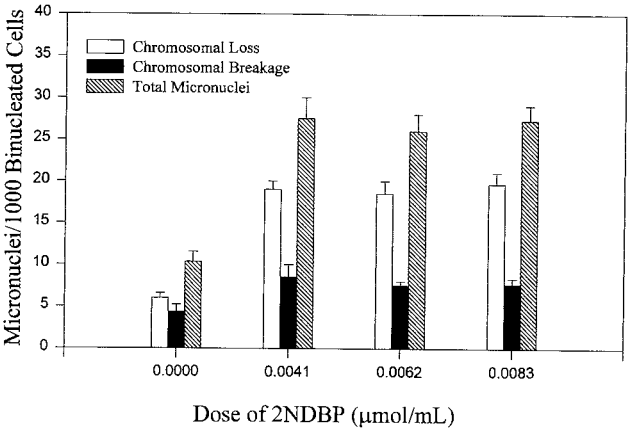

Figure 16. Micronuclei per 1,000 binucleated cells induced by exposure of AHH-1 1A1 human lymphoblasts to indicated doses of 2NDBP.

LACK OF MUTAGENICITY BY βNA IN MCL-5 AND AHH-1 1A1

β-Naphthylamine was tested for mutagenic activity in MCL-5 and AHH-1 1A1 since it has been observed as a metabolic product following exposure to 2NN (Poirier and Weisburger 1974; Johnson and Cornish 1978). We postulated that the production of βNA could account for the genotoxicity of 2NN in MCL-5 and AHH-1. Although experiments were not performed for βNA at doses higher than 0.350 μmol/mL, only a slight (< 50%) or no increase in mutations in MCL-5 or AHH-1 1A1 at either the *tk* or *hprt* locus was observed following βNA treatment (Figures 17 and 18). These results suggest that the genotoxicity of 2NN in these cells is not likely to be attributable to the metabolic conversion of 2NN to βNA.

GENOTOXICITY OF 2NN, βNA, AND 2NDBP COMPARED IN THREE RELATED CELL LINES

To further elucidate the metabolic activation pathways of 2NN, βNA, and 2NDBP, these compounds were additionally tested in L3, the parent cell line of MCL-5, which contains no transfected enzymatic activities. L3 does exhibit endogenous low-level CYP 1A1 activity, as does MCL-5. The activities in both cell lines are lower than the transfected CYP 1A1 activity in AHH-1 1A1 (Penman et al. 1994); MCL-5 expresses high levels of transfected 1A2 activity. No increase in mutation frequency was seen at the *tk* locus in L3 cells by exposure to 2NN, βNA, or 2NDBP (Figures 19, 20, and 21). These results indicate that oxidative metabolism is required for the mutagenic effects of 2NN and 2NDBP. Endogenous nitroreductase activity, if present in

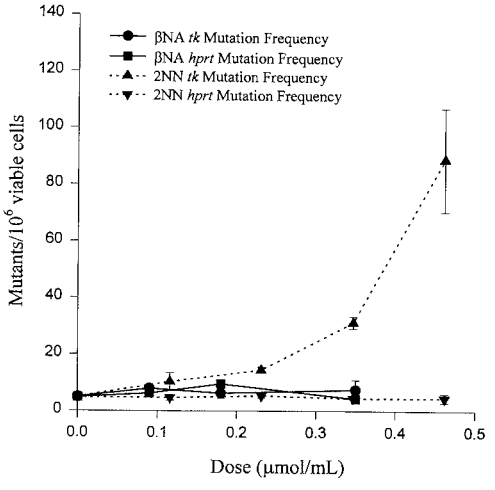

Figure 17. Comparative induction of mutations at *tk* and *hprt* by 2NN and βNA in MCL-5 human lymphoblasts.

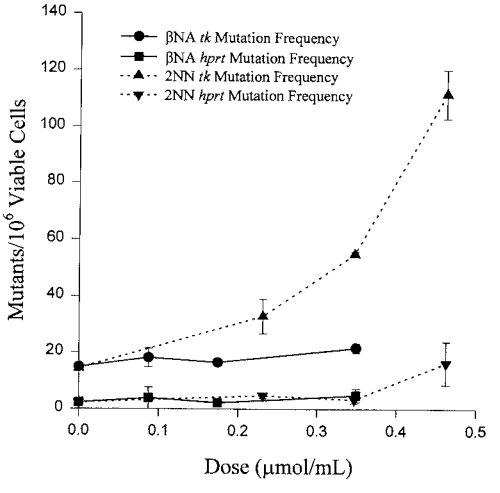

Figure 18. Comparative induction of mutations at *tk* and *hprt* by 2NN and βNA in AHH-1 1A1 human lymphoblasts.

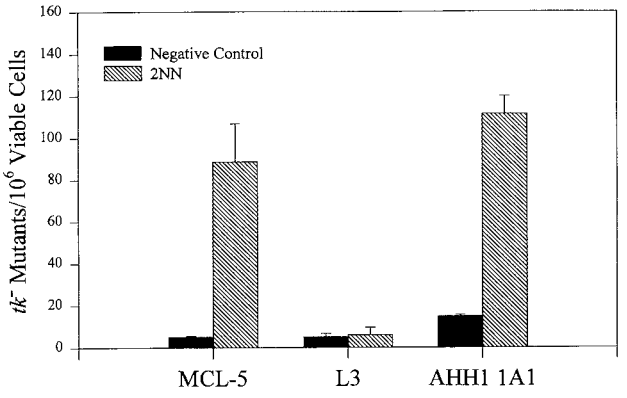

Figure 19. Comparative induction of tk^- mutations by 2NN in MCL-5, L3, and AHH-1 1A1 human lymphoblasts.

these lymphoblastoid cell lines, does not appear to account for the mutagenicity induced by these nitro-PAH compounds.

DISCUSSION AND CONCLUSIONS

In this investigation, a broad-based evaluation of genotoxicity was performed for naphthalene, phenanthrene, and their atmospheric reaction products. Genotoxicity was assessed using metabolically competent human lymphoblastoid cells and endpoints that were sensitive to both chromosomal-scale and point mutations. 2-Nitronaphthalene and 2NDBP were the most active genotoxic compounds of the eight chemicals surveyed, demonstrating both that atmospheric reaction products can be significantly more mutagenic and clastogenic than their parent compounds in human cells, and that the evaluation of chromosomal scale mutagenicity is essential for a comprehensive determination of genotoxic risk. The results further indicate that the genotoxicity of these compounds in human cells is evident at the chromosomal scale, but the compounds do not appear to induce intragenic mutations recoverable as *hprt*$^-$ mutants. Finally, comparison of genotoxicity in related cell lines with varying ectopic cytochrome P450 activities indicates that 2NN and 2NDBP are activated to genotoxic species through oxidative rather than nitroreduction pathways.

2-Nitronaphthalene has previously been reported to be mutagenic in the plate-incorporation *S. typhimurium* assay with higher activity in strain TA100 than TA98 (McCann et al. 1975; Rosenkranz and Mermelstein 1983; Tokiwa and Ohnishi 1986; International Agency for Research on Cancer 1989). 2-Nitronaphthalene has also been reported to be substantially more mutagenic in strain TA98 using the microsuspension modification of the assay, which has been shown to enhance sensitivity particularly for semi-volatile mutagens such as the nitronaphthalenes and methylnitronaphthalenes (Kado et al. 1983, 1992; Arey et al. 1992; Gupta et al. 1996). 2-Nitrodibenzopyranone was first reported to be highly mutagenic in TA98 in the microsuspension assay (Arey et al. 1992; Helmig et al. 1992a). Subsequent studies have confirmed the high activity of 2NDBP toward TA98 in the microsuspension assay and shown that the microsuspension assay is significantly more sensitive to 2NDBP than the plate incorporation assay. Interestingly, with the plate incorporation assay, the addition of S9 to provide metabolic activation enhances the activity of 2NDBP, although the addition of S9 decreases the activity of 2NDBP in the microsuspension assay (Watanabe et al. 1995, 1996). In the plate incorporation assay, the addition of the pKM101 plasmid enhanced frameshift and base-substitution activities of 2NDBP, suggesting the induction of "complex frameshifts" (frameshifts with associated base substitutions) in strain TA98 (Watanabe et al. 1995).

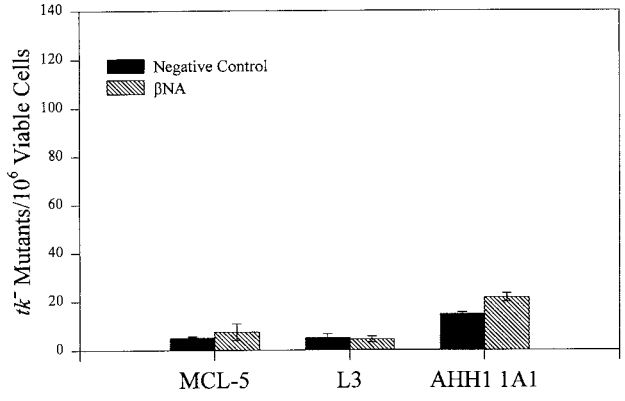

Figure 20. Comparative induction of tk^- mutations by βNA in MCL-5, L3, and AHH-1 1A1 human lymphoblasts.

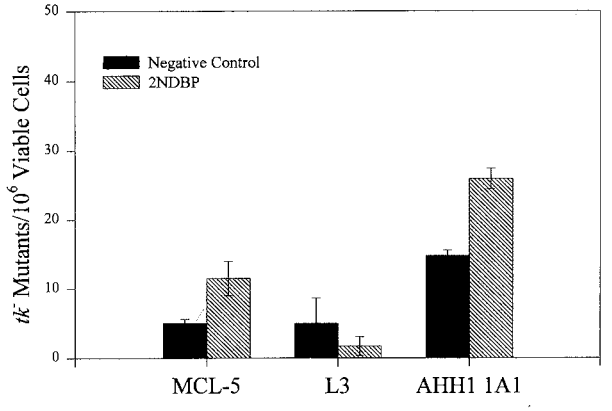

Figure 21. Comparative induction of tk^- mutations by 2NDBP in MCL-5, L3, and AHH-1 1A1 human lymphoblasts.

In general, for the nitro-PAH class of compounds, activity is greatest in the TA98 strain, without exogenous S9 activation, suggesting that these compounds are metabolically activated via nitroreductase pathways and induce frameshift mutations. In contrast to bacterial reversion assays, analysis of tk^- and $hprt^-$ mutations in MCL-5 cells allows for detection of both chromosomal and intragenic mutations. In addition, the endogenous and transfected enzymatic activities in MCL-5 are human in origin, whereas the S9 mixtures used in bacterial assays are derived from rodents. Because both 2NN and 2NDBP were mutagenic at *tk* but not *hprt*, these data suggest that these compounds (or metabolites of these compounds) are inducing chromosomal scale mutational events that would not be detectable in the *S. typhimurium* assay. Moreover, the difference in prokaryotic versus mammalian cell response to the compounds (i.e., point mutations versus chromosomal scale mutations) indicates that there may be fundamental differences in lesion processing and metabolic activation pathways responsible for the mutants recovered in the two systems.

Recent reports (Durant et al. 1996; Busby et al. 1997) found no induction of tk^- mutants in AHH-1 1A1 following exposure to 2NDBP. This is in contrast to our results (Figures 13 and 14). This apparent difference is probably attributable to procedural differences. Unlike the previous reports, we did not include serum in the culture medium during the exposure period, and we also extended the phenotypic expression period from 3 days to 5 days. Both of these parameters were found to have large effects on the recovery of tk^- mutants in control experiments (Table 2). Additionally, the doses employed in the previous work were substantially higher than those we used, and it is possible that incomplete dissolution contributed to the lack of observed mutagenicity.

Micronucleus data also indicated that chromosomal scale events can be induced by exposure to 2NN and 2NDBP (Figure 4). The induction of micronuclei by these and other test compounds and mixtures (Figures 4 and 6) was largely attributable to chromosomal loss (CREST antigen positive). The CREST $^+$ response may be mechanistically more closely related to cytotoxicity than to mutagenicity, since our previous results indicate that whole chromosomal loss may not allow for the survival of viable mutants in a clonogenic assay. For example, in extensive investigation of tk^- LOH mutants in TK6 human lymphoblasts conducted by our laboratory and others (Kronenberg and Little 1989; Yandell et al. 1990; Amundson and Liber 1992; Grosovsky et al. 1995), as well as more limited analysis of tk^- LOH mutants in MCL-5 and AHH-1 cells (Dobo et al. 1995), no instance of a whole chromosome 17 loss has ever been reported in a viable tk^- mutant. This may explain the induction of a significant increase in chromosome loss by 1,4-naphthoquinone (Figure 4, Table 5) that was not detected as mutagenic at *tk* or *hprt* (Figure 3, Table 3). Although chromosome loss may only rarely be a mechanism for the production of viable mutants in diploid or near-diploid cells, it can play a significant role in tumor progression in aneuploid cells (Kallioniemi et al. 1992). On the other hand, only a limited correlation was established between chromosome breakage as detected in the micronucleus assay and mutagenesis at *tk*. A significant increase in chromosomal breakage was observed for 2NDBP (Figure 4), but 2NN induced a smaller elevation in chromosomal breakage than the parental compound naphthalene, which was not found to be mutagenic (Figure 3).

Nitro-PAHs have been reported to induce carcinogenicity in rats and mice (Rosenkranz and Mermelstein 1983; Tokiwa and Ohnishi 1986; International Agency for Research on Cancer 1989). However, the isomers most commonly studied as model compounds for the nitro-PAHs include electrophilic nitration products of PAHs such as 1-nitropyrene, which are present in direct emission sources such as exhaust from diesel engines (Schuetzle et al. 1981; International Agency for Research on Cancer 1989; Health Effects Institute 1995). The mutagenicity of 1-nitropyrene at the *tk* locus of MCL-5 cells has been reported (Busby et al. 1994), and the mutagenicity of 1-nitropyrene is compared with that of 2NN and 2NDBP in Table 4. The mutagenic potency of 1-nitropyrene and 2NN are similar when compared on a molar basis, and are approximately tenfold lower than the mutagenic potency of 2NDBP. Typical ambient atmospheric concentration ranges of these compounds measured in California (Atkinson et al. 1988; Helmig et al. 1992b) are given in Table 4, and these data show that 2NN and 2NDBP are consistently more abundant than 1-nitropyrene (Table 4). The ambient concentration data and the induction of chromosomal-scale events by 2NN and 2NDBP in human cells reported here, (Figures 3 and 4), suggest that the health effects of atmospherically formed nitro-PAHs and nitro-PAH lactones should be considered along with the nitro-PAHs derived from direct emission sources.

Previous investigations in bacteria and in rodent carcinogenicity assays suggest that the metabolic activation of many nitro-PAHs, and specifically 2NN, occurs via reductive mechanisms (Tokiwa and Ohnishi 1986). Three lines of evidence presented in this investigation indicate that P450-mediated oxidative metabolism, rather than nitroreduction, is the metabolic pathway for the production of genotoxic metabolites in human lymphoblastoid cell lines. Genotoxicity has been shown (Figures 19 and 21) to correlate with the expression of cytochrome P450 monooxygenases; isogenic cell lines that do not contain plasmid-encoded cytochrome P450 activities do not

exhibit elevated frequencies of mutation following exposure to 2NN or 2NDBP. Secondly, βNA, the reduced analog of 2NN, induces no mutagenicity in any of the human lymphoblastoid cell lines tested (Figures 17, 18, and 20). Finally, 2NN and 2NDBP are active in bacterial reversion assays that by definition are restricted to the recovery of intragenic point mutations. In contrast, 2NN and 2NDBP induce mutations at the *tk* but not the *hprt* locus, and, therefore, the mutational spectrum appears to be predominated by chromosomal-scale mutations rather than intragenic point mutations (Giver et al. 1995; Nelson et al. 1995). This mutational specificity may also reflect a spectrum of potentially genotoxic metabolites that differs from bacterial systems.

2-Nitronaphthalene induced a substantial increase in mutation frequency and micronuclei, but only in cell lines that contained transfected, elevated levels of cytochrome P450 activity (MCL-5 and AHH-1 1A1). Because the cytochrome P450s are monooxygenases, these findings indicate that oxidation is an important step in the metabolic activation of 2NN. AHH-1 1A1 has been transfected with plasmids that support the high-level, constitutive expression of CYP 1A1 activity (Penman et al. 1994). In contrast, L3 cells express only a lower-level and inducible CYP 1A1 activity (Penman et al. 1994); the effectiveness of 2NN in inducing CYP 1A1 has not been determined. Like L3, MCL-5 expresses only an endogenous and inducible CYP 1A1 activity. However, the presence of transfected, constitutively expressed CYP 1A2, CYP 2E1, CYP 3A4, CYP 2A6, and epoxide hydrolase in MCL-5 may provide alternative bioactivation pathways for the metabolic processing of specific nitro-PAHs.

Previous in vivo and in vitro rodent studies have detected βNA as a high-abundance metabolite in cells exposed to 2NN (Johnson and Cornish 1978; Poirier and Weisburger 1974). Because βNA has been identified as a bladder carcinogen in mammals and humans (Conzelman et al. 1970; Conzelman and Moulton, 1972; Garner et al. 1984), it has been postulated that the carcinogenicity and mutagenicity of 2NN is attributable to the production of βNA (Fu et al. 1985; Tokiwa and Ohnishi 1986). However, βNA was not mutagenic in any of the cell lines tested (Figure 20). These results cannot exclude the possibility that exogenously supplied βNA is not incorporated efficiently into the test cells. Consequently, intracellular production of βNA following 2NN exposure may still be significant. However, this argument cannot account for the requirement for monooxygenase activity for the genotoxicity of 2NN.

In this investigation two marker loci were used for the detection of mutagenicity. Both the *hprt* and *tk* loci encode salvage pathway enzymes that are inessential for cell survival in ordinary culture medium. Therefore, a wide range of inactivating intragenic mutations will result in viable mutants at both loci, which are resistant to medium supplemented with the appropriate toxic nucleotide analogs. The *hprt* locus is hemizygous, and therefore the chromosomal-scale mutations are restricted to deletions of no more than three to four megabases (Mb) (Nelson et al. 1995). On the other hand, *tk* is heterozygous and viable tk^- mutants can result from a broader set of events, including recombinationally mediated loss of heterozygosity and multi-locus mutations extending for at least 35 centimorgans (cM) (Dobo et al. 1995). The recovery of mutations at *tk* but not *hprt* (Figures 7, 12, and 13) indicates that 2NN and 2NDBP are inducing a class of mutations that can be recovered only at *tk*, which is most likely to originate from extensive, chromosomal-scale loss of heterozygosity. The presumptive absence of intragenic point mutations is in sharp contrast to the mutagenicity of 2NN and 2NDBP in bacteria.

The results presented here indicate that the genotoxicity of nitro-PAHs in human cell lines may not be appropriately modeled by bacterial systems. The spectrum of metabolites, the required metabolic activities, and the targets for mutagenesis all appear to distinguish human and bacterial cells, and underscore the importance of genotoxicity testing in human systems to obtain useful mechanistic information about the mutagenic effects of xenobiotics.

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Sasaki JC, Arey J, Eastmond DA, Parks KK, Phousongphouang P, Grosovsky AJ. The Role of Oxidative Metabolism in the Genotoxicity of 2-Nitronaphthalene in Human Lymphoblastoid Cell Lines. Submitted for publication.

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ABBREVIATIONS

AHH-1 1A1 (also h1A1v2)	AHH-1 cell line transected with CYP 1A1
βNA	β-naphthylamine
B[*a*]P	benzo[*a*]pyrene
cM	centimorgan
$CREST^+$	centromere is present, indicating micronucleus formed from chromosome loss
$CREST^-$	centromere is absent, indicating micronucleus formed from chromosome fragment
CPD	cumulative population doubling
CYP 1A1	cytochrome P450 1A1
DMSO	dimethylsulfoxide
GC/MS	gas chromatography/mass spectrometry
h1A1v2 (also AHH-1 1A1)	AHH-1 cell line transected with CYP 1A1
HPLC	high-performance liquid chromatography
hprt	hypoxanthine phosphoribosyl transferase gene
LOH	loss of heterozygosity
Mb	megabases
MCL-5	human B-lymphoblastoid cell line
2NDBP	2-nitro-6*H*-dibenzo[*b*,*d*]pyran-6-one or 2-nitrodibenzopyranone
2NN	2-nitronaphthalene
NO_3	nitrate radical
OH	hydroxyl radical
PAH	polycyclic aromatic hydrocarbon
POM	polycyclic organic matter
S/S_0	survival fraction
tk	thymidine kinase gene

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs)* and their nitrated and oxygenated derivatives (nitro- and oxy-PAHs) are ubiquitous environmental pollutants that comprise a portion of atmospheric pollutants classified as polycyclic organic matter (POM). Polycyclic organic matter originates as an emission product from the combustion of organic materials such as the burning of fuels to power motor vehicles. Many of the atmospheric PAHs and their derivatives, which are present as components of POM, are genotoxic. They exhibit variable mutagenic potency in bacterial and mammalian assays and are rodent carcinogens (reviewed by Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986; International Agency for Research on Cancer 1989). Thus, the presence of these compounds in ambient air may pose a significant risk to human health.

Because of concerns about the potential health consequences of POM originating from diesel exhaust, the U.S. Environmental Protection Agency (EPA) set emissions standards for diesel engines and vehicles under Section 202 of the Clean Air Act (CAA), first amended in 1990. These emissions standards were set for POM and four other pollutant classes (total hydrocarbons, nonmethane hydrocarbons, carbon monoxide, and oxides of nitrogen) originating from diesel engines and vehicles. These standards continue to evolve, as evidenced by the final regulations for highway heavy-duty engines and the proposed regulations for nonroad diesel engines and locomotives (EPA 1997a,b,c). The process of reviewing and revising emissions standards requires an understanding not only of the health effects of the individual compounds present in diesel exhaust but also of their atmospheric transformation products.

Because of the need for better information on the potential health effects of PAHs and other air toxics, the Health Effects Institute (HEI) conducted a workshop in 1992, "Research Priorities for Mobile Air Toxics," during which research needs for compounds designated as Air Toxics under the CAA of 1990 (for example, benzene, 1,3-butadiene, formaldehyde, and POM) and methanol vapors were identified (Health Effects Institute 1993). The research priorities for POM included: (1) examination of the atmospheric transformation reactions of PAHs; (2) characterization of the polar transformation products of POM, such as nitro- and oxy-PAHs; and (3) investigation of the biological significance of PAH derivatives, including their mutagenic spectra.

In response to the workshop proceedings, Dr. Arey and collaborators submitted a preliminary application in January of 1994 to assess the mutagenicity of two abundant gas-phase PAHs, naphthalene and phenanthrene, and their atmospheric transformation products in mammalian cells. The following Commentary on the Investigators' Report is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the Investigators' Report into scientific and regulatory perspective.

SCIENTIFIC BACKGROUND

Polycyclic aromatic hydrocarbons are ubiquitous air pollutants containing two or more benzene rings; they are formed largely from the incomplete combustion of fossil and biogenic fuels (International Agency for Research on Cancer 1989). Once emitted into the atmosphere, a complex set of processes affects PAHs, including atmospheric transport, chemical transformation, and dry and wet deposition. These transport and transformation processes are important because (1) they influence the ambient concentrations of specific emission constituents and their reaction products; (2) they alter the toxic, mutagenic (damaging to genes), or carcinogenic properties of the original emission constituents; and (3) they can create new, potentially toxic, mutagenic or carcinogenic products.

Some PAHs are transformed in the atmosphere through reactions with other environmental pollutants to yield nitro-PAHs and oxy-PAHs (Pitts et al. 1978; Pitts 1987). For example, naphthalene and phenanthrene, two of the simplest and most abundant gas-phase PAHs, can react with hydroxyl radicals and nitrate radicals; phenanthrene can also react with ozone (Winer and Busby 1995). The majority of ambient nitro-PAHs are formed from the gas-phase reactions of PAHs with hydroxyl or nitrate radicals in the presence of oxides of nitrogen (Atkinson and Arey 1994).

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

† Drs. Andrew Grosovsky and Janet Arey's 3-year study, *Evaluation of the Potential Health Effects of the Atmospheric Reaction Products of Polycyclic Aromatic Hydrocarbons*, began in July 1994 and had total expenditures of $179,000. The Investigators' Report from Arey and colleagues was received for review in July 1997. A revised report, received in March 1998, was accepted for publication in April 1998. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary.

The formation of oxy-PAHs may involve the atmospheric reaction of PAHs with ozone; few details are known about these reactions, however (Winer and Busby 1995).

Concentrations of particle-bound PAHs in ambient air typically range from less than 1 $\mu g/m^3$ in rural areas to 1 to 10 $\mu g/m^3$ in urban areas (Grimmer 1983). Concentrations of napathalene as high as 6 $\mu g/m^3$ have been reported in the Los Angeles air basin (Arey et al. 1989). Concentrations of nitro-PAHs in ambient air are generally 10 to 100 times lower than concentrations of PAHs (Ramdahl et al. 1986; MacCrehan et al. 1988; Hayakawa et al. 1995). Oxy-PAHs, though less well studied than PAHs and nitro-PAHs, have been reported at concentrations in the range of 0.05 to 4 $\mu g/m^3$ in urban air (Konig et al. 1983; Ligocki and Pankow 1989).

Many PAHs found in the atmosphere are mutagenic and carcinogenic in experimental settings (International Agency for Research on Cancer 1989), and nitro-PAHs are especially potent bacterial mutagens. For example, when tested in *Salmonella typhimurium* strain TA98, without exogenous metabolic activation, 1,8-dinitropyrene was up to 100,000 times more potent than benzo(*a*)pyrene (Rosenkranz and Mermelstein 1985). Relatively few studies have been published on the mutagenicity and carcinogenicity of oxy-PAHs. However, some oxy-PAHs were reported to be mutagenic (Durant et al. 1996; Enya et al. 1997).

Because cancer is a multistage process involving multiple mutational and nonmutational events, scientists often measure the ability of chemicals to cause genetic mutations in cultured cells and laboratory animals as a possible indicator for predicting the risk of cancer in humans (Harris 1991). Genotoxicity assays may be performed in vivo (in laboratory animals) or in vitro (in cellular systems). Although in vivo assays that take into account variations in the tissue distribution and metabolism of the chemical are most appropriate for evaluating human health risks, the simplicity of in vitro cell assays renders them ideal for screening the mutagenic potential of numerous chemicals quickly and inexpensively.

Bacterial test systems have traditionally been used to characterize the mutagenic potential of test compounds. Bacterial systems typically require the addition of exogenous metabolizing enzymes prepared from rat liver to activate some procarcinogens. In vitro tests using human cell lines, which express metabolic capabilities similar to those of human cells in vivo, provide genotoxicity information that may be more appropriate than the information derived from bacterial systems. Scientists have manipulated a human lymphoblastoid cell line (designated L3) (Crespi and Thilly 1984) to express an array of biotransformation enzymes necessary for the activation of promutagenic PAHs (Crespi et al. 1991; Penman et al. 1994). The cell line designated AHH-1 expresses human cytochrome P450 1A1 (CYP 1A1), an enzyme known to be important in the activation of many PAH compounds. Another cell line, designated h1A1v2 (or AHH-1 1A1), constitutively expresses CYP 1A1 at a level that is approximately 50 times higher than the basal level present in the AHH-1 cell line. MCL-5 expresses epoxide hydrolase and other P450 isozymes (CYP 1A2, CYP 2A3, CYP 2E1, and CYP 3A4) in addition to CYP 1A1. Because of these metabolic differences, a variety of cell lines can be selected to determine the contribution of individual metabolic pathways to the genotoxicity of test chemicals. In this study, Arey and colleagues used some of these human cell lines to obtain information on the spectrum of genotoxicity selected PAHs were capable of producing.

RATIONALE FOR THE STUDY

HEI funded this study because it addressed a number of the research priorities for POM identified in the proceedings of the workshop, "Research Priorities for Mobile Air Toxics," and in the Institute's review, "Diesel Exhaust: A Critical Analysis of Emissions, Exposure, and Health Effects." The underlying premise of the study was that atmospheric transformation products of PAHs may be more potent mutagens than the parent molecules and, therefore, should be considered in genotoxicity assessments of air pollutants (Winer and Busby 1995). The investigators' goal of evaluating the mutagenic spectrum of atmospheric PAHs along with their more polar transformation products would provide important information concerning the potential long-term health consequences of these major combustion products of diesel fuels.

OBJECTIVES AND SPECIFIC AIMS

The objective of this study was to evaluate the genotoxicity, both mutagenesis and clastogenesis, of selected PAHs and their atmospheric transformation products using metabolically competent human cells. There were three specific aims: (1) to screen naphthalene and phenanthrene, and extracts of their gas-phase atmospheric reaction products, for mutagenic activity; (2) to fractionate the gas-phase reaction products of naphthalene, characterize the activity of these fractions, and attempt to identify the compounds responsible for any mutagenic activity observed; and (3) to compare the mutagenic activity of selected fractions or identified products in human lymphoblastoid cell lines expressing different cytochrome P450 isozymes. Information on the technical aspects of these human cell assays can be found in the accompanying sidebar.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The investigators successfully evaluated the genotoxicity of naphthalene and phenanthrene as well as a subset of their atmospheric reaction products using metabolically competent human lymphoblastoid cells. All major elements of the three specific aims were completed. The investigators have considerable expertise in their multiple disciplines, and have together produced a cohesive and well executed study. Overall, the study report is clearly written and documents several interesting and important findings with broader scientific implications.

METHODS AND STUDY DESIGN

The investigators evaluated naphthalene, phenanthrene, and a subset of their atmospheric transformation products (1-nitronaphthalene, 2NN, 1-hydroxy-2-nitronaphthalene, 2-hydroxy-1-nitronaphthalene, 1,4-naphthoquinone, and 2NDBP) for mutagenic activity in the human lymphoblastoid cell line MCL-5. This cell line expresses several P450 isozymes as well as epoxide hydrolase. Gene mutations were evaluated using both the thymidine kinase (*tk*) gene locus and the hypoxanthine-guanine phosphoribosyltransferase (*hprt*) gene locus. The CREST modified micronucleus assay was used to characterize chromosomal breakage and loss.

The investigators also performed studies on the fractionated components of simulated atmospheric reactions of naphthalene. In addition, they determined the role of P450-mediated oxidative biotransformation in the activation of 2NN and 2NDBP to species capable of inducing mutations at the *tk* locus using three related cell lines with multiple (MCL-5), single (AHH-1 h1A1v2), or no (L3) P450 isozymes.

The authors provided a plausible rationale for the selection of naphthalene as the most appropriate starting reagent, an adequate characterization of the methods used to produce and fractionate atmospheric reaction products of naphthalene, and suitable justification for the selection of mutagenicity test system and cell types. The statistical analyses appear to have been appropriately conducted.

RESULTS AND INTERPRETATIONS

Collectively, the results indicated that 2NN and 2NDBP exhibit greater genotoxic potency than their parent compounds. Interestingly, both compounds induced significant increases in mutation frequency at the *tk* locus, but not at the *hprt* locus. In addition, some of the other compounds tested produced chromosomal damage in the absence of damage to individual genes. The genotoxicity of 2NN and 2NDBP in human cells, together with their high concentrations in ambient air, validates the underlying premise of this work: that atmospheric reaction products of PAHs should be considered in genotoxicity assessments of air pollutants.

Studies performed on the fractionated components of simulated atmospheric reaction products of naphthalene were less revealing, because no active fractions were identified. Even the fraction containing the active parent compound was inactive at the concentrations used in the assay. The investigators did not use additional positive controls, such as testing the extract prior to fractionation to determine whether mutagenic activity was present in the whole extract, to guide the fractionation experiments. This flaw does not substantively affect the interpretation that the investigators have placed on their

GENOTOXICITY TESTS USING HUMAN CELL LINES

hprt and _tk_ Assays

These assays are useful to examine genetic damage at one locus (that is, the nonessential *hprt* or *tk* gene). The *hprt* gene contains the genetic information to produce the hprt enzyme, a protein that aids the cell in utilizing biochemicals called purines. This protein is not essential for cell growth and survival. If the purine is a normal substrate (for example, hypoxanthine or guanine), the hprt enzyme converts it into a nucleotide that is used to synthesize new nucleic acids. If the purine is a toxic analogue (for example, 6-thioguanine), the hprt enzyme converts it into a nucleotide derivative that leads to a toxic effect or death of the cell. When induced mutations have destroyed the ability of the *hprt* gene to produce the normal hprt enzyme, it is not available to convert 6-thioguanine into the toxic derivative. Therefore, only the cells with the *hprt* mutation, which lack the functional hprt enzyme, can grow and form visible colonies that can be counted in the presence of 6-thioguanine. The *tk* mutation assay functions in a manner similar to that described for the *hprt* assay, using trifluorothymidine as the toxic analog rather than 6-thioguanine.

CREST Modified Micronucleus Assay

This assay is used to examine clastogenicity, the ability of an agent to produce large-scale chromosomal damage such as breakage and loss. In this assay, human cells are microscopically examined for chromosomal damage after treatment with a test agent by staining the exposed cells with an antibody that recognizes proteins at the centromere-associated kinetochore of chromosomes.

results, however, which were largely negative in this aspect of the study. The fact that the concentrations of known mutagens in the various fractions were present at concentrations appreciably lower than required for activity as pure agents provides a plausible explanation for the negative results in the fractionation experiments.

Finally, the role of biotransformation in the activation of these PAHs to genotoxic species was determined for 2NN and 2NDBP. In contrast to the situation with the MCL-5 and AHH-1 h1A1v2 lines, these compounds were not mutagenic in the L3 line, indicating that oxidative activation mediated by cytochrome P450 was necessary to induce genotoxicity at the *tk* locus. These findings demonstrate that conclusions derived from various test systems will be driven by the particular array of metabolic processes present in the cells used in the assay.

IMPLICATIONS FOR FUTURE RESEARCH

Some of the PAHs tested by the investigators were genotoxic at the chromosomal level in the absence of the induction of gene mutations. Because large-scale chromosomal damage is not detectable in the standard bacterial mutagenesis assays, these studies reinforce the view that the use of bacterial test systems alone may not be adequate for evaluating the genotoxicity of gas-phase PAHs and their atmospheric reaction products. Therefore, screening methods for evaluating the genotoxicity of motor vehicle emissions need to be expanded to include multiple assays focused on variable types and levels of genetic damage.

The results of this study also demonstrate that atmospheric reaction products may be more genotoxic than their parent hydrocarbons in human cells—as has been demonstrated in nonmammalian test systems. These findings point to a need to continue to characterize and identify atmospheric reaction products and the effects they may exert on human health. It should be noted that the results of this study are based on in vitro testing alone. Extrapolation of in vitro genotoxicity results from a given test to populations where tremendous heterogeneity exists for the regulation, expression, and activity of metabolizing enzymes will not be straightforward and probably will require confirmation in in vivo systems. This problem is not unique to atmospheric PAHs, however.

CONCLUSIONS

The investigators examined the genotoxicity of two abundant gas-phase PAHs, naphthalene and phenanthrene; a subset of their atmospheric transformation products; and fractions of simulated atmospheric reaction products of naphthalene using metabolically competent mammalian cell lines. The study reports several interesting and important findings that have implications reaching beyond the specific details of this project. The results demonstrate that atmospheric reaction products of PAHs, including 2NN and 2NDBP, may be more mutagenic and clastogenic than their parent molecules in human cells. In addition, the study demonstrates that these compounds require oxidative metabolism to exert their genotoxic activity. These findings reinforce the need to identify atmospheric reaction products of PAHs further and to characterize their genotoxicity in test systems using metabolically competent human cells and multiple genetic endpoints.

The findings of this study raise interesting questions, answers to which are essential to protect public health better. It is not possible, however, on the basis of these results alone, to conclude definitively that the atmospheric presence of these PAHs presents a risk to human health, since extrapolation from in vitro testing to humans may not always be accurate. This type of information can, however, focus the more complicated in vivo studies on the most significant atmospheric PAH compounds.

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