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Biomonitoring of Nitropolynuclear Aromatic Hydrocarbons via Protein and DNA Adducts

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Includes the Commentary of the Institute's Health Review Committee

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HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe.

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HEI Statement

Synopsis of Research Report Number 64

Development of Biomarkers to Monitor Carcinogen Exposure

BACKGROUND

Exposure to polycyclic aromatic hydrocarbons (PAHs) and their nitro-substituted derivatives (nitro-PAHs), products of incomplete combustion, is widespread. This is of concern because individual PAHs and PAH-containing mixtures cause tumors in animals and they are suspected to contribute to human cancer. To assess their carcinogenic potential in humans, however, requires more accurate exposure information than can be obtained from either air measurements or surrogate exposure estimates such as employment records. Biomarkers of PAH exposure that measure the internal dose or the effective dose would enable better risk assessments than currently exist.

The possibility of utilizing a biomarker to measure exposure to PAHs or other carcinogens is not far-fetched. In the last few years, scientists have taken advantage of the fact that many toxic chemicals are highly reactive and bind tightly to large cellular molecules such as DNA or proteins. These pollutant-biomolecule structures, called adducts, are often unique to the particular pollutant, and researchers are beginning to use them to monitor specific pollutant exposures. Both DNA adducts and protein adducts have the potential to serve as biomarkers of PAH exposure, but work to develop reliable and sensitive assays is needed. This study is one of a series of studies supported by HEI to advance the development of biomarkers of exposure to constituents of diesel engine exhaust, especially nitro PAHs.

APPROACH

Dr. Karam El-Bayoumy and his associates set out to develop methods for measuring DNA and protein adducts formed in animals after exposure to nitro-PAH derivatives. To begin the development process, they treated laboratory rats with two important PAHs: 1-nitropyrene, which is the most abundant nitro-PAH, and the highly mutagenic and tumorigenic 1,6-dinitropyrene. At intervals, tissue and blood samples were taken from the treated rats and examined for the presence of DNA and protein adducts. In addition, Dr. El-Bayoumy determined the structures of several of these adducts and began to investigate the mechanisms involved in their formation.

RESULTS AND INTERPRETATION

Following nitropyrene treatment, stable nitro-PAH adducts were formed with hemoglobin, with plasma albumin, and with DNA from white blood cells and other tissues. One striking finding was that, contrary to the original assumption that 1-nitropyrene adducts would form on the globin subunit of hemoglobin, the adducts were located exclusively in the heme subunit. Because of the complexity of heme adduct chemistry, the investigators were unable to determine the structure of the 1-nitropyrene–hemoglobin adducts. However, their results with both hemoglobin and albumin adducts suggest that measuring 1-nitropyrene–protein adducts may be a practical method for measuring recent nitro-PAH exposure. The DNA adduct studies were inconclusive. Treatment of rats with 1-nitropyrene resulted in the formation of multiple DNA adducts, only one of which could be identified. 1,6-Dinitropyrene administration produced a much simpler DNA adduct pattern, suggesting that 1,6-dinitropyrene–DNA adducts might be better biomarkers than 1-nitropyrene–DNA adducts. Whether this has practical application in humans is not clear because dinitropyrenes are only 1% as abundant in environmental emissions as 1-nitropyrene.

In summary, results of this investigation provide a good foundation for the future development of quantitative assays for monitoring exposure to diesel engine emissions and other air pollutant mixtures containing nitro PAHs. However, before either DNA or protein adducts of nitropyrenes can be used as biomarkers in epidemiological studies, more work is needed to determine the nature of the adducts and to develop sensitive, specific, and inexpensive methods for their detection.

This Statement is a summary, prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1987 to 1991. This study was conducted by Dr. Karam El-Bayoumy and associates of the American Health Foundation, Valhalla, NY. 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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Biomonitoring of Nitropolynuclear Aromatic Hydrocarbons via Protein and DNA Adducts

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INVESTIGATORS' REPORT

Biomonitoring of Nitropolynuclear Aromatic Hydrocarbons via Protein and DNA Adducts

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ABSTRACT

Nitropolynuclear aromatic hydrocarbons (nitro-PAHs) are widely distributed in the environment. For several chemicals in this class of compounds, mutagenic activity in bacterial and mammalian systems and tumorigenic activity in laboratory animals have been clearly documented. Procedures for assessing the risk to humans from exposure to nitro-PAHs have not been clearly defined, despite the widespread occurrence of such agents in the environment and their possible involvement in the etiology of some human cancers.

Several methods are available for determining exposure, uptake, and metabolic activation of genotoxic carcinogens in humans. DNA adducts currently are regarded as the most direct markers of genotoxicity. However, several proteins are equally capable of forming adducts with electrophiles derived from xenobiotics. We focused on developing methods to detect and quantify adducts of 1-nitropyrene and 1,6-dinitropyrene with proteins and with DNA. 1-Nitropyrene is the most abundant nitro-PAH in emissions from combustion sources such as diesel engines. Although 1,6-dinitropyrene is far more mutagenic and more tumorigenic than 1-nitropyrene, it is present in the environment at lower levels. Seeking a highly sensitive method, we have utilized the ^{32}P-postlabeling technique to establish the pattern of the DNA adducts formed in rat tissues, as well as in peripheral blood lymphocytes, following administration of both 1-nitropyrene and 1,6-dinitropyrene. We also present results on hemoglobin and albumin adducts formed after administration of these nitro-PAHs.

[^{3}H]1-Nitropyrene was given to male or female Fischer-344 or Sprague-Dawley rats by gavage at five dose levels ranging from 0.1 to 1,000 μg/kg of body weight. This led to stable hemoglobin adducts, which accounted for 0.08% ± 0.05% of the dose. The radioactivity associated with hemoglobin following administration of [^{3}H]1-nitropyrene was cleared with a half-life of 13.6 days. This is faster than the clearance of unmodified erythrocytes in the rat (half-life of 30 days). Treating the hemoglobin with 1% hydrochloric acid in acetone, to precipitate the globin, released the radioactivity so that none remained bound to the globin. Rather, the radioactivity remained bound to the heme moiety. To obtain structural information about the heme adducts, we incubated [^{3}H]1-nitrosopyrene and [^{3}H]4,5-epoxy-4,5-dihydro-1-nitropyrene with rat hemoglobin. In each case, [^{3}H] was bound mainly to globin and, to a lesser extent, to the heme moiety. These findings contrasted with those obtained in vivo, thereby suggesting that these metabolites may not be required in the binding of 1-nitropyrene to the heme moiety in vivo. However, the contribution of such metabolites to the in vivo binding cannot be excluded solely on the basis of in vitro findings. Although the structures of the 1-nitropyrene–heme adducts have not been determined, the stability of these adducts may make them useful as dosimeters of human exposure to 1-nitropyrene.

Preliminary studies indicated that [^{3}H]1,6-dinitropyrene binds to rat hemoglobin at a level that is 0.04% of the dose given by gavage. As with 1-nitropyrene, the [^{3}H] was bound to the heme moiety. This observation and the fact that the level of 1,6-dinitropyrene present in the environment is only 1/100 that of 1-nitropyrene directed our focus toward 1-nitropyrene adducts as more suitable dosimeters.

[^{3}H]1-Nitropyrene was found to bind to albumin to approximately half the extent that it binds to hemoglobin, or 0.04% ± 0.01% of the dose given by gavage. The radioactivity associated with albumin following the administration of [^{3}H]1-nitropyrene was cleared with a half-life of 2.5 days, which is not very different from the half-life of unmodified albumin in the rat. Additional in vitro experiments were conducted to learn more about the nature of the 1-nitropyrene–albumin adducts. From incubations of [^{3}H]1-nitrosopyrene or [^{3}H]4,5-epoxy-4,5-dihydro-1-nitropyrene with rat albumin or whole rat plasma, we learned that the major 1-nitropyrene–albumin adduct formed in vivo could not be derived from either metabolite. We then performed pronase digestion of the albumin fraction containing the major adduct, and nitroreduction. The outcome of this treatment suggests that the nitro group is already modified during

* 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 64, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Karam El-Bayoumy, American Health Foundation, 1 Dana Road, Valhalla, NY 10595.

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the initial process of binding of 1-nitropyrene to albumin in vivo.

Mild acid hydrolysis of 1-nitropyrene–albumin adducts yielded a phenolic derivative, which, upon acetylation, yielded a material with a mass spectrum similar to that of 1-acetylamino-*x*,*y*-diacetoxypyrenes, where *x* and *y* are undefined isomers. With high-performance liquid chromatography, this compound also eluted closer to a synthetic mixture of 1-acetylamino-*x*,*y*-diacetoxypyrenes. These findings were further supported by gas chromatography and mass spectrometry analysis using the selected ion monitoring mode. Thus, 1-acetylamino-*x*,*y*-dihydroxypyrene, released from albumin, may be a suitable indicator for monitoring human exposure to and metabolic activation of 1-nitropyrene, provided that human metabolism leads to the same metabolite or metabolites as that of the rat, and that the metabolite is capable of binding with albumin.

Upon administration of 1,6-dinitropyrene to rats, and analysis with ^{32}P-postlabeling, only one adduct derived from nitroreduction, namely *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, was observed in the liver, mammary glands, and peripheral blood lymphocytes. However, treating rats with 1-nitropyrene resulted in the formation of multiple adducts in all of these tissues. The one adduct resulting from nitroreduction, *N*-(deoxyguanosin-8-yl)-1-aminopyrene, was only a minor constituent among all adducts. Incubating calf thymus DNA with mutagenic, ring-oxidized metabolites of 1-nitropyrene in vitro, followed by ^{32}P-postlabeling analysis, resulted in the formation of multiple adducts. The chromatographic behavior of the DNA adducts derived from such metabolites suggests that these adducts may have been formed in vivo; however, this needs to be confirmed. Comparing the rather complex DNA adduct pattern formed with 1-nitropyrene with the simple adduct pattern formed with 1,6-dinitropyrene in the rat suggests that *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene, derived from 1,6-dinitropyrene, may be a better dosimeter, even though 1,6-dinitropyrene is present in the environment in far lower concentrations than 1-nitropyrene. This will require further methods development.

The results obtained to date demonstrate the potential of utilizing protein adducts as well as DNA adducts of 1-nitropyrene and 1,6-dinitropyrene as dosimeters of the uptake and metabolic activation of these nitro-PAHs. These indicators are prerequisites for assessing cancer risk related to exposure to nitro-PAHs.

INTRODUCTION

Nitropolynuclear aromatic hydrocarbons are widely distributed in the environment (reviewed in International Agency for Research on Cancer 1989). Sources include emissions from diesel engines (Schuetzle 1983; Salmeen et al. 1984), airplanes, and kerosene and other fuel heaters (Tokiwa et al. 1985; McCartney et al. 1986). Nitro-PAHs are also found in airborne particulates (Pitts et al. 1978; Gibson 1983; Arey et al. 1988, 1989a,b; Atkinson 1990), coal fly ash (Hanson et al. 1983; Harris et al. 1984), and food (Dennis et al. 1984; Kinouchi et al. 1986). Although an earlier investigation suggested that cigarette smoke contains nitro-PAHs (McCoy and Rosenkranz 1982), findings in our laboratory (El-Bayoumy et al. 1985) do not indicate the presence of 1-nitronaphthalene, 1-nitropyrene (1-NP), or 6-nitrochrysene in mainstream cigarette smoke (detection limit, 1 to 10 ng/cigarette). The mutagenic activities in bacterial and mammalian systems and the tumorigenic activities in laboratory animals of several nitro-PAHs have been clearly documented (Beland et al. 1985; Rosenkranz and Mermelstein 1985; Tokiwa and Ohnishi 1986; Hart et al. 1988).

The degree of carcinogenic risk from human exposure to nitro-PAHs has not been determined, despite the widespread occurrence of such agents in the environment and their possible involvement in the etiology of some human cancers (Hecht and El-Bayoumy 1990). Further research is needed to determine whether nitro-PAHs contribute to human cancer. This research necessitates the development of sensitive analytical methods for detecting and quantifying nitro-PAHs and their metabolites in biological fluids and for assessing their adducts with proteins or DNA in humans. Such analytical methods should allow the quantification of an individual's exposure and uptake of nitro-PAHs (exposure assessment). Risk assessment would be the ultimate goal. Such assessment can best be determined on the basis of biological activity (for example, tumor data in laboratory animals) of nitro-PAHs and epidemiological studies combined with data obtained from exposure assessment studies.

DNA adducts are currently regarded as the most direct markers of genotoxicity (Wogan 1988). Determining the presence and extent of DNA adducts of genotoxic agents is considered a feasible method for monitoring human exposure. Several methods, including ^{32}P-postlabeling, are available for detecting and quantifying such adducts (Hemminki et al. 1990; Schoket et al. 1991). The studied exposures of humans include tobacco smoke (Savela and Hemminki 1991), polynuclear aromatic hydrocarbons (PAHs) in the occupational environment (Herbert et al. 1990), coal tar in psoriasis treatment (Zhang et al. 1990), and cisplatin in cancer chemotherapy (Santella 1991). The cited studies demonstrate that DNA isolated from white blood cells can be used for monitoring human exposure. However, some of these studies lack evidence for a correlation between PAH-DNA adducts detected by ^{32}P-postlabeling analysis of ad-

ducts in white blood cells and levels of PAHs in the environment. In addition, the absence of knowledge regarding the structures of adducts and the inconsistency of labeling efficiency are drawbacks to this technique.

Some proteins are as capable as DNA of forming adducts with electrophilic compounds (Skipper and Tannenbaum 1990). Measurements of hemoglobin adducts have been used for monitoring occupational exposures to acetaldehyde (Stevens et al. 1981), ethylene oxide (Calleman et al. 1978; Farmer et al. 1986; Törnqvist et al. 1986), and propylene oxide (Osterman-Golkar et al. 1984), and lifestyle exposures such as tobacco smoking (Foiles et al. 1992) and excessive consumption of alcohol (Stevens et al. 1981). Therefore, protein adducts can be used reliably to monitor human exposure and the metabolic activation of carcinogens such as nitro-PAHs. The advantages and drawbacks of each method have been described by Ashby (1988).

Detection and quantification of carcinogens and their metabolites in human urine also have been applied to exposure assessments (Groopman et al. 1984; El-Bayoumy et al. 1986a; Murray et al. 1989; Groopman and Zarba 1990; Stettler et al. 1992). In this research project, we focused on developing methods to detect and quantify adducts of nitro-PAHs with proteins as well as with DNA. Two representative compounds were used in this study: 1-NP and 1,6-dinitropyrene (1,6-DNP). 1-Nitropyrene is the most abundant nitro-PAH in sources such as diesel engine emissions; it is both mutagenic and tumorigenic. Dinitropyrenes are also found in diesel engine emissions, albeit at much lower concentrations than 1-NP. However, dinitropyrenes are more mutagenic in bacterial and mammalian systems and more tumorigenic in laboratory animals than 1-NP (Tokiwa and Ohnishi 1986; King 1988; Imida et al. 1991).

Establishing the metabolic profiles of 1-NP and 1,6-DNP in laboratory animals is essential for developing sensitive methods for detecting and quantifying these compounds in humans. In the mammalian system, 1,6-DNP is metabolized mainly via nitroreduction; however, 1-NP is metabolized by nitroreduction, ring oxidation, and a combination of both pathways (Roy et al. 1988) (Figure 1). 1-Nitropyrene is activated in bacterial systems via nitroreduction to yield a major DNA adduct, *N*-(deoxyguanosin-8-yl)-1-aminopyrene (*N*-dG-AP) (Messier et al. 1981; Howard et al. 1983). Studies in our laboratory and elsewhere indicate that simple nitroreduction of 1-NP to yield *N*-dG-AP cannot account for the observed DNA adducts in vivo and in vitro (Jackson et al. 1985; Stanton et al. 1985; El-Bayoumy et al. 1988a,b; Mitchell 1988; Roy et al. 1989). To define more clearly the metabolic activation of 1-NP, the determination of the structure of DNA adducts is required. Therefore, some of our efforts in this project were directed toward identifying DNA adduct markers that are derived from ring-oxidized metabo-

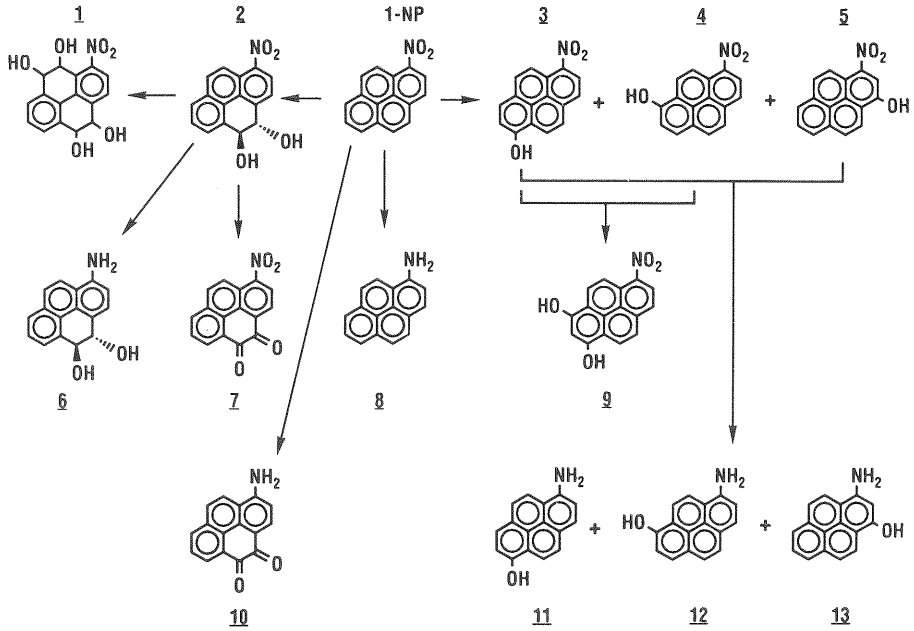

Figure 1. 1-Nitropyrene metabolites identified in vivo, or in vitro, or both. Acetylation of the amino functionality and conjugation (glucuronides and sulfates) of C-hydroxylated metabolites have also been demonstrated in vivo in F344 and Sprague-Dawley rats.

lites of 1-NP. In contrast, 1,6-DNP is metabolically activated in vivo via nitroreduction to yield *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (*N*-dG-ANP) (reviewed by Fu 1990). Seeking a highly sensitive method, we utilized the ^{32}P-postlabeling technique (Randerath et al. 1981) to establish maps of the DNA adducts obtained from rat tissues such as liver, mammary tissues, and peripheral blood lymphocytes following the administration of 1-NP and 1,6-DNP. In this report, we also present results on the binding of 1-NP and 1,6-DNP to hemoglobin and albumin.

SPECIFIC AIMS

The following are two of the major aims we originally proposed:

1. To develop sensitive analytical methods for measuring human uptake of 1-NP and 1,6-DNP via two approaches: detection and quantification of DNA adducts using ^{32}P-postlabeling, and detection and quantification of protein adducts by gas chromatographic (GC) and mass spectrometric (MS) analysis.
2. Having established these methods in animal models, we proposed pilot studies with individuals exposed to diesel engine emissions. However, because of the enormous amount of work proposed in Specific Aim 1, the Health Effects Institute Research Committee and ad hoc reviewers suggested that animal studies should take precedence over the proposed studies in humans.

METHODS

CHEMICALS

Commercial 1-NP (Aldrich Chemical Co., Milwaukee, WI) was purified by column chromatography on silica gel with elution by 10% benzene in hexane. The purity of the 1-NP, assessed by GC with electron capture detection (El-Bayoumy et al. 1988b) and by GC and MS analysis, was greater than 99.9%; no dinitropyrenes were detected. 1-Nitrosopyrene (1-NOP) was synthesized by oxidation of 1-aminopyrene using *m*-chloroperoxybenzoic acid and the method described by El-Bayoumy and Hecht (1983). 1-Nitro-3-hydroxypyrene, 1-nitro-6-hydroxypyrene, and 1-nitro-8-hydroxypyrene were obtained by nitration of 1-acetoxypyrene,followed by deacetylation (El-Bayoumy and Hecht 1983). Treating a mixture of acetylamino-6(8)-acetoxypyrene with lead tetraacetate at 20°C for 30 minutes led to a mixture of 1-acetylamino-*x*,*y*-diacetoxypyrenes in less than 10% of the yield. The *x* and *y* designations are used here because isomers have not been defined in this mixture. Mass spectrometry analysis results were as follows (mass-to-charge ratio [*m*/*e*], relative intensity): 375 (M^-, 5), 333 (M^-, -42, 10), 291 (M^-, -84, 40), 249 (M^-, -126, 100).

Refluxing of 1-NP with *m*-chloroperoxybenzoic acid in methylene chloride yielded 4,5-epoxy-4,5-dihydro-1-nitropyrene (1-NP-4,5-oxide) and 9,10-epoxy-9,10-dihydro-1-nitropyrene (1-NP-9,10-oxide) (Fifer et al. 1986a). The *cis*- and *trans*-4,5-dihydro-4,5-dihydroxy-1-nitropyrene (*cis*- and *trans*-1-NP-4,5-DHD) were also synthesized (El-Bayoumy et al. 1986b). The 1,6-DNP was acquired commercially (Chemsyn, Lenexa, KS) but was also synthesized in our laboratory by a method described by Hashimoto and Shudo (1984). 1-Nitroso-6-nitropyrene was prepared by the method described by Fifer and associates (1986b). The [^{14}C]1-NP was synthesized in our laboratory with a radiochemical purity greater than 99%, and a specific activity of 5 mCi/mmol (El-Bayoumy and Hecht 1984). The [^{3}H]1-NP (13.7 Ci/mmol) and [^{3}H]1,6-DNP (13 Ci/mmol) were acquired from Chemsyn, and pure samples were obtained by silica gel thin-layer chromatography (TLC) with benzene as an eluent; both radiochemicals were more than 99% pure, as ascertained by reverse-phase high-performance liquid chromatography (HPLC) and by monitoring radioactivity with a Flow-One radioflow detector.

The [^{3}H]1-NOP and [^{3}H]1-NP-4,5-oxide were also prepared in our laboratory (El-Bayoumy et al. 1988a; Roy et al. 1991), as were *N*-dG-AP and *N*-dG-ANP. The three major N^2-deoxyguanosine adducts derived from 1-NP-4,5-oxide were synthesized and structurally characterized (Roy et al. 1991). Briefly, calf thymus DNA was dissolved (2 mg/mL in 50-mM citrate buffer, pH 5.8), and the solution was purged with nitrogen (10 minutes). An aliquot of [^{3}H]1-NP-4,5-oxide (23.6 mCi/mmol) or 1-NP-4,5-oxide (from a 4.0-mM stock solution in nitrogen-purged dimethylsulfoxide was added to the DNA solution for a final concentration of 20M to 40M. The solution was incubated for three hours at 37°C. The DNA was purified by solvent extractions and enzymatically hydrolyzed by the method described by Martin and colleagues (1982). The adducts were analyzed by reverse-phase HPLC. The experiment was repeated, and 50 to 100 µg of each adduct was obtained for nuclear magnetic resonance analysis (see the Results section).

Calf thymus DNA (type I), enzymes, and other biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Carrier-free [γ-^{32}P]adenosine 5′-triphosphate (approximately 5,000 Ci/mmol) (Amersham, Chicago, IL) and T_4 polynucleotide kinase (30 units/mL) (U.S. Biochemical Corp., Cleveland, OH) were purchased from commercial sources. The antibodies to rat serum albumin for enzyme-linked immunosorbent assays (ELISAs), as well as the electrophoretically pure rat serum albumin, were provided by Dr. Richard Feldhoff, University of Louisville, KY.

INSTRUMENTATION

High-performance liquid chromatography was performed with a Waters Associates high-performance liquid chromatograph (Millipore Waters Division, Milford, MA) equipped with a model 510 solvent delivery system, a model U6K septumless injector, a model 440 ultraviolet/visible range detector, and a model 680 automated gradient controller. The HPLC conditions are described in the figure legends. A radioflow detector (Flo-One/Beta Radiomatic Instruments & Chemicals Co., Tampa, FL) was employed in the radiochromatography. Quantitative measurements of radioactivity were made on a Beckman LS 9800 series liquid scintillation counter (Beckman, Pittsburgh, PA). Mass spectra were recorded with a Hewlett-Packard (Charlotte, NC) 5988 dual-source mass spectrometer. Proton nuclear magnetic resonance analyses were recorded at 360 MHz with a Bruker AM-360 MHz spectrometer (Bruker Instruments, Billerica, MA) and at 500 MHz on a Bruker AM-500 spectrometer in dimethylsulfoxide-d_6, in dimethylsulfoxide-d_6 and deuterium oxide (deuterated water), and in deuterated methanol; experimental conditions and typical data acquisition were reported by Roy and associates (1991).

PROTEIN ADDUCT STUDIES

1-Nitropyrene–Hemoglobin Adducts

Acute in Vivo Dose Response. All bioassays were carried out in the laboratories of the American Health Foundation, which is accredited by the American Association for the Accreditation of Laboratory Animal Care, and protocols were approved by the American Health Foundation's Institutional Committee for the Humane Use of Laboratory Animals.

Five groups of three male Fischer-344 (F344) rats weighing 272 ± 6 g (Charles River Breeding Laboratories, Kingston, NY) were given [^{3}H]1-NP in predistilled trioctanoin by gavage. They were housed under standard conditions (El-Bayoumy et al. 1988b) and given tap water and NIH-07 diet (Dyers Inc., Bethlehem, PA) ad libitum. The compound was administered about mid-morning at dose levels of 0.1, 1.0, 10, 100, and 1,000 µg/kg of body weight. Each rat was given 15 µCi, except with the lowest dose, for which 2.2 µCi was administered.

Twenty-four hours after gavage, the rats were anesthetized with ether or halothane, and blood was obtained (average 8 mL/rat, representing approximately 50% of the total blood volume); rats were killed by cardiac puncture (Baker et al. 1980). The blood was drawn into syringes containing a 0.1-mL ethylene diaminetetraacetic acid solution (0.25 M, pH 7.4) to prevent clotting. The blood was then transferred into polypropylene centrifuge tubes (35 mL) and placed on ice. All subsequent steps were carried out at 4°C. The blood was centrifuged at 900 × *g* to pack the red blood cells. The supernatant plasma was drawn off with a Pasteur pipette and processed as described below. The red blood cells were washed twice with 15-mL portions of 0.9% saline and centrifuged at 900 × *g* each time. The supernatant was kept at normal freezer temperatures. Cell rupture and release of hemoglobin was minimal, as evidenced by the slight coloration of the supernatant. The red blood cells were then lysed by vortexing with 15 mL of ice-cold distilled water for five minutes. Ten milliliters of 0.67 M potassium *ortho*phosphate buffer (pH 6.5) was added, and the solution was centrifuged at 27,000 × *g* for 20 minutes. The supernatant containing the hemoglobin was placed into dialysis tubes (Spectra/Por 4, 14,000 molecular weight cutoff, Spectrum Medical Industries, Los Angeles, CA) and was dialyzed against distilled water (2.5 L with four changes during four days.

The hemoglobin solutions were frozen at −20°C until analysis. The hemoglobin obtained from each rat was analyzed individually. Five-milliliter aliquots of each test solution were placed in scintillation vials containing 0.5 mL of 10 N sodium hydroxide; these vials were brought up to 50°C for two hours to hydrolyze the hemoglobin. After cooling, 5 mL of 50% hydrogen peroxide was added to bleach the solution. The solutions were neutralized with 6 N hydrochloric acid (HCl), and scintillation fluid (Monofluor, National Diagnostic, Manville, NJ) was added. The samples were stored at 4°C in the dark for several days to minimize chemiluminescence and then were counted. This set of experiments was also performed with female F344 rats and male and female Sprague-Dawley rats. The latter were selected because they are susceptible to tumor induction by nitro-PAHs and can be used in experiments intended to compare levels of DNA adducts in target tissues to levels of hemoglobin adducts. These data will be valuable as baseline studies for future investigations in humans.

Adduct Persistence in Vivo. Three male F344 rats (162 ± 9 g of body weight) were gavaged with 2 mCi 1-NP (0.71 µmol) in trioctanoin. Approximately 0.5 mL of blood was drawn from the orbital sinus of each rat at the following intervals: 1, 3, 6, 8, 14, 28, 35, 42, 49, 56, 63, and 68 days. The hemoglobin was isolated as described above, and the bound radioactivity was measured.

Chronic in Vivo Exposure. Five male F344 rats (187 ± 5 g of body weight) were gavaged three times/week on Mondays, Wednesdays, and Fridays for five weeks with [^{3}H]1-NP (0.1 mCi, 0.006 µmol; total dose 1.5 mCi, 0.009 µmol) in trioctanoin. Blood (0.5 mL/rat) was drawn twice weekly via the orbital sinus on Tuesdays and Thursdays; the hemoglobin was isolated, and the bound radioactivity was quantified.

Isolation and Chromatographic Analysis. The thawed hemoglobin samples were added dropwise to 200 mL of rapidly stirred 1% HCl in acetone on ice. The globin precipitate was filtered, dried, and kept frozen at −20°C for further analysis. The acidic acetone solution was evaporated to dryness under reduced pressure. The residue was resuspended in tetrahydrofuran, the radioactivity was measured, and an aliquot was analyzed by reverse-phase HPLC. The heme fraction that contained radioactivity was treated with sulfuric acid and methanol (CH_3OH) to derivatize the carboxylic acid groups on the heme moiety to methyl esters and was treated with zinc acetate to form methylated zinc metalloporphyrins (Ortiz de Montellano and Kunze 1980, 1981; Ortiz de Montellano and Mathews 1981; Grab et al. 1988); the heme fraction was then analyzed by HPLC. As an alternative approach, the whole hemoglobin was treated in a similar manner; this simultaneously precipitated the protein and derivatized the heme moiety to the corresponding methyl ester.

In Vitro Response. Both 1-NOP and 1-NP-4,5-oxide are 1-NP metabolites that bind to DNA. They also represent both metabolism pathways of 1-NP: nitroreduction and ring oxidation. Freshly isolated rat hemoglobin was incubated at room temperature with both compounds for 4.5 hours (Green et al. 1984). The [^{3}H]1-NOP (0.7 μCi, 0.08 pmol) was incubated under both aerobic and anaerobic conditions. The anaerobic conditions were employed to minimize further oxidation of 1-NOP to 1-NP. The [^{3}H]1-NP-4,5-oxide (0.94 μCi, 0.013 μmol) was incubated under aerobic conditions only.

1-Nitropyrene–Plasma Protein Adducts

Isolation and Chromatographic Analysis. The plasma obtained from the dose-response study described above (2 to 3 mL each time for optimal separation) was placed on top of the 2 × 60-cm Cibacron Blue F3GA dye column, which was bound to agarose to function as an affinity column (Reactive Blue 2-Sepharose, Sigma Chemical Co.). The column was washed with 125 to 150 mL of 0.01 M Tris-HCl buffer (pH 7.5) containing 0.01 M sodium azide; elution was at a rate of 2 mL/min (Travis et al. 1976; Kellehner and Smith 1979). The buffer was then switched to Tris buffer with 0.2 M sodium thiocyanate. Protein content of the eluent was monitored with a Coomassie Blue assay (Pierce Chemical Co., Rockford, IL). The albumin started eluting approximately 50 mL after switching buffers, and the next 200 mL was collected (see Figure 6; Results section).

Fractionation of plasma using reverse-phase HPLC on a wide-pore C_4 column yielded the same results; three peaks were obtained. Dialysis of each peak indicated that the radioactivity eluted in peak 1 was not associated with protein, whereas peaks 2 and 3 were associated with protein; only peak 3 coeluted with the standard rat albumin. Because peak 2 was associated with considerable radioactivity, we examined the nature of this protein, as described below.

Immunocharacterization of Peak 2. Peak 2 was collected from the Cibacron Blue column, dialyzed, and further purified by HPLC. The solvent was evaporated, and the residue was suspended in 2 mL of phosphate-buffered saline (PBS) (pH 7.2 to 7.4); 100 μL of this solution measured 2,400 dpm. This solution was added to 12 wells (50 μL/well) of a microtiter plate, and a solution of pure rat serum albumin was placed in another 12 wells (50 μL, concentration was 1 μg/mL PBS). The plates were incubated overnight at 4°C and then washed twice with PBS and polyoxyethylene sorbitan monooleate (Tween) and twice with distilled water. The antibody dilutions were made in a 5% solution of nonfat dry milk in PBS, which served as a blocking agent and prevented nonspecific binding of the antibody to the well. The antibodies to rat serum albumin were diluted in 1 mL of PBS, and 1:100 and 1:1000 dilutions were made in the milk solution. Of each diluted solution, 50 μL was placed in wells coated with either peak 2 or electrophoretically pure rat serum albumin. After one hour at room temperature, the wells were washed, and 100 μL of a 1:1000 dilution of alkaline phosphatase–labeled goat anti–rabbit IgG was added to each well. After one hour at room temperature, the plate was washed again, and 100 μL of *p*-nitrophenyl phosphate (1 mg/mL) was added. The absorbance at 405 nm was read after 1 hour and after 2.5 hours.

Enzymatic Digestion of Modified Rat Albumin (Peak 2). Using a Cibacron Blue affinity column, modified rat albumin (peak 2) was isolated from the plasma of male Sprague-Dawley rats that had been gavaged with 2 mCi of [^{3}H]1-NP (13.7 Ci/mmol). After dialysis and lyophilization, aliquots of peak 2 were dissolved in buffer (1 to 2 mg/mL in 0.1 M phosphate buffer) and incubated with Pronase E (protease type XXV, Sigma Chemical Co.) for 24 hours (Turesky et al. 1987; Sabbioni et al. 1990). The ratio of protein in peak 2 to the enzymes was 10:1. The digest was run through a C_{18} solid-phase column (solid-phase octadecyl cartridges) (Baxter Health Care Corp., Burdick and Jackson Division, Muskegon, MI), and the column was washed, first with water, then with CH_3OH; the CH_3OH fraction contained more than 90% of the radioactivity.

An aliquot of the digest was treated with zinc and ammonium chloride to reduce the nitro (NO_2) group. Unlabeled 1-NP was added to the reaction mixture to monitor the reduction conditions.

Acid Hydrolysis of Modified Albumin (Peaks 2 and 3). Peak 2, obtained by Cibacron Blue affinity chromatography, was dialyzed and further purified by HPLC. The peak was hydrolyzed for one hour at 80°C in 0.3 M HCl. Ethyl acetate

extracts of the hydrolysis mixture were analyzed by HPLC; fractions were collected and monitored for radioactivity. Acid hydrolysis of peak 3 was also performed under the conditions described above.

Adduct Persistence in Vivo. A dose-clearance study was carried out to determine the rate of disappearance of the radioactive peaks from the plasma. Eighteen female Sprague-Dawley rats were gavaged with [^{3}H]1-NP in trioctanoin (0.5 mCi each, 13.5 Ci/mmol, average 180.6 nmol/kg of body weight, 44.6 μg/kg of body weight). Groups of three animals each were bled via the orbital sinus 24, 32, 48, 72, 96, and 168 hours after dosing. At each time point the plasma was processed as described above, and levels of peaks 2 and 3 were quantified.

In Vitro Response. Pure rat serum albumin (approximately 40 mg) was dissolved in 10 mL of phosphate buffer (0.1 M, pH 7.5) and incubated with [^{3}H]1-NP-4,5-oxide (0.94 μCi, 0.013 μmol) or with [^{3}H]1-NOP (0.66 μCi, 0.076 pmol) overnight, in a nitrogen atmosphere. The solutions were then chromatographed on a Cibacron Blue affinity column, and radioactivity and protein levels were monitored.

1,6-Dinitropyrene–Hemoglobin Adducts

The hemoglobin binding of 1,6-DNP was investigated using the conditions described above for 1-NP; only two female Sprague-Dawley rats were used. As described in the Results section, levels of binding were low, and radioactive material was also bound to the heme. Because the analyses of the adducts were not straightforward, we decided to focus only on 1-NP.

DNA ADDUCT STUDIES

In Vitro Response

Calf thymus DNA, which had been modified with 1-NP-4,5-oxide (El-Bayoumy et al. 1990) or 1-NP in the presence of xanthine oxidase (Roy et al. 1989), was enzymatically hydrolyzed to 3′-monophosphates; this was followed by ^{32}P-postlabeling. The *trans*-4,5-dihydro-4,5-dihydroxy-1-nitropyrene, 1-nitro-3-hydroxypyrene, 1-nitro-6-hydroxypyrene, or 1-nitro-8-hydroxypyrene, and 1-NP-9,10-oxide each were incubated with calf thymus DNA in the presence or absence of xanthine oxidase (Howard et al. 1983; Colvert and Fu 1986). DNA that had been modified with ring-oxidized metabolites of 1-NP and 1-nitroso-6-nitropyrene (Fifer et al. 1986b; Djurić et al. 1988) was also hydrolyzed to 3′-monophosphates; this was followed by ^{32}P-postlabeling.

In Vivo Response

Female Sprague-Dawley rats (average body weight 100 g at age seven to eight weeks) (Charles River Breeding Laboratory), 7 to 9 weeks old, were gavaged with 1-NP, 1,6-DNP, 7,12-dimethylbenz[*a*]anthracene (DMBA), or pyrene in trioctanoin (Roy et al. 1989). The potent mammary carcinogen DMBA and the noncarcinogenic pyrene were used in this experiment as positive and negative controls, respectively. Blood was obtained 24 hours after treatment by cardiac puncture. By this procedure, the rats were killed, and mammary glands (with surrounding fat pads from the inguinal region) and livers were collected and stored at −80°C until analysis. The livers and mammary glands were homogenized, and the DNA was isolated and purified by the method described by Roy and colleagues (1989). To isolate DNA from peripheral blood lymphocytes, the same animal protocol as that described above was employed. Mononuclear cells were isolated with the aid of Leucoprep separation tubes (Becton-Dickson, Mountain View, CA). Lymphocyte DNA was isolated by the method described by Harris and associates (1985).

Digestion of DNA. DNA was hydrolyzed to deoxyribonucleoside-3′-monophosphates by incubation with equal amounts of micrococcal endonuclease and spleen phosphodiesterases in 10-mM sodium succinate (pH 6.0) containing 5-mM calcium chloride (Gupta et al. 1982). The adducts were enriched by extracting the digests with 1-butanol in the presence of ammonium formate and tetrabutyl ammonium chloride according to the method described by Gupta (1985).

^{32}P-Postlabeling of Isolated Adducts. The adducts were dissolved in water and ^{32}P-postlabeled with carrier-free [γ-^{32}P]adenosine 5′-triphosphate (5,000 Ci/mmol) in the presence of T_4 polynucleotide kinase. Four-directional systems were used, and the adducts were localized by screen-enhanced autoradiography (Roy et al. 1989).

STATISTICAL ANALYSIS

Each point in Figures 2, 3a, 5, and 7 represents the mean ± SD. Group means ± SD were plotted with linear regression lines using graphics software (Grapher, version 1.75, Golden Software, Golden, CO; Statview II, Abacus Concepts, Berkeley, CA). Biological half-lives were calculated by the method of Rumack and Lovejoy (1991).

RESULTS

PROTEIN ADDUCTS

1-Nitropyrene–Hemoglobin Adducts

After dialysis, the hemoglobin was extracted with ethyl acetate. A putative 1-NP metabolite was covalently bound to the hemoglobin, as evidenced by the absence of radioactiv-

ity in the ethyl acetate layer. [^{3}H]1-Nitropyrene formed hemoglobin adducts at 0.08% ± 0.05% (mean ± SD, n = 3 rats) of the dose given orally in trioctanoin. The dose-response data were obtained by measuring the radioactivity in the whole hemoglobin compartment, i.e., after dialysis but before acidic acetone treatment. Using linear regression analysis, the dose-response curve is linear over 5 orders of magnitude ($p<0.01$, r^2 = 0.963) (Figure 2).

As indicated by the disappearance of radioactivity after the administration of a single dose of [^{3}H]1-NP, these adducts appeared to have cleared (by first order kinetics with a half-life of 13.6 days) faster than unmodified rat erythrocytes (zero order kinetics with a half-life of 30 days) (Neumann 1984a; Rumack and Lovejoy 1991) (Figure 3a). Thus, these adducts would be expected to accumulate as a result of chronic exposure; this was, in fact, the case (Figure 3b). After cessation of dosing (after 35 days), the adducts decreased rapidly, as indicated in Figure 3b. The rate of disappearance of the adducts in Figure 3b seems to be faster than that observed in Figure 3a. This is probably due to several factors, including differences between acute and chronic treatment (Fennell et al. 1992).

The hemoglobin was treated with 1% HCl in acetone to precipitate the globin; this released 80% of the initial radioactivity in the fraction containing the heme; the globin had no measurable radioactivity. Ethanol precipitation (Birner and Neumann 1988) of the globin, followed by successive washings with ethanol, ethanol ether, and, finally,

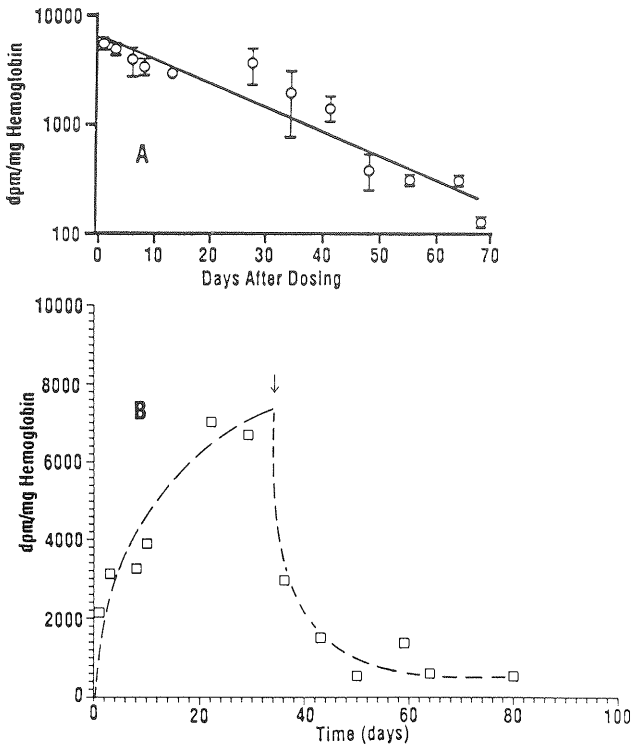

Figure 3. A: Clearance of radioactivity ([^{3}H]1-NP) bound to hemoglobin in rats after a single oral dose of [^{3}H]1-NP. B: Accumulation of radioactivity bound to rat hemoglobin after dosage of [^{3}H]1-NP three times weekly. Blood was drawn twice weekly for five weeks; the arrow marks the point of termination of the dosage regimen. Each datapoint in the figure is the mean ± SD for 3 rats.

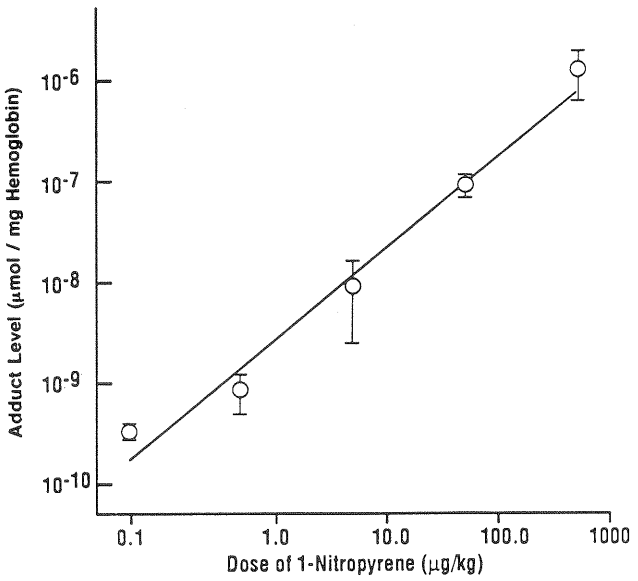

Figure 2. In vivo formation of 1-NP–hemoglobin adducts as a function of a single oral dose of [^{3}H]1-NP. The protein was isolated 24 hours after 1-NP administration. Adduct levels were calculated from the total radioactivity bound to purified hemoglobin. Each datapoint is the mean ± SD from 3 rats.

ether, indicated that the radioactivity still remained bound tightly to the heme fraction, whereas globin had no measurable radioactivity. As shown in Figure 4a, using a C_{18} column and a water and methanol gradient, radioactive material eluted slightly after the unmodified heme; the arrow indicates the elution of the unmodified heme. This radioactive material did not coelute with any of the known 1-NP metabolites. It was not possible to separate the radioactivity from the dark brown heme by silica gel column chromatography. Upon analysis of the radioactive material, using a reverse-phase C_4 column and a water and acetonitrile gradient containing 0.1% trifluoroacetic acid, two radioactive peaks eluted after the unmodified heme (Figure 4b). Treatment of the radioactive fraction with 6 N and 12 N HCl, or with concentrated hydrogen bromide, concentrated hydrogen iodide, or 5 N and 10 N sodium hydroxide did not release a recognizable derivative of 1-NP. In fact, such treatment did not alter the retention time of the original radioactive peak in the C_{18} system (see Figure 4a). This indicated that a radioactive metabolite of 1-NP was covalently bound to the heme.

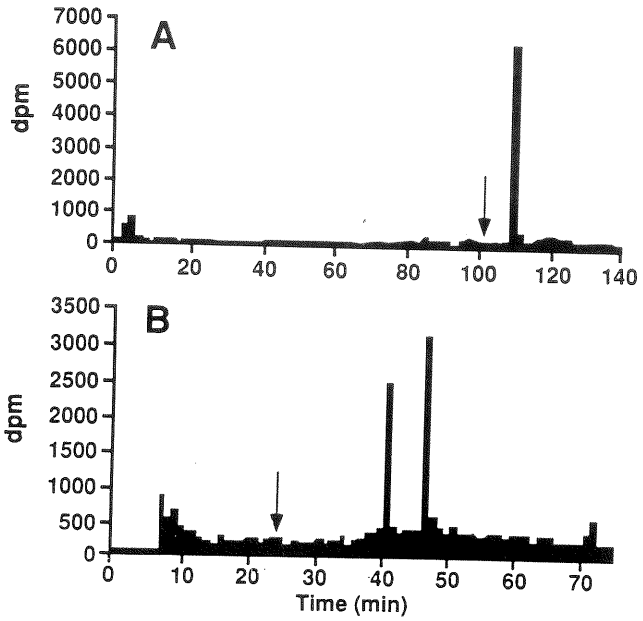

Figure 4. A: HPLC radiochromatogram of the heme fraction obtained by treating 1-NP–hemoglobin adducts with 1% HCl in acetone. HPLC conditions: 0% to 100% CH_3OH in H_2O at 1 mL/min for 110 minutes using a 10-µm Vydac C_{18} column (4.6 × 250 mm) (Separation Group, Hesperia, CA). **B: The heme fraction was also analyzed** using the following HPLC conditions: 23% to 38% solvent A in solvent B in 25 minutes, then into 100% solvent B in 30 minutes at 2 mL/min using a Vydac large-pore C_4 column (10 × 250 mm). Solvent A is 20% acetonitrile in water, and solvent B is 80% acetonitrile in water. Both solvents A and B contain 0.1% trifluoroacetic acid. The arrows indicate the retention time of unmodified heme.

Following a general procedure described by Ortiz de Montellano and Mathews (1991), the heme fraction was derivatized to the methyl ester. The hemoglobin that contained the adducted heme was treated with 10% sulfuric acid in CH_3OH. This simultaneously precipitated the proteins, removed the iron ligand, and methylated the free carboxylic acid groups on the porphyrin. The methylated porphyrins were then isolated by extraction and complexed with zinc using zinc acetate. The zinc-complex was purified by means of silica gel chromatography. The HPLC analysis of the methylated zinc-complex on a C_4 column indicated the presence of two major radioactive peaks that eluted slightly later than those observed before derivatization (data not shown). This is consistent with ester formation. When the in vivo experiments were repeated using [^{14}C]1-NP instead of [^{3}H]1-NP, similar results were obtained. More of the zinc-complex is needed to enable field desorption MS analysis for structural information.

In vitro studies were performed to gain insight into the nature of the binding and to provide ample material for spectral analysis. We incubated [^{3}H]1-NOP and [^{3}H]1-NP-4,5-oxide with rat hemoglobin. Both compounds bound to globin, the former more so than the latter (37% versus 25% of the initial radioactivity). In addition, radioactivity from both compounds was found to be associated with the heme fraction (approximately 20%). The in vitro results do not exclude the contribution of these two metabolites to adduct formation in vivo. However, we did not investigate further whether the radioactivity that was covalently bound to the heme moiety was similar to that observed in vivo.

1,6-Dinitropyrene–Hemoglobin Adducts

The hemoglobin binding of [^{3}H]1,6-DNP was investigated in two rats. Approximately 0.04% (average) of the dose was bound to hemoglobin. The hemoglobin was treated with 1% HCl in acetone, and the acidic acetone fraction was analyzed by reverse-phase HPLC. Neither 1-amino-6-nitropyrene nor any other derivative of 1,6-DNP was detected. As in the case of 1-NP, the radioactivity appeared to be bound to the heme moiety. Because the results were similar to those with 1-NP, and because 1,6-DNP is present at such low levels in the environment, we decided to continue our studies only with 1-NP.

1-Nitropyrene–Plasma Protein Adducts

1-Nitropyrene binds to albumin at a level of 0.04% ± 0.01% (mean ± SD, n = 3 rats) of the dose given by gavage. The binding was linear over 5 orders of magnitude ($p < 0.01$, r^2 = 0.931) (Figure 5). The whole plasma was analyzed by

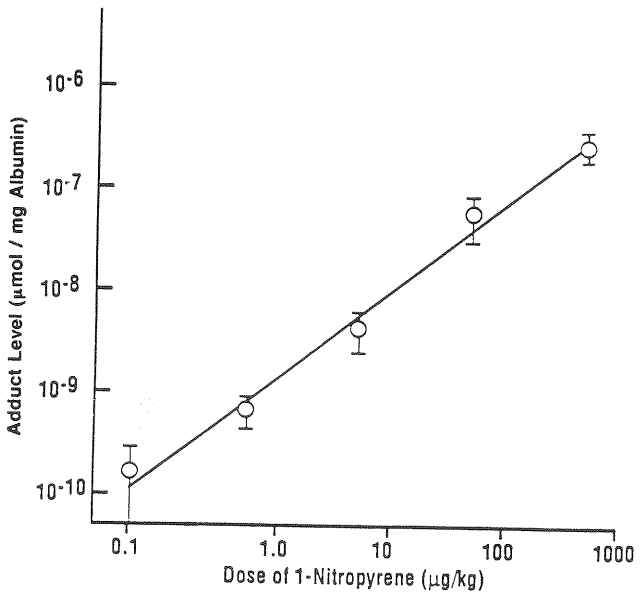

Figure 5. In vivo formation of 1-NP albumin adducts as a function of a single oral dose of [^{3}H]1-NP. The protein was isolated 24 hours after 1-NP administration. Albumin was purified by Cibacron Blue affinity chromatography (see Figure 6) followed by dialysis of the individual peaks, and then the bound [^{3}H] was determined as described in the Methods section. Each datapoint is the mean ± SD for 3 rats.

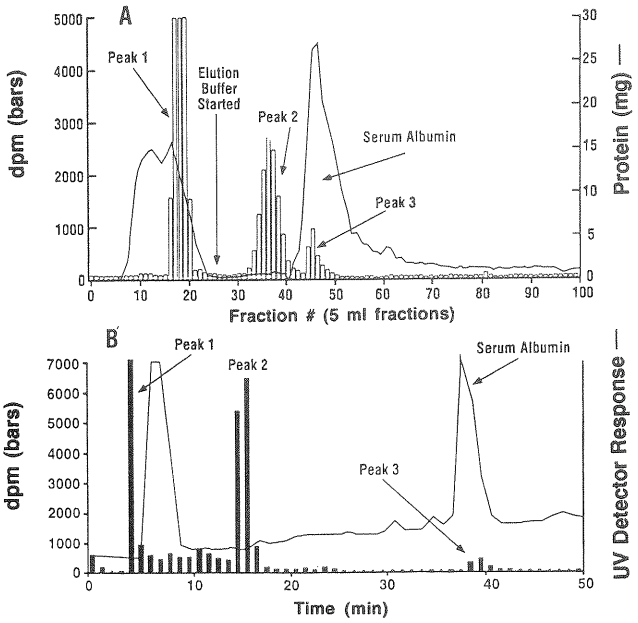

Figure 6. Isolation of albumin adducts of 1-NP. The plasma (from four rats) was obtained by centrifugation of whole blood at 900 × g. The plasma was analyzed on (A) a 2.5- × 60-cm column (Cibacron Blue F3GA bound to agarose); or (B) by a reverse-phase 10- × 250-mm Vydac-C_4 semipreparative column.

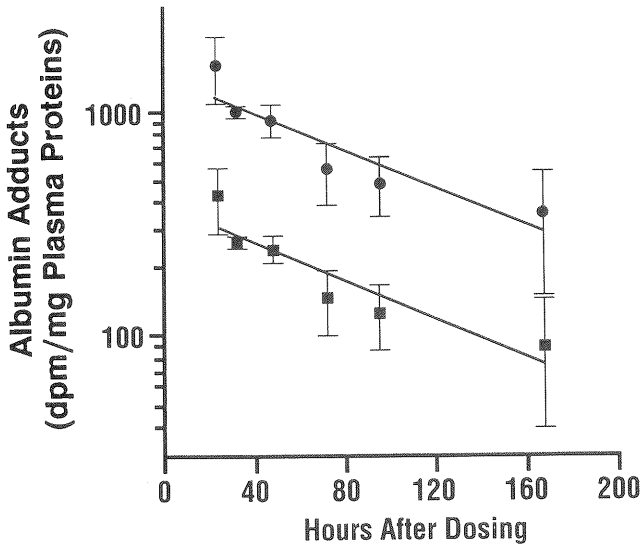

Figure 7. Clearance of radioactivity ([^{3}H]) from albumin adducts (see Figure 6). ●- - -● = peak 2 from Figure 6; ■- - -■ = peak 3 from Figure 6. Measurements were taken following a single dose of [^{3}H]1-NP to rats. Each datapoint is the mean ± SD for 3 rats. Half-life of [^{3}H]1-NP is approximately 60 hours.

Cibacron Blue affinity column chromatography to isolate albumin. Fractions were assayed for both radioactivity and protein content. Figure 6a shows a typical result. The majority of the radioactivity resided in peak 1 (79%), peak 2 contained 16%, and peak 3 contained 4.5%. Aliquots of each peak were dialyzed (14,000 molecular weight cutoff) against water for two days. The results are given below. Radioactivity in peak 1 was not associated with protein; only 2.7% was bound to protein and 97.3% was unbound. However, considerable amounts of the radioactivity associated with peaks 2 and 3 were bound to protein: peak 2, 74.6% bound and 25.4% unbound; peak 3, 47.3% bound and 52.3% unbound.

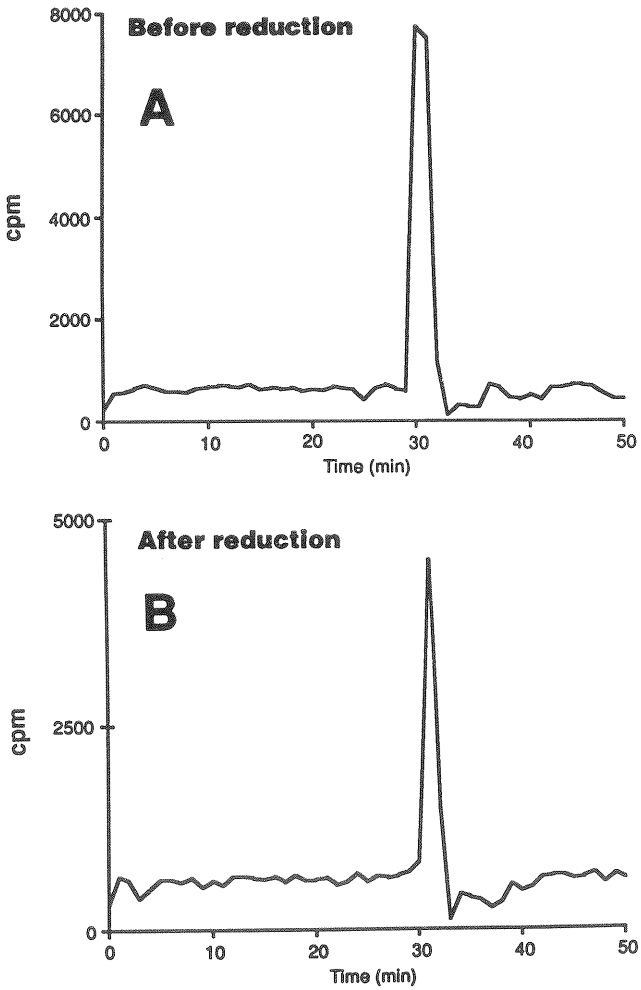

Figure 8. A: Radiochromatogram obtained upon HPLC analysis of pronase digest of 1-NP albumin adduct from peak 2 (in Figure 6). B: The analysis of the Pronase digest after treatment with zinc and ammonium chloride to reduce nitro groups. In this experiment, unlabeled 1-NP was added to the Pronase digest prior to treatment with zinc and ammonium chloride to monitor reduction conditions. A linear gradient from 0% to 100% CH_3OH in water in 50 minutes (buffered with 50-mM ammonium formate, pH 7.4) at 1 mL/min on a Vydac C_{18} reverse-phase column was used in this HPLC analysis.

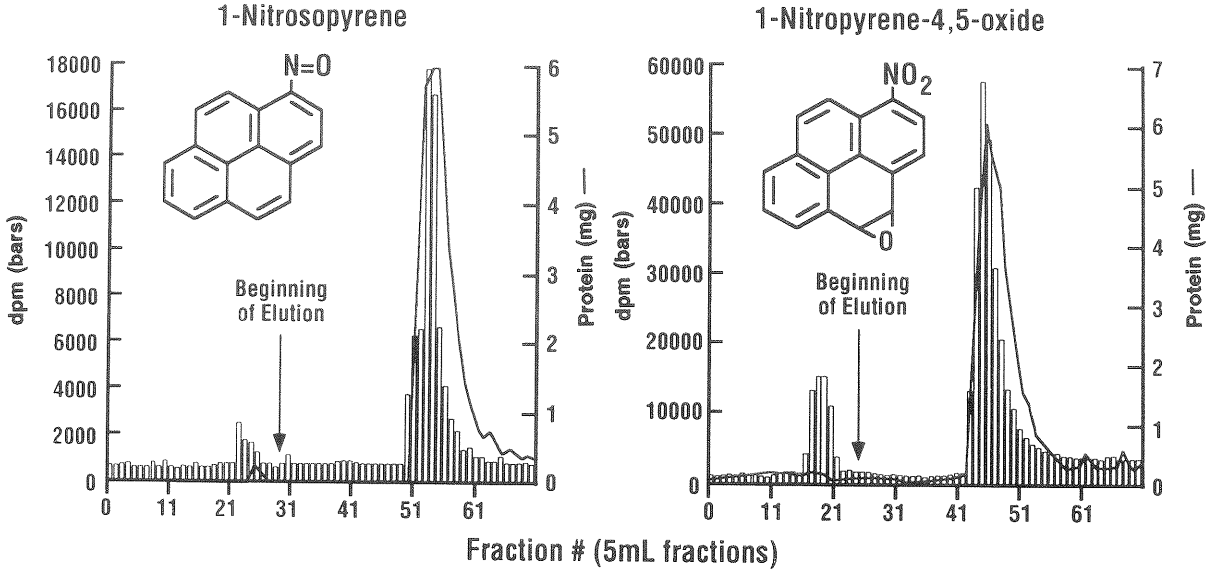

Figure 9. In vitro incubation of rat albumin with [^{3}H]1-NOP and [^{3}H]1-NP-4,5-oxide. The bound radioactivity was analyzed by Cibacron Blue affinity chromatography as described for Figure 6.

Figure 6a does not suggest the presence of any protein associated with peak 2; however, when aliquots of this peak were concentrated, a second protein assay confirmed that peak 2 was also associated with protein.

An HPLC method using a reverse-phase wide-pore C_4 column was developed to analyze plasma. Figure 6b shows an aliquot of whole plasma injected and chromatographed in this program, with monitoring of the ultraviolet absorbance at 254 nm, as well as the radioactivity. As with the Cibacron Blue column analysis, three peaks were detected: only peaks 2 and 3 were associated with protein. However, peak 2 eluted before the standard rat albumin in both systems. On the basis of this finding, we proposed that peak 2 might be associated with proteins other than albumin. Consequently, we determined the identity of this protein by collecting it from the Cibacron Blue column, dialyzing and further purifying it by HPLC, then subjecting it to an ELISA for albumin. As with peak 3, the protein associated with peak 2 was an albumin.

A dose-clearance study was carried out to determine the rate of disappearance of peaks 2 and 3 following a single dose of [^{3}H]1-NP. The plasma was processed at each time point, and the results are shown in Figure 7. Although peak 2 had greater radioactivity, it disappeared at about the same rate as peak 3 (half-life of 2.5 days).

To obtain information about the structural identity of 1-NP–albumin adducts, the following experiments were conducted. Pronase digestion of peak 2, under conditions described by Turesky and associates (1987) and Sabbioni and colleagues (1990), yielded a peak eluting at 31 minutes (Figure 8a). Treating the digest with zinc ammonium chloride under conditions that reduced 1-NP when it was added to the reaction mixture did not alter the retention time of this peak (Figure 8b).

Pure rat albumin was incubated overnight with either [^{3}H]1-NOP or [^{3}H]1-NP-4,5-oxide. Then, albumin adducts were analyzed by the method described above. Monitoring both protein levels and radioactivity (Figure 9) showed that most of the radioactivity was bound to the albumin. Incubation of each of the metabolites with whole rat plasma produced similar results.

Acid hydrolysis of peak 2 (or peak 3), followed by HPLC analysis, essentially yielded a major radioactive peak eluting after 35 to 37 minutes (Figure 10a). Partitioning of this radioactive material between sodium hydroxide and ethyl acetate indicated its phenolic nature. Acetylation with an acetic anhydride and 4-dimethylaminopyridine mixture yielded a derivative that eluted at 52 to 53 minutes (Figure 10b). Figure 10c shows the HPLC analysis of a synthetic mixture of 1-acetylamino-*x*,*y*-diacetoxypyrenes. The inset represents the clearer resolution achieved by optimal HPLC conditions. Although this synthetic mixture eluted one to two minutes after the acetylated hydrolysis product, we suspected that the radioactivity released by the acid also could have been 1-acetylamino-*x*,*y*-dihydroxypyrene. On the basis of MS (Figure 11) and GC and MS analysis with selected ion monitoring (Figure 12), our prediction is justified. Figure 11 compares the MS of the synthetic mixture with the MS of material isolated after the treatment of peak 2 with HCl and subsequent acetylation. In both spectra, we ob-

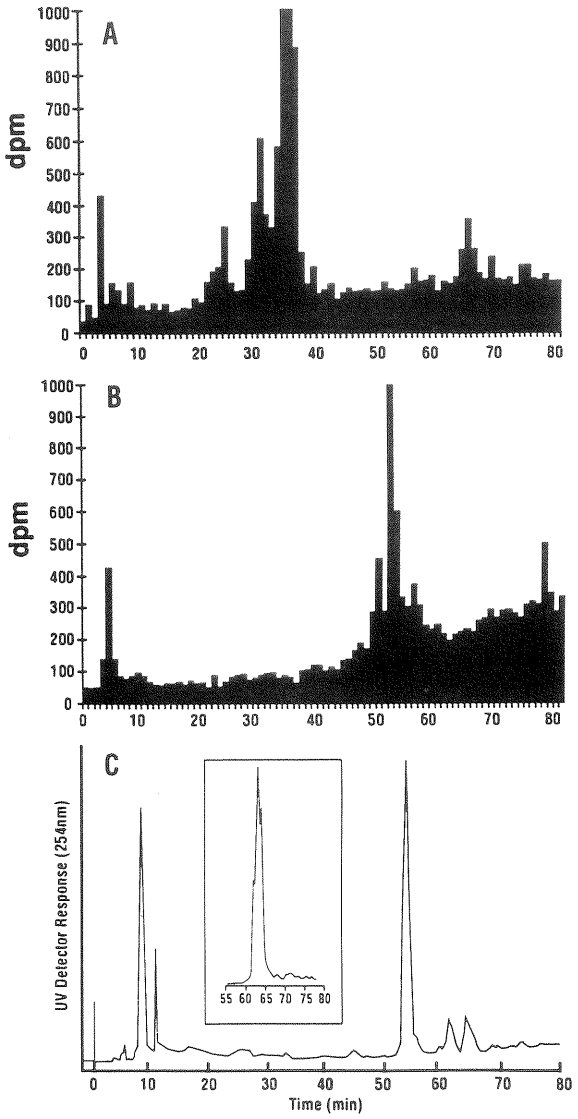

Figure 10. A: Radiochromatogram obtained upon reverse-phase HPLC analysis of the acid hydrolysates of a 1-NP–albumin adduct (peak 2 in Figure 6). **B: Radiochromatogram obtained upon reverse-phase analysis of the acetylated acid hydrolysates of peak 2 in Figure 6. C: Reverse-phase HPLC analysis of synthetic 1-acetylamino-x,y-diacetoxypyrenes.** Better resolution of the major peak was achieved by optimizing HPLC conditions (see inset).

served the molecular ion peak (*m/e* 375), as well as peaks due to the loss of one, two, and three ketene moieties (*m/e* 333, 291, 249). Selected ion monitoring demonstrated that these ions were present only at the GC retention times of the synthetic sample and were isolated from rats treated with 1-NP (Figure 12).

2-Nitrofluorene–Hemoglobin Adducts

Although studying hemoglobin adducts of 2-nitrofluorene (2-NF) was not proposed in the original application, these adducts were examined in this study. 2-Nitrofluorene

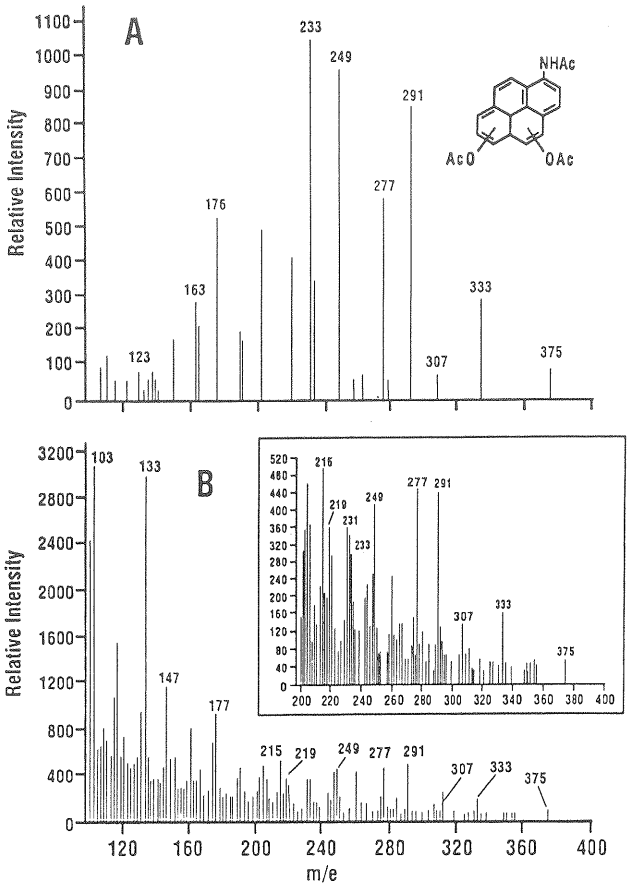

Figure 11. Mass spectrometry of (A) standard 1-acetylamino-x,y-diacetoxypyrenes; and (B) material isolated from acid treatment of rat albumin adduct (peak 2 of Figure 6) followed by acetylation of the phenolic product eluted between 35 and 37 minutes (see Figure 10a). The inset, showing *m/e* 200 to 400, is included for clarity.

is another abundant nitro-PAH present in concentrations close to or exceeding those of 1-NP (Schuetzle 1983). 2-Nitrofluorene is carcinogenic; its metabolism leads to compounds identical to those produced by the potent and well-studied carcinogen 2-acetylaminofluorene (Moller et al. 1987). The major metabolites of 2-NF are 5- and 7-hydroxy-2-acetylaminofluorene; other hydroxylated derivatives of 2-acetylaminofluorene are minor metabolites. During the course of this study, we suggested to the Health Effects Institute Research Committee that 2-NF would be another suitable compound to examine. Accordingly, we initiated a preliminary study. 2-Acetylaminofluorene has been shown to bind to hemoglobin at a level of approximately 108 pmol/g hemoglobin/μmol of 2-acetylaminofluorene administered (Pereira and Chang 1981). Given the similarity of metabolism between 2-NF and 2-acetylaminofluorene, we expected similar results for hemoglobin binding. We gavaged three female Sprague-Dawley rats with [^{3}H]2-NF in trioctanoin (6.25 ×

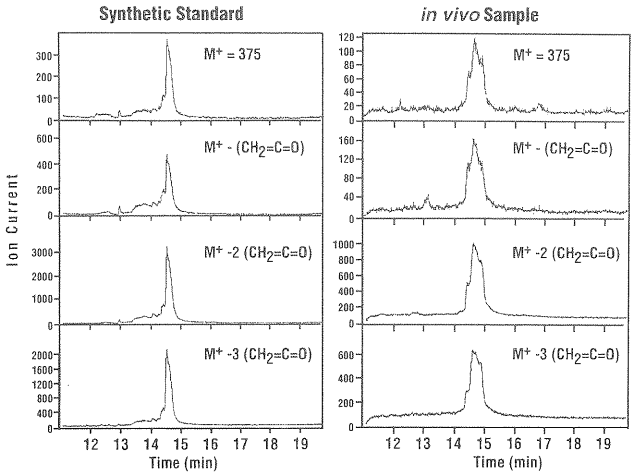

Figure 12. Gas chromatography–mass spectrometric analysis of synthetic 1-acetylamino-x,y-diacetoxypyrenes and the in vivo sample obtained by acid treatment of peak 2 of Figure 6, followed by acetylation of the phenolic product eluted between 35 and 37 minutes (see Figure 10a). Selective ion monitoring was utilized in this analysis. Ion current is given in electron volts.

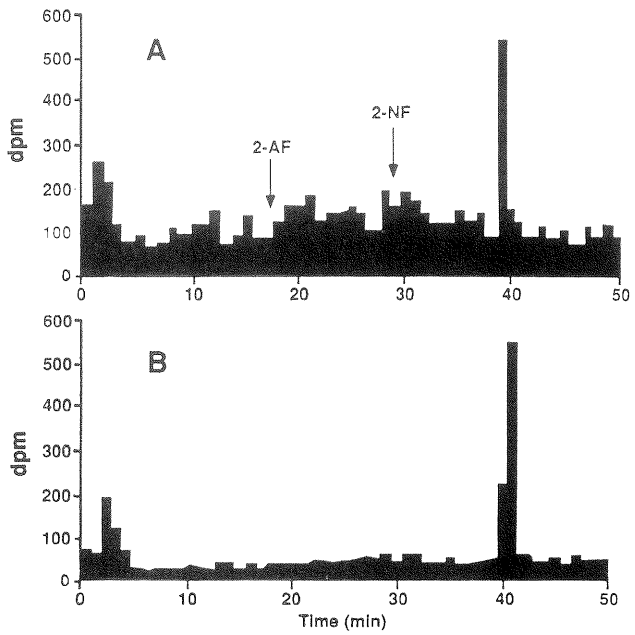

Figure 13. A: Radiochromatogram obtained with reverse-phase HPLC analysis of the acidic acetone fraction of hemoglobin obtained from rats treated with 2-NF. HPLC conditions were 40% to 100% CH_3OH in water in 45 minutes at 1 mL/min, using a Perkin-Elmer (Wilton, CT) HS-5-μm C_{18} column (4.6 × 125 mm). **B: The acidic acetone fraction was chromatographed on a silica gel column and eluted with an ether-benzene mixture.** This was then evaporated, and the residue was analyzed under HPLC conditions identical to those described above.

10^{-5} mmol, average dose 0.29 μmol/kg of body weight). Upon isolating the hemoglobin, we found binding to be 290 pmol/g hemoglobin/μmol of 2-NF administered, almost three times that of 2-acetylaminofluorene. The level of binding of 2-NF (0.06% ± 0.02% of the given dose, mean ± SD, for 3 rats) was comparable to that observed for 1-NP.

Treatment of the hemoglobin solution with 1% HCl in acetone released 37% to 40% of the radioactivity. The HPLC analysis of the released radioactivity showed one major peak at 41 minutes (Figure 13a). In this HPLC program, 2-NF elutes at 28 minutes, 2-AF at 19 minutes, and the azoxy derivative at 48 minutes. The radioactivity was not associated with the heme moiety; when the acidic acetone solution was fractionated on a silica gel column, this compound eluted with the ether-benzene mixture, whereas the heme did not elute under these conditions (Figure 13b).

Future studies will include further characterization of the peak that eluted at 41 minutes, which was the major 2-NF metabolite released via HCl treatment. Enzymatic and mineral acid hydrolysis of the globin will be conducted to release the bound metabolites that constitute the greater amount of bound material (63%). The plasma will also be examined to determine the extent of albumin binding of 2-NF.

DNA ADDUCTS

1-Nitropyrene–DNA Adducts

A major DNA adduct, N-dG-AP, has been obtained by nitro reduction of 1-NP, which is catalyzed by xanthine oxidase; the same adduct was detected by incubating 1-NP with *Salmonella typhimurium*. This adduct is derived from the nitroreduction of 1-NP. We synthesized it by incubating 1-NP with calf thymus DNA in the presence of xanthine oxidase (Roy et al. 1989) and used the adduct as a marker for in vivo studies. A portion of the modified DNA was hydrolyzed to deoxyribonucleosides and then analyzed by HPLC (Roy et al. 1989). This adduct eluted at 28.5 minutes (Figure 14a); upon treatment with sodium hydroxide, the resulting radioactive material eluted at 21.5 minutes (Figure 14b). The other portion of the modified DNA was hydrolyzed to deoxyribonucleoside 3′-monophosphates and analyzed by HPLC using a phosphate-buffered gradient of CH_3OH in water (Roy et al. 1989). Under these conditions, *N*-(deoxyguanosin-8-yl)-1-aminopyrene-3′-monophosphate eluted at 27 minutes. It was collected and analyzed by the ^{32}P-post-labeling technique; the results are illustrated in Figure 15. Only one adduct spot, corresponding to *N*-dG-AP was detected after a five-second exposure of DNA to 1-NP and xanthine oxidase (Figure 15a). By extending the exposure to one minute, two more adduct spots ("b" and "c") were observed (Figure 15b). The adduct spot "a" was absent in control incubations (namely, without xanthine oxidize), as shown in Figure 15c.

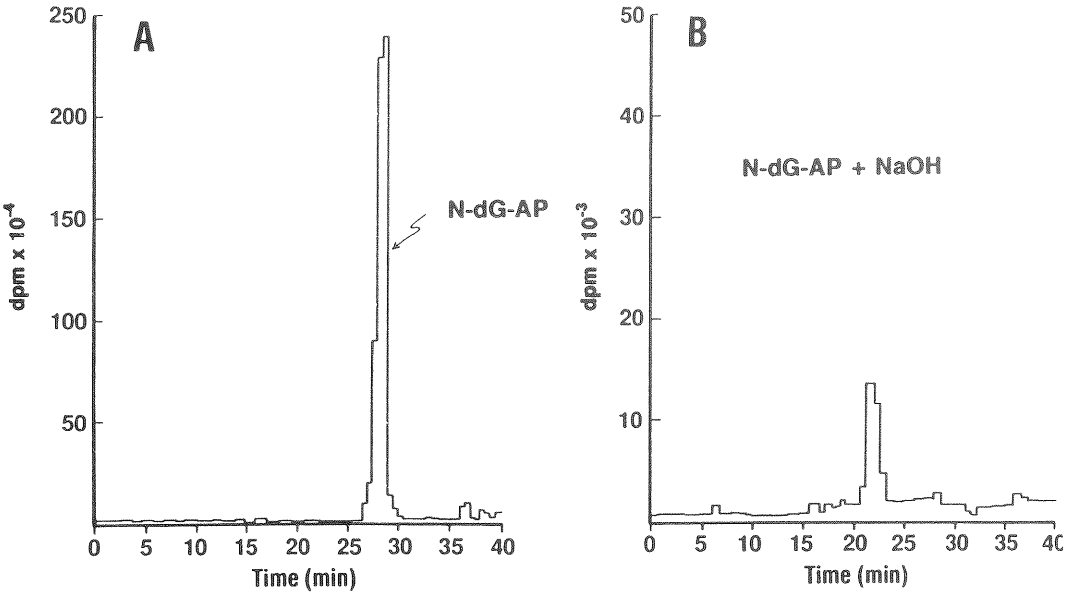

Figure 14. Radiochromatogram obtained (A) upon reverse-phase HPLC analysis of the synthetic marker *N*-dG-AP, and (B) following treatment with sodium hydroxide.

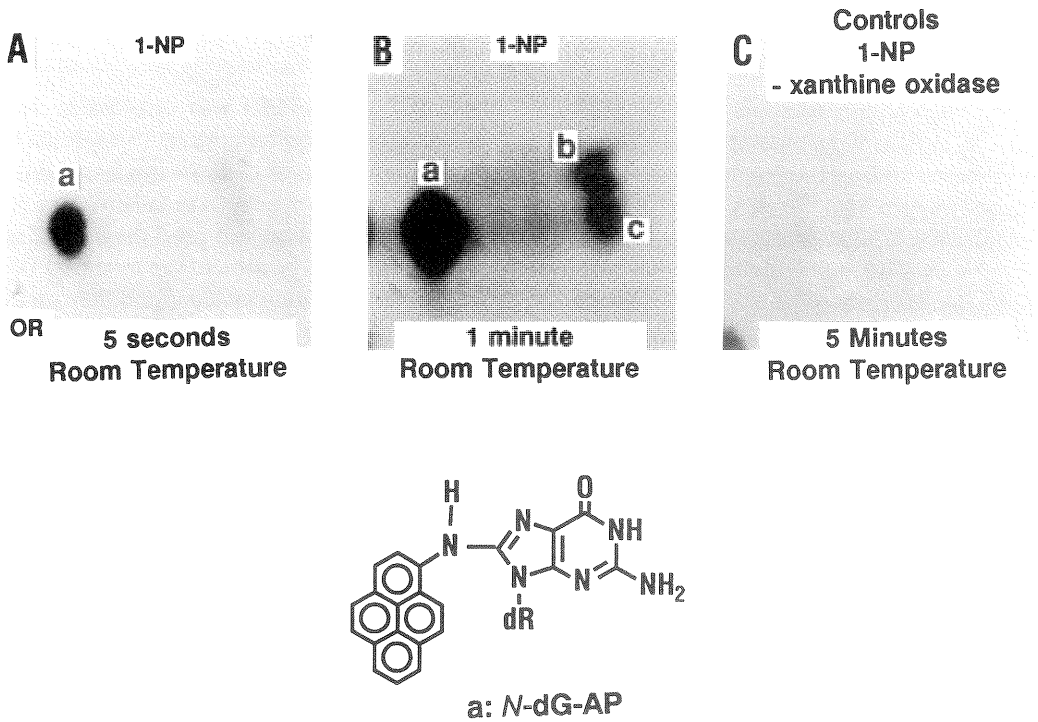

Figure 15. Polyethyleneimine-cellulose TLC maps of [^{32}P]-postlabeling analysis of digests of DNA modified with 1-NP in the presence of xanthine oxidase after film exposures of (A) five seconds, (B) one minute, and (C) in the absence of xanthine oxidase controls after film exposure of five minutes. Solvent systems used for four-directional TLC (10- × 10-cm polyethyleneimine-cellulose plates; Brinkmann Instruments Co., Westbury, NY) were 1 M sodium phosphate, pH 6.8 (D1); 2.5 M ammonium formate, pH 3.5 (D2); 3 M lithium formate and 7 M urea, pH 3.5 (D3); and 0.8 M lithium chloride, 7 M urea, and 0.5 M Tris-buffer, pH 8.0 (D4). *N*-(Deoxyguanosin-8-yl)-1-aminopyrene is labeled as "a"; "b" and "c" are unknown adducts. OR = origin of chromatogram.

In the next phase, we examined whether this adduct could be detected in vivo as a result of treatment with 1-NP. The ^{32}P-postlabeling technique was used to establish the qualitative pattern of DNA adduct formation from 1-NP in the liver, mammary tissues, and peripheral blood lymphocytes of female Sprague-Dawley rats in vivo. The mammary carcinogen DMBA was included in this study as a positive control; pyrene, a noncarcinogenic analogue of 1-NP, was used as a negative control. The ^{32}P-postlabeling maps of DNA obtained from lymphocytes and mammary tissues were similar to those obtained from the liver (Figure 16). The spot labeled "a" in Figures 15a and 16a was excised from the polyethyleneimine-cellulose plates and was contact-transferred to a new plate using button-type magnets (Reddy and Randerath 1987). After cochromatography, which was followed by autoradiography, only a single spot was detected. Thus, multiple adducts were observed upon treatment of rats with 1-NP, including only one minor adduct spot that was derived from nitroreduction. To examine the nature and origin of the other adducts, we prepared markers derived from ring-oxidized metabolites of 1-NP.

1-Nitropyrene-4,5-oxide was synthesized and incubated with calf thymus DNA. The DNA was enzymatically hydrolyzed to deoxyribonucleosides, which were analyzed by HPLC. Three major peaks were obtained in yields of less than 5% (Figure 17a). The structural assignment of these adducts was made by comparing their proton nuclear magnetic resonance spectra with those of *cis*- and *trans*-1-NP-4,5-DHD, and by long-range coupling constants, decoupling experiments, deuterium oxide (deuterium water) exchange, partitions, and acid hydrolysis. Two *trans* adducts (peaks 1 and 2) resulted from the *trans*-1-NP-4,5-DHD addition and one *cis* adduct (peak 3) from the *cis*-1-NP-4,5-DHD addition of the N^2-exocyclic amino group of deoxyguanosine to the C-5-benzylic carbon of the epoxide ring. The proposed structures are shown in Figures 17b and 17c. The ^{32}P-postlabeling maps of DNA that had been modified with 1-NP-4,5-oxide are shown in Figure 18. By comparing chromatographic behaviors, we deduced that these oxide adducts may be responsible for the formation of some of the putative 1-NP–DNA adducts in vivo (El-Bayoumy et al. 1990). Although perhaps coincidental, it should be pointed out that adducts with an R_f value close to that of spots "X" and "Y" observed in vivo (Figure 16) were also found in control animals; this requires further study. The ^{32}P-postlabeling maps of DNA modified with 1-NP-4,5-oxide in the presence of xanthine oxidase showed additional adduct spots, including those that had been observed in the absence of xanthine oxidase. The maps of DNA modified with 1-NP-9,10-oxide in the presence and absence of xanthine oxidase were more complex. Multiple adducts of DNA that had been modified with 1-nitro-3-hydroxypyrene, 1-nitro-6-hydroxypyrene, or 1-nitro-8-hydroxypyrene (Figure 19) or with *trans*-4,5-dihydro-4,5-dihydroxy-1-nitropyrene in the presence of xanthine oxidase were also observed in the ^{32}P-postlabeling assays (Figure 20). The complexity of the ^{32}P-postlabeling maps observed in vitro and after the administration of 1-NP to rats prompted us to examine the ^{32}P-postlabeling map af-

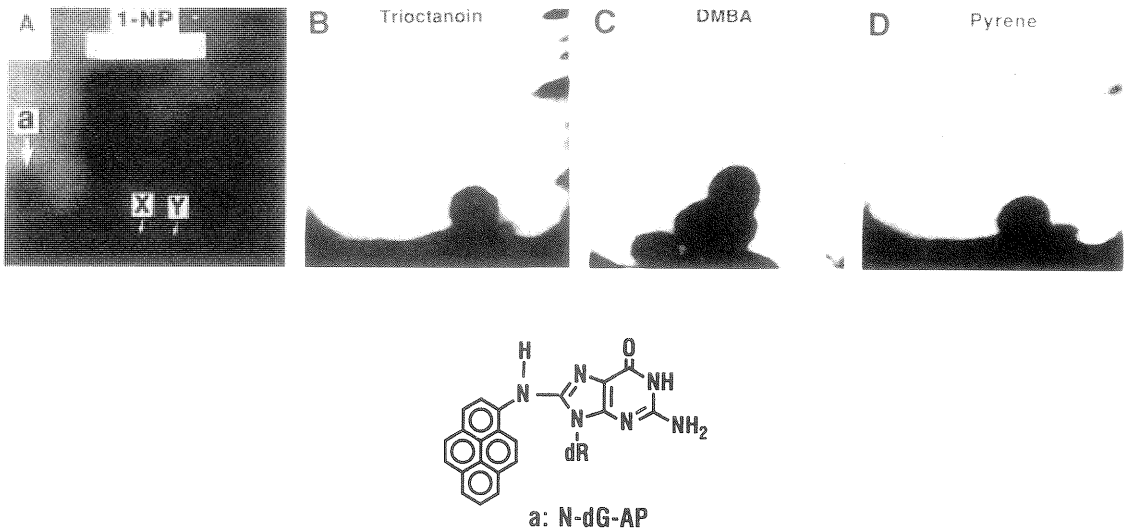

Figure 16. Polyethyleneimine-cellulose thin-layer chromatography maps of ^{32}P-postlabeling analysis (after 24 hours of film exposure at −80°C) of digests of DNA isolated from rat liver after treatment with (A) 1-NP, (B) trioctanoin, (C) DMBA, or (D) pyrene. Similar patterns were obtained with rat mammary glands and rat peripheral lymphocytes. See legend to Figure 15 for TLC solvent systems. *N*-(Deoxyguanosin-8-yl)-1-aminopyrene is labeled as "a" in panel A. dR = deoxyribonucleic acid.

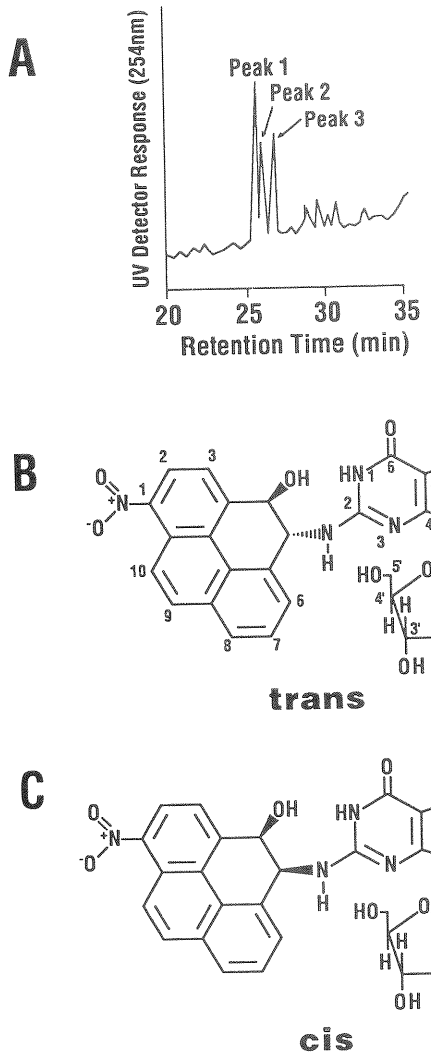

Figure 17. A: HPLC profile of an enzymatic hydrolysate of calf thymus DNA incubated with 1-NP-4,5-oxide. The HPLC conditions were 0% to 75% CH_3OH in water in 30 minutes at a flow rate of 5 mL/min; a Vydac C_{18} 10-μm reverse-phase column (10 × 250 mm) was used. **B and C: Proposed structures of adducts (*trans* for peaks 1 and 2; *cis* for peak 3) obtained from 1-NP-4,5-oxide with calf thymus DNA in vitro.** HN and NH = imino group; OH and HO = hydroxy group.

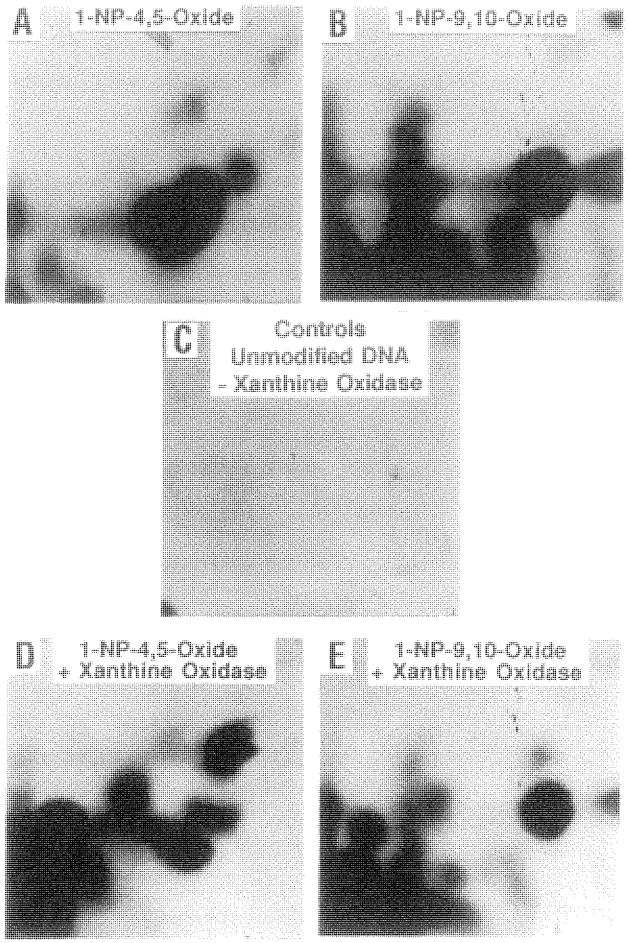

Figure 18. ^{32}P-Postlabeling polyethyleneimine-cellulose TLC maps of DNA (A) modified with 1-NP-4,5-oxide in the absence of xanthine oxidase; (B) modified with 1-NP-9,10-oxide in the absence of xanthine oxidase; (C) unmodified DNA in the absence of xanthine oxidase; (D) modified with 1-NP-4,5-oxide in the presence of xanthine oxidase; and (E) modified with 1-NP-9,10-oxide in the presence of xanthine oxidase. See legend to Figure 15 for TLC solvent systems.

ter the administration of 1,6-DNP because the major metabolic pathway of the latter occurs via simple nitroreduction (Djurić et al. 1988); metabolites derived from ring oxidation were not observed.

1,6-Dinitropyrene–DNA Adducts

Using ^{32}P-postlabeling analysis, only one adduct was observed in the liver following the administration of 1,6-DNP to rats (Figure 21). The same adduct spot was observed in mammary tissues, as well as in peripheral blood lymphocytes. This single adduct coeluted with the synthetic standard *N*-dG-ANP, indicating that upon in vivo administration of 1,6-DNP, the major adduct was derived from simple nitroreduction. The standard, *N*-dG-ANP, was prepared by incubating 1-nitroso-6-nitropyrene with calf thymus DNA in the presence of ascorbic acid (Figure 22) (Djurić et al. 1988). However, the detection and quantification of the adducts obtained in vivo with ^{32}P-postlabeling have not been consistently reproducible. Accordingly, we intend to collaborate with Dr. Roger Giese, at Northeastern University, in a dose-response study using [^{3}H]1,6-DNP. Reducing the resulting [^{3}H]N-dG-ANP with hydrazine should yield [^{3}H]1,6-diaminopyrene, which will be amenable to derivatization to a fluoro-derivative, which can be analyzed by GC with negative ion chemical ionization and MS detection. This will be the focus of future studies.

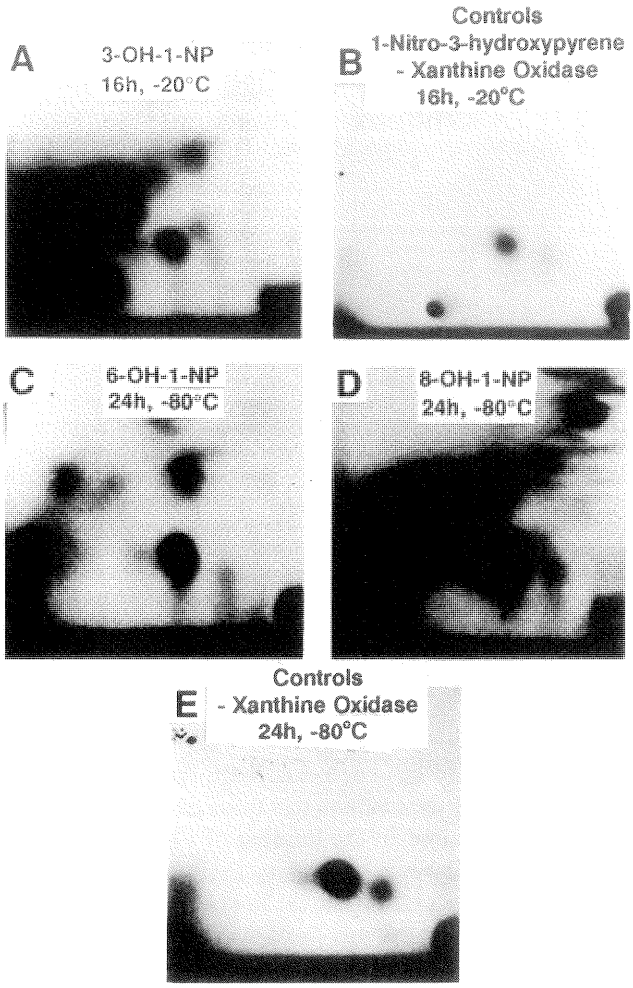

Figure 19. ^{32}P-Postlabeling polyethyleneimine-cellulose TLC maps of DNA (A) modified with 1-nitro-3-hydroxypyrene in the presence of xanthine oxidase; (B) modified with 1-nitro-3-hydroxypyrene in the absence of xanthine oxidase; (C) modified with 1-nitro-6-hydroxypyrene or (D) 1-nitro-8-hydroxypyrene in the presence of xanthine oxidase; or (E) unmodified in the absence of xanthine oxidase. See the legend to Figure 15 for TLC solvent systems.

DISCUSSION

The International Agency for Research on Cancer has classified diesel engine emissions as a possible human carcinogen in category 2A (1989). Findings from epidemiological studies on the effect of diesel engine emissions on human lung cancer are ambiguous (Garshick et al. 1987; Boffetta et al. 1990). Some investigators have concluded that, in contrast with lung tumor induction in the rat (Heinrich et al. 1986; Mauderly et al. 1987), the incidence of lung cancer in diesel-exposed workers cannot be accounted for by diesel exhaust particulate matter (Garshick et al. 1988). Yet, the effects of organic or gas-phase components should not be underestimated (Oberdörstar and Yu 1990). In general, the exposure of laboratory animals to diesel engine emissions was performed at relatively high dose levels that were well out of proportion with levels to which humans are exposed. Thus, to relate human exposure to risk in a reliable manner, the epidemiologist must have a sensitive and selective biomarker of the uptake of representative components in diesel engine emissions.

HEMOGLOBIN STUDIES

To assess the risks associated with human exposure to carcinogenic nitro-PAHs in the environment, one must be able to estimate the effective doses of these agents. One of our approaches was to use hemoglobin as a dosimeter. The measurement of covalent adducts formed between hemoglobin and carcinogens or their metabolites was introduced by Ehrenberg and associates (1974) and has been utilized successfully in many studies (reviewed by Skipper and Tannenbaum 1990). As far as we are aware, prior to the study reported here, there have been no other reported attempts to develop sensitive analytical methods for detecting nitro-PAH–protein adducts.

We have ascertained that 1-NP binds to hemoglobin of both male and female F344 rats in a manner related to dose over 5 orders of magnitude, at a level of 0.08% of the dose; levels of binding were the same in male and female Sprague-Dawley rats (data not shown). With the exception of 4-aminobiphenyl binding to hemoglobin (approximately 5%) (Green et al. 1984), most carcinogenic xenobiotics studied have been found to bind to hemoglobin at levels comparable to those measured in the present study (Skipper and Tannenbaum 1990). After the administration of a single dose of 1-NP, clearance of hemoglobin-associated radioactivity (half-life of 13.6 days) occurred at a rate faster than that of unmodified rat erythrocytes (half-life of 30 days). However, our results indicated that 1-NP–hemoglobin adducts in the rat appeared to be stable and that they accumulated with chronic exposure. Thus, it can be concluded that 1-NP–hemoglobin adducts are suitable for dosimetry studies. The stability of hemoglobin adducts varies depending on their structure. For example, the stability of adducts derived from ethylene oxide, dimethylnitrosamine, vinyl chloride, methylmethane sulfonate, benzo[*a*]pyrene, fluoranthene, and benzene has been demonstrated (Osterman-Golkar et al. 1976; Osterman-Golkar et al. 1977; Segerbäck et al. 1978; Segerbäck 1985; Hutchins et al. 1988; Gorelick et al. 1989). In contrast, Neumann (1984b) reported that several aromatic amines form unstable hemoglobin adducts in the rat. Carmella and Hecht (1987) reported a half-life of 9.1 days for adducts formed with metabolites of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

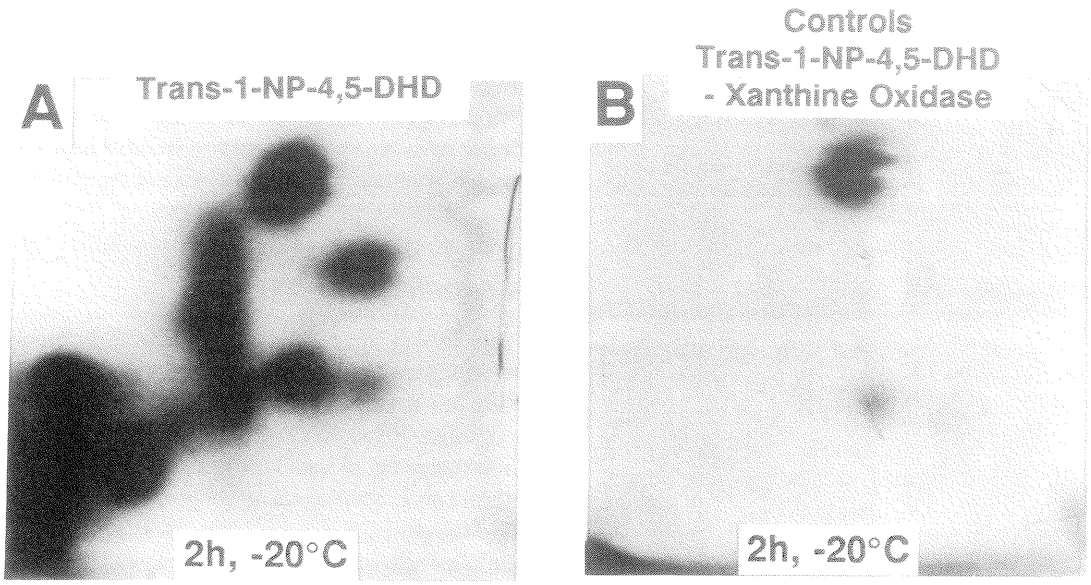

Figure 20. ^{32}P-Postlabeling polyethyleneimine-cellulose TLC maps of DNA modified with trans-1-NP-4,5-DHD (A) in the presence of xanthine oxidase, and (B) in the absence of xanthine oxidase. See the legend to Figure 15 for TLC solvent systems.

Attempts were made to define the structure of 1-NP–hemoglobin adducts. Knowledge gained during the past decade on the metabolism of 1-NP led us to reason initially that nitroreduction could be responsible for hemoglobin binding via the formation of 1-NOP. The latter compound would be expected to react with cysteine residues on hemoglobin. The adduct containing the sulfinamide bond would be labile under the acidic conditions used in this study for the hydrolysis of 1-NP–hemoglobin adducts. The acid hydrolysis of hemoglobin adducts revealed that none of the radioactivity remained bound to the globin, and more than 80% of the initial radioactivity was recovered in the heme fraction. Concentrated acid and base treatments of the heme fraction did not release any detectable derivative of 1-NP, suggesting that the radioactivity was strongly bound to the heme moiety. Other compounds (e.g., phenylhydrazine, 3-alkylsydnones, diethylnitrosamine, aminobenzotriazole, and norethindrone) also have been shown to form adducts with the heme moiety of hemoglobin, as well as with enzymes containing heme (Ortiz de Montellano and Kunze 1980 1981; Ortiz de Montellano and Mathews 1981; White 1981; White et al. 1983; Hirota et al. 1987; Grab et al. 1988).

Although we have evidence to suggest that binding of 1-NP to heme forms stable adducts, the exact nature of this binding is still unknown. To learn more about the structure of the heme adducts, we incubated [^{3}H]1-NOP and [^{3}H]1-NP-4,5-oxide with rat hemoglobin. In each case, [^{3}H] was bound mainly to globin, and, to a lesser extent, to the heme moiety. These findings contrasted with those obtained in vivo, thus leading us to believe that these particular metabolites are not required in the binding of 1-NP to the heme moiety in vivo. However, one cannot exclude that such metabolites possibly contribute to the in vivo binding solely on the basis of findings in vitro. Even though the structures of the 1-NP–heme adducts have not been defined, the fact that they are stable may render them useful as dosimeters for human exposure to 1-NP. On the basis of knowledge reported in the literature, the stability of such adducts is not surprising. The stability of many porphyrin chelates with copper, nickel, zinc, iron, and vanadyl underscores this (Rho et al. 1973).

As described in the literature (Ortiz de Montellano and Mathews 1981) and briefly in the Results section, the heme adducts can be derivatized to the corresponding methyl esters. The latter may be amenable to a quantitative assay by GC and MS with selective ion monitoring, in view of the availability of high-temperature glass capillary columns that withstand temperatures up to 430°C. Such columns were successfully employed in analyzing several metal-porphyrin complexes by GC (Blum and Eglinton 1989). This approach may provide an appropriate tool for monitoring exposure to 1-NP in humans.

Although the ratio of 1,6-DNP to 1-NP is about 1:100 in sources containing nitro-PAHs, we proposed to investigate the binding of 1,6-DNP with hemoglobin. This consideration was based on the facts that 1,6-DNP has far greater tu-

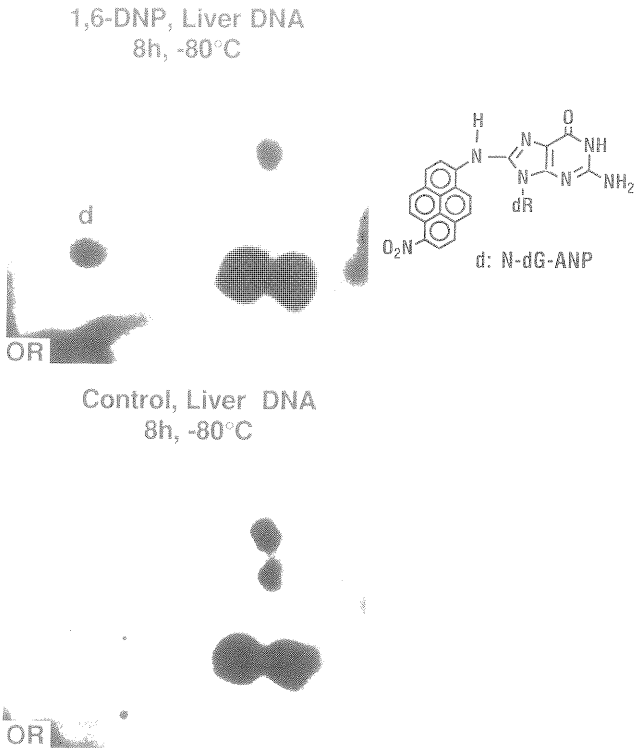

Figure 21. Polyethyleneimine-cellulose TLC maps of ^{32}P-postlabeling analysis of digests of DNA isolated from the livers of female Sprague-Dawley rats treated orally with 1,6-DNP in trioctanoin. Similar patterns were obtained following the analysis of DNA obtained from rat mammary glands and peripheral lymphocytes. *N*-(Deoxyguanosin-8-yl)-1-amino-6-nitropyrene is labeled "d" in the top panel on the basis of a comparison with a synthetic standard (see Figure 22). OR = origin of chromatogram.

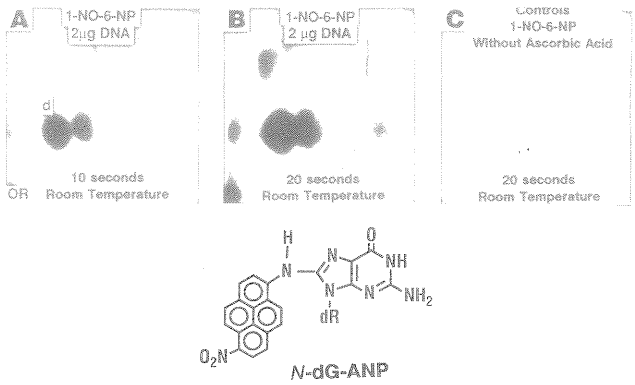

Figure 22. Polyethyleneimine-cellulose TLC maps of ^{32}P-postlabeling analysis of digests of DNA (A) modified with 1-nitroso-6-nitropyrene in the presence of ascorbic acid at room temperature **after 10 seconds, and (B) after 20 seconds; or (C) unmodified and in the absence of ascorbic acid.** The spot labeled "d" in panel A is *N*-dG-ANP. See Figure 15 legend for TLC solvent systems.

morigenic and mutagenic activity than 1-NP and that it is activated by simple nitroreduction (Djurić et al. 1988). Thus, 1-nitroso-6-nitropyrene would be expected to be a suitable candidate for hemoglobin binding; acidic acetone hydrolysis would yield 1-amino-6-nitropyrene. Unfortunately, the outcome of the in vivo study was not encouraging. We found that the binding of 1,6-dinitropyrene to hemoglobin was at best in the range of that of 1-NP. As in the case of 1-NP, 1,6-DNP was found to bind to heme with no measurable binding to globin chains. Thus, hemoglobin studies on 1,6-DNP were discontinued.

ALBUMIN STUDIES

We selected albumin instead of other plasma proteins because of its abundance; it constitutes approximately half of the plasma proteins. Because serum albumin is readily available in serum banks, its utilization in future studies of biochemical epidemiology is fully justified. Although albumin has a relatively short lifetime in rodents (half-life of 2 to 3 days in the rat), a half-life of 20 to 25 days in humans is a sufficiently long span for its use as a dosimeter. Like hemoglobin, albumin contains nucleophilic centers that are capable of covalent binding with active electrophiles derived from carcinogenic compounds (Skipper and Tannenbaum 1990). 1-Nitropyrene binds to albumin at approximately half the level observed with hemoglobin.

An ELISA indicated that peak 2 (in Figure 6) contained an adducted albumin; however, it eluted somewhat earlier than the standard albumin. This suggested that the chemical nature of the adducts associated with peak 2 was different from that of adducts associated with peak 3 (in Figure 6). This may account for the differences in chromatographic behavior observed with peaks 2 and 3. Despite these differences, albumin adducts appear to be stable and can, therefore, be used in dosimetry. This suggestion is justified because peaks 2 and 3 disappeared at a rate (half-life of 2.5 days) comparable to that of unmodified rat albumin. Because enzymatic digestion of peak 2 yielded a single labeled peptide fragment, we were interested in determining whether or not the nitro group was involved in the binding. This peptide fragment was not amenable to nitroreduction under conditions that normally ensured reduction of the nitro group in 1-NP. We hypothesized, therefore, that the nitro group could be involved in the binding or that it had already been reduced. However, there was not sufficient material of the peptide fragment for MS analysis at this time. This question will be addressed in future studies.

Further experiments were conducted in the area of structural characterization of albumin adducts. Incubation of 1-NOP and 1-NP-4,5-oxides with either albumin or whole rat plasma suggested that neither metabolite is involved in

the in vivo formation of albumin adducts present in peak 2. However, such metabolites may contribute to the formation of some of the albumin adducts present in peak 3. We currently are developing an immunoassay for peak 2 to be utilized in human studies.

Acid hydrolysis, followed by HPLC analysis of peak 2 (or peak 3), yielded a major radioactive peak that led to 1-acetylamino-*x*,*y*-diacetoxypyrene upon acetylation with a mixture of acetic anhydride and 4-dimethylaminopyridine.

Figure 10c suggests that acid hydrolysis of peak 2 (or peak 3) followed by acetylation yields an isomer or isomers eluting close to that of the synthetic mixture of 1-acetylamino-*x*,*y*-dihydroxypyrenes. On the basis of these results, we propose the scheme shown in Figure 23. Intermediates proposed in Figure 23 are likely candidates because all were formed in vivo via nitroreduction, ring oxidation, and acetylation of the amino functionality; oxidation at the 9- and 10-positions of 1-NP to 9,10-oxide is also feasible. K-region oxides of PAHs and 1-NP are good substrates for compounds containing thiol (e.g., glutathione and albumin). It should be emphasized that activation of 1-NP to intermediates capable of binding to albumin can occur via either route A or B, (as indicated in Figure 23). Our results cannot differentiate between the two routes; for the sake of simplicity we have considered only the 4,5-oxide derivative as the substrate employed for albumin binding. Several nucleophilic centers are available in albumin, including a free thiol group in its cysteine-34 residue; this site may be amenable to nucleophilic attack at the K-region oxide (4 or 5 position). Although the structures of 1-NP–albumin adducts could not be elucidated in this study, we suggest that 1-acetylamino-*x*,*y*-dihydroxypyrenes can be utilized as potential markers for dosimetry of human exposure to 1-NP, provided that human metabolism leads to the same metabolite as that of the rat and that the metabolite is capable of binding with albumin. Our proposed strategy, as described below, is similar to strategies used in previous studies for monitoring the exposure to 4-aminobiphenyl, ethylene oxide, and the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Törnqvist et al. 1986; Bryant et al. 1987; Carmella and Hecht 1987). Derivatization of the phenolic compounds with hexafluoroacetic anhydride or related derivatizing agents (released via acid treatments and followed by GC and MS analysis with negative ion chemical ionization as means of detection and quantification) will be performed.

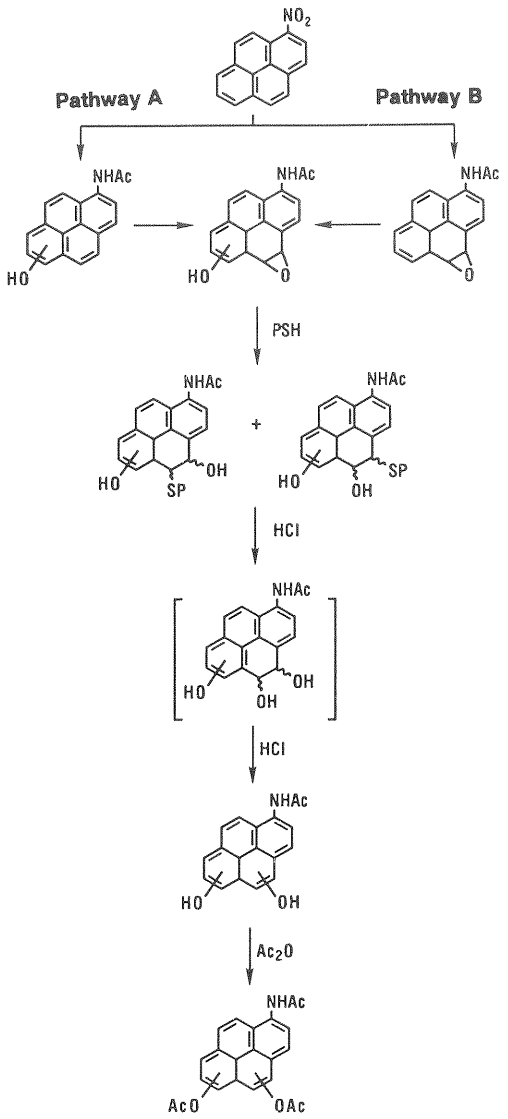

Figure 23. Proposed scheme for 1-NP binding to albumin in vivo. For simplicity, we considered only the 4,5-oxide derivative as the substrate employed for albumin binding, although oxidation to yield the 9,10-oxide is also feasible. SP = albumin minus hydrogen; PSH = albumin; NHAc = imino acetate; Ac_2O = acetic anhydride; AcO and OAc = acetate. The bracketed structure in the center indicates a proposed intermediate.

DNA ADDUCT STUDIES

There is convincing evidence that the formation of carcinogen-DNA adducts is one of the primary events in chemical carcinogenesis and mutagenesis. Quantification of specific carcinogen adducts in humans is required for assessing their possible significance in and contribution to human cancer. Sensitivity and selectivity are essential for reliable dosimetry. Although several analytical methods are available, each has its own merits and drawbacks. As proposed, we used the ^{32}P-postlabeling technique to analyze DNA samples from rats treated with compounds that are constituents of diesel engine emissions, 1-NP and 1,6-DNP. Our aim was to compare the ^{32}P-postlabeling maps in vivo with those obtained from DNA samples in vitro following the in-

cubations of metabolites derived from nitroreduction and ring oxidation of 1-NP and from nitroreduction of 1,6-DNP. Comparing our results with those obtained by Jeffrey and colleagues (1990) and Bond and associates (1988), who exposed rats to diesel engine emissions, should provide insights regarding the utility of the adduct markers. After standardizing the analytical methods, one should be able to utilize these markers in human exposure assessment. These analytical methods should not be limited to monitoring nitro-PAHs in diesel emissions, but could also be applied to assessing exposure from other environmental sources.

The ^{32}P-postlabeling technique was used initially to establish qualitatively the pattern of DNA adduct formation in mammary tissue, liver, and peripheral blood lymphocytes following administration of 1-NP to female Sprague-Dawley rats. The animal model used in this study was selected because nitro-PAHs in general are capable of inducing mammary tumors in female Sprague-Dawley rats. In addition, this model would allow the comparison of DNA adducts in the target tissue (mammary) with protein adducts. Such comparison will provide valuable information for future studies in humans. Lymphocyte-DNA was analyzed because blood was the most likely physiologic specimen for human dosimetry. In addition to determinations in blood, lymphocyte-DNA adducts and other adducts could also be detected and quantified in target tissues during surgical procedures or autopsies. Our studies have indicated the presence of multiple putative adducts in the tissues analyzed. In a previous study, Smith and colleagues (1990) demonstrated the presence of *N*-dG-AP at the injection site and in mammary glands of Sprague-Dawley rats, as well as the presence of unidentified adducts in mammary glands as a result of subcutaneous administration of 1-NP. In addition to *N*-dG-AP, Smith and associates (1990) detected *N*-dG-ANP at the injection site; however, this was related to DNP contamination. Although our protocol differs from that used by Smith and coworkers (1990) in dose, route of administration, and age of the animals, our results appear to correspond to theirs with regard to the identification of *N*-dG-AP in the mammary glands.

The major adducts detected in vivo appeared not to originate from simple nitroreduction of 1-NP, suggesting that other metabolic pathways, such as ring oxidation, either alone or in combination with nitroreduction, may be responsible for the putative 1-NP–DNA adducts observed in vivo. Thus, the need for additional DNA adduct markers was apparent. Therefore, we aimed to characterize the DNA adducts formed from 1-NP-4,5-oxide, a metabolite derived from ring oxidation of 1-NP. Several metabolites other than 1-NP-4,5-oxide, but also derived from ring oxidation, have been shown to bind to DNA in vitro (Djurić et al. 1986). The structures of three major DNA adducts derived from 1-NP-4,5-oxides were characterized as N^2-deoxyguanosine adducts (see the Results section). These adducts were also analyzed by ^{32}P-postlabeling (El-Bayoumy et al. 1990). Because these ^{32}P-postlabeling assays were conducted under identical conditions, it can be concluded that adducts derived from 1-NP-4,5-oxide are chromatographically different from the major adduct obtained by the nitroreduction of 1-NP. On the other hand, the adducts derived from 1-NP-4,5-oxide were chromatographically similar to some of the adduct spots observed in vivo; this needs to be examined further. Also, multiple DNA adducts were observed following the modification of DNA in vitro with other mutagenic ring-oxidized metabolites of 1-NP in the presence or absence of xanthine oxidase. Consequently, such metabolites also could be responsible for some of the adducts observed in the rat. Jeffrey and associates (1990) and Bond and colleagues (1988) demonstrated that animals exposed to diesel engine emissions had higher adduct levels in the lungs than did unexposed rats. Although the chemical identity of these adducts had not been determined, the adducts were chromatographically different from the major adduct spots derived from (±)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide. It appears that adducts derived from 1-NP may be present in the lungs of rats after exposure to diesel engine emissions; however, the DNA from such exposed animals and the DNA modified with metabolites of 1-NP in vitro should be analyzed under identical conditions.

We also analyzed by ^{32}P-postlabeling the DNA in rat mammary and liver tissue and in peripheral blood lymphocytes following administration of 1,6-DNP. The ^{32}P-postlabeling maps were much simpler than those found in the case of 1-NP; i.e., only one major adduct, *N*-dG-ANP, was detected. This is consistent with results obtained in another laboratory (Djurić 1988). Detection and quantification of this adduct have not been reproducible (see the Results section). Thus, we decided to follow another analytical approach using [^{3}H]1,6-DNP (see the Results section). This will be part of our efforts in future studies.

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Roy A, El-Bayoumy K, Hecht SS. 1989. ^{32}P-Postlabeling analysis of 1-nitropyrene–DNA adducts in female Sprague-Dawley rats. Carcinogenesis 10:195–198.

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ABBREVIATIONS

2-AF	2-aminofluorene
ATP	adenosine 5′-triphosphate
CH_3OH	methanol
cis-1-NP-4,5-DHD	*cis*-4,5-dihydro-4,5-dihydroxy-1-nitropyrene
DMBA	7,12-dimethylbenz[*a*]anthracene
1,6-DNP	1,6-dinitropyrene
dpm	disintegrations per minute
ELISA	enzyme-linked immunosorbent assay
GC	gas chromatography
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
m/e	mass-to-charge ratio
MS	mass spectrometry
N-dG-AP	*N*-(deoxyguanosin-8-yl)-1-aminopyrene
N-dG-ANP	*N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene
2-NF	2-nitrofluorene
nitro-PAH	nitropolynuclear aromatic hydrocarbon
1-NOP	1-nitrosopyrene
1-NP	1-nitropyrene
1-NP-4,5-oxide	4,5-epoxy-4,5-dihydro-1-nitropyrene
1-NP-9,10-oxide	9,10-epoxy-9,10-dihydro-1-nitropyrene
^{32}P	phosphorus-32
PAH	polynuclear aromatic hydrocarbon
r^2	correlation coefficient to the square
R_f	ratio of the movement of solute to the movement of the solvent front
SD	standard deviation
trans-1-NP-4,5-DHD	*trans*-4,5-dihydro-4,5-dihydroxy-1-nitropyrene

INTRODUCTION

Protecting humans from the adverse health effects of air pollution is difficult because of the complex mixture of air pollutants emitted from numerous sources, including industry, motor vehicles, public utilities, residential heating, and tobacco products. Among the pollutants of concern are the polycyclic aromatic hydrocarbons (PAHs)* and their nitro-substituted derivatives (nitro-PAHs). These components are ubiquitous, with persons living in industrialized regions or working in enclosed spaces receiving the highest PAH exposures, generally through exposures to coal tar, creosote, cokeoven emissions, and the products of incomplete combustion of fossil fuel. Such exposures are of concern because PAHs and nitro-PAHs cause tumors in a variety of animal organs, including the lungs, skin, mammary glands, and liver, and are suspected to be carcinogenic in humans (International Agency for Research on Cancer 1983, 1989).

Since 1983, the Health Effects Institute (HEI) has conducted a multidisciplinary research program on the biological effects of some of the PAHs found in diesel engine exhaust, with the ultimate goal of providing regulators with the necessary information to conduct sound risk assessments. Because a major weakness in most cancer risk assessments is a lack of information regarding human exposures to suspected and known carcinogens, a part of HEI's program has been directed toward the development of better methods for assessing human exposure to PAHs and related compounds. The study described in this report was funded to address that need.

In 1986, HEI issued a Request For Applications (RFA 86-2) that solicited proposals for studies on "Health Effects of Diesel Emissions." In response, Dr. Karam El-Bayoumy, from the American Health Foundation, in Valhalla, New York, submitted a proposal entitled "Assessment of Human Exposure to Nitroaromatics." After a competitive external peer-review process, the HEI Research Committee approved the three-year project, which began in August 1987. Because of unexpected results requiring further research, Dr. El-Bayoumy requested and was granted a one-year extension. Experimental work concluded in July 1991. Total expenditures were $540,215. The draft Investigators' Report was received at HEI for review in October 1991. A revised report was received in August 1992 and was accepted by the Health Review Committee in November 1992. During the review of the Investigators' Report, the Review Committee and the investigators had an opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. This Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of HEI and to the public.

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

REGULATORY BACKGROUND

Diesel engines, which are more efficient than spark-ignited engines, are used in heavy-duty applications such as trucks, buses, construction equipment, locomotives, and ships. Light-duty diesel engines are used in passenger cars and trucks, especially in Europe; however, light-duty diesel engines currently account for less than 2% of the vehicles sold in the United States. In addition to being fuel efficient, diesel engines emit 10% to 20% less carbon dioxide than gasoline engines. Both factors account for the renewed interest in diesel engines in this country.

Despite the advantages of diesel engines, their use has potential environmental and health consequences. Diesel engines operating without emission controls emit higher levels of nitrogen oxides than gasoline engines, as well as a highly complex mixture of gases and carbonaceous particles. The particles in diesel exhaust are particularly troublesome because they are of respirable size and hundreds of toxic chemicals, including PAHs, are adsorbed on their surfaces.

PAHs and their chemical derivatives are well-known mutagens in bacteria and mammalian cells and are carcinogens in laboratory animals (International Agency for Research on Cancer 1983, 1989). Moreover, several long-term bioassays have shown that chronic inhalation of high concentrations of diesel engine exhaust causes lung tumors in rats, and there is suggestive evidence that workers exposed to high concentrations of diesel exhaust have a higher incidence of lung cancer than their nonexposed counterparts (reviewed by Mauderly 1992 and International Agency for Research on Cancer 1989). Because of concerns about the potential health consequences of inhaling diesel engine exhaust, the U.S. Environmental Protection Agency (EPA) sets emissions standards for diesel engines and vehicles under Section 202 of the Clean Air Act, as amended in 1990.

Section 202(a)(1) of the Act directs the Administrator of the EPA to "prescribe (and from time to time revise) . . . standards applicable to the emissions of any air pollutant from any class or classes of new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a)(3)(A)(i) of the Act specifically directs the Administrator to set standards for the "emissions

of carbon monoxide, hydrocarbons, oxides of nitrogen and particulate matter from classes of heavy-duty vehicles and engines . . ."

The EPA has taken a variety of regulatory actions with respect to diesel engines and vehicles under the authority given it by Sections 202(a)(1) and 202(a)(3)(A)(i). The EPA has, for instance, set emissions standards for hydrocarbons, carbon monoxide, nitrogen oxides, and particulate matter for heavy-duty and light-duty trucks. These emissions standards initially applied to all engines and vehicles produced in a given model year. Engines and vehicles of the same class that are produced in succeeding years must also comply with these existing standards unless the EPA establishes a new set of standards.

Determining appropriate emissions standards for particles and hydrocarbons depends, in part, on an assessment of the health risks they present, and that process requires a better understanding of the levels of pollutants to which individuals are exposed than currently exists. Dr. El-Bayoumy and coworkers were funded by HEI to develop techniques for measuring individual exposures to selected PAHs. Such research is essential to the informed regulatory decision-making required by the Clean Air Act.

SCIENTIFIC BACKGROUND

A central problem for environmental epidemiologists is determining the dose of potentially toxic substances delivered to individuals in a study population. The weakest link in most epidemiological studies of air pollution is exposure estimation. Because it is expensive and time-consuming to monitor individual exposures using personal air samplers, surrogate measures of exposure are generally used in epidemiological studies. For example, in studies of the long-term effects of occupational exposure to diesel engine exhaust, researchers have used employment records to identify workers at risk and to estimate diesel engine exhaust exposures (Schenker et al. 1984; Garshick et al. 1987, 1988). Such surrogate exposure estimates are unsatisfactory for several reasons. First, employment records may not be available or may be incorrect. Second, work patterns vary widely among individuals, and employees with similar job histories may actually have received very different doses of pollutant because of undocumented differences in individual behavior. Finally, interindividual differences in chemical absorption, metabolism, and elimination may lead to wide individual variations in response.

To improve the accuracy of exposure estimates, epidemiologists and toxicologists have begun to explore the possibility of using biological markers as indicators of past contact with pollutants (Wogan 1992). The levels of some air pollutants, such as lead or other heavy metals, can be measured directly in body tissues or fluids. Alternatively, because many toxic chemicals (or their metabolites) react with cellular macromolecules to produce modified structures called adducts, there has been considerable interest in developing techniques to analyze DNA and protein adducts (reviewed by Groopman and Skipper 1991; U.S. Department of Health and Human Services 1993).

Initially, researchers focused on developing methods to measure DNA adducts because they are thought to reflect mutagenic processes that are generally regarded as pivotal events in chemical carcinogenesis. In animals, the levels of persistent DNA adducts in tissues reflect the steady state levels that are produced by the competing steps of DNA adduct formation and repair and generally correlate with the susceptibility of target tissues to tumor formation. However, it is often difficult to obtain sufficient quantities of DNA adducts from target tissues to conduct qualitative or quantitative analyses. For this reason, nontarget cells, such as blood lymphocytes or cells shed into body fluids, have been used as sources of DNA adducts. Protein adducts are also frequently used as molecular dosimeters (Skipper and Tannenbaum 1990a,b; Farmer 1991). They have advantages over DNA adducts in that they can be obtained in relatively large quantities and are not subject to repair processes. Hemoglobin and serum albumin adducts have proved to be useful human protein dosimeters. Both proteins reside in the body for relatively long and predictable periods (human hemoglobin has a half-life of 120 days and human serum albumin of 20 to 25 days) and can be obtained in relatively large quantities from human body fluids by minimally invasive techniques.

There have been several successful efforts to use DNA or protein adducts as biomarkers to follow environmental exposures to air-borne pollutants. For example, cigarette smoking in humans can be monitored by measuring the appearance of covalent adducts between blood proteins and 4-aminobiphenyl, a prominent cigarette combustion product (Jahnke et al. 1990; Weston et al. 1991). 4-Aminobiphenyl adduct levels in albumin and hemoglobin were related to dose and declined when a subject ceased smoking (Maclure et al. 1990; Skipper and Tannenbaum 1990). Human hemoglobin has been successfully used as a molecular dosimeter of exposure to PAHs (Day et al. 1990), and work on PAH–serum albumin adducts has been reported (Day et al. 1991). In other studies of human populations, individuals exposed to high levels of pollution had higher DNA adduct levels than control populations (Hemminki et al. 1990; Lewtas et al. 1993; Mumford et al. 1993).

Thus, there is a body of evidence indicating that adducts formed by interactions between chemical pollutants or their metabolites and DNA or proteins are useful measures of ex-

posure. However, before such biomarkers can be used in epidemiology studies, inexpensive and rapid measurement techniques must be developed. For any DNA or protein adduct to be useful as a tissue biomarker, it should have the following characteristics:

1. stability, with a relatively long life span;
2. levels that relate to exposure over a wide dose range;
3. presence in easily collectible samples (blood, urine, skin);
4. suitability for inexpensive and rapid chemical analyses;
5. specificity for individual pollutants (or classes of pollutants);
6. detectability following exposures to low levels of pollutants.

Many researchers have worked to develop simple methods to measure combustion-related adducts in body fluids from exposed animals and humans (Beland and Poirier 1993; Lewtas et al. 1993). For example, diesel engine emissions contain a large number of well-characterized reactive combustion products. Among these products are PAHs such as benzo[*a*]pyrene and several nitrated PAHs, including 1-nitropyrene and 1,6-dinitropyrene. Naylor and associates (1990) demonstrated the feasibility of measuring benzo[*a*]pyrene-protein adducts using an in vitro cell culture system. Metabolites of 1-nitropyrene have been shown to react efficiently with both proteins and DNA, leading to the formation of adducts in several tissues from rodents treated with 1-nitropyrene or diesel engine exhaust (Wong et al. 1986; Bond et al. 1988, 1990; Beland 1991).

JUSTIFICATION FOR THE STUDY

In 1986, the HEI Research Committee identified two major research areas in which critical gaps in knowledge limited the understanding of the carcinogenic potential of inhaled diesel engine exhaust pollutants. First, research was needed to clarify the role of diesel engine exhaust particles, particularly with respect to the heavy burdens of particles found in the lungs of exposed animals in which lung tumors developed. Second, research was needed to develop techniques that would estimate the effective dose of diesel engine emissions in laboratory animals and exposed human populations. The Research Committee was particularly interested in improving the methodology for detecting and quantifying DNA and protein adducts, possibly by using 1-nitropyrene and dinitropyrenes as model compounds for diesel emissions. Methods for evaluating exposure of selected human populations were also solicited.

Dr. El-Bayoumy proposed to develop techniques for measuring protein and DNA adducts in tissues isolated from rats treated with nitro-PAHs and, eventually, from humans exposed to these compounds. The investigator is a recognized expert in the analysis of adducts. His methods were regarded as sensitive and specific for nitro-PAH adducts and had the potential to be used on blood samples from exposed human volunteers. Because diesel engine emissions carry high levels of nitro-PAHs, the proposed study would fulfill one of the HEI goals, that of estimating human exposure to diesel emissions.

OBJECTIVES AND STUDY DESIGN

Dr. El-Bayoumy's original application to HEI proposed two specific objectives. The first was to develop methods for detecting DNA and protein adducts of 1-nitropyrene and 1,6-dinitropyrene in rat models. DNA adducts were to be detected using an established postlabeling technique. Protein adducts were to be detected in hemoglobin using gas chromatography and mass spectrometry. The second objective was to apply these new techniques to blood samples taken from humans exposed to high concentrations of diesel engine emissions, a known source of 1-nitropyrene and 1,6-dinitropyrene. Because the HEI Research Committee expected that a large amount of effort would be required to meet the first objective, it recommended that the second objective, the human study, be deleted from the project.

As a consequence, the following specific aims were adopted for this study:

1. To determine the relationship between the administered dose of 1-nitropyrene or 1,6-dinitropyrene and the levels of hemoglobin adducts in male Fischer-344 (F344) rats. This was to be accomplished by the administration (by gavage) of ^{3}H-labeled 1-nitropyrene or 1,6-dinitropyrene, by isolating hemoglobin from blood samples, and determining the amount of radioactivity associated with the heme and globin portions of the molecule.
2. To determine the kinetics of 1-nitropyrene–hemoglobin and 1,6-dinitropyrene–hemoglobin adduct formation and elimination after their oral administration to male F344 rats.
3. To identify the 1-nitropyrene–hemoglobin adducts using chromatographic methods.
4. To compare 1-nitropyrene–hemoglobin adducts generated in vitro by several known metabolites of 1-nitropyrene with those formed in vivo.
5. To identify and quantify DNA adducts formed in the mammary gland, liver, and white blood cells of female Sprague-Dawley rats after oral administration of 1-nitropyrene or 1,6-dinitropyrene. Conventional ^{32}P-postlabeling techniques were to be used for DNA adduct identification.

During the first year of the project, it became clear that additional work would be required because of unexpected results in the hemoglobin studies as will be discussed below. Therefore, the following additional goals were established:

6. To conduct further work to identify the nature of the 1-nitropyrene–hemoglobin adduct.
7. To determine the relationship between 1-nitropyrene or 1,6-dinitropyrene dose and plasma albumin adduct levels.
8. To conduct in vitro studies with known nitropyrene metabolites to determine possible mechanisms of plasma protein adduct formation.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

Dr. El-Bayoumy and his coworkers carried out an ingenious and impressive series of experiments that led to the discovery of unsuspected properties of the metabolism and nature of 1-nitropyrene metabolites and adducts. The quality of the work is excellent and reflects carefully controlled and well-executed experiments. The principal investigator embarked on what seemed to be a straightforward problem: namely, to determine the nature of the DNA and hemoglobin adducts formed upon exposure to 1-nitropyrene and, secondarily to 1,6-dinitropyrene. Indeed, DNA and protein adducts could be identified in both the in vitro and the in vivo studies. However, when the in vivo exposure (by gavage) was carried out, there were a number of unexpected findings. In particular, the original assumption that 1-nitropyrene–hemoglobin adducts would be restricted to the globin subunit of the protein was found to be incorrect. In fact, the 1-nitropyrene adducts were located exclusively in the heme subunit. Thus, the goals of the study were appropriately modified to include investigation of the heme adducts. The scope of the study was also expanded to include an examination of plasma protein adducts. The DNA adduct studies yielded substantial new information about the nature of 1-nitropyrene–DNA adducts; however, the investigators did not achieve their objective of developing a quantitative assay to measure 1-nitropyrene–DNA adducts, primarily due to the complexity of the adduct profile in vivo.

In summary, although the overall goal of this study, to develop biomonitoring techniques for 1-nitropyrene and 1,6-dinitropyrene, was not completely achieved, the investigators' results support the feasibility of using 1-nitropyrene–serum albumin adducts and 1-nitropyrene–hemoglobin adducts as biomarkers of PAH exposure.

METHODS AND STUDY DESIGN

The methods used in this study were generally appropriate, and the study design was excellent. The one exception was the dialysis procedure used to determine the amount of [^{3}H]1-nitropyrene bound to hemoglobin. It is not clear whether the investigators' procedure removed all of the unbound label. Other laboratories have demonstrated residual unbound material after exhaustive dialysis of proteins treated with radiolabeled PAHs (Naylor et al. 1990). In addition, the ethyl acetate extraction procedure used to measure the magnitude of covalent binding may not have adequately solubilized the unbound radioactivity. Consequently, the extent of covalent binding of 1-nitropyrene to hemoglobin and albumin may have been overestimated.

The results of this study were largely qualitative and generally did not require statistical description or inference. In those instances in which statistical analysis was applied, however, it was not fully or correctly realized. The lack of thorough statistical analysis with regard to Figures 2, 3a, 5, and 7 in the Investigators' Report may be attributable to the fact that the regression lines were obtained by the use of graphics software, rather than statistical software. No standard errors were provided, and p-values were used incorrectly. Thus, these regression lines were more in the nature of illustration than true statistical analysis.

RESULTS AND INTERPRETATION

The studies reported by Dr. El-Bayoumy fall into three categories. First were basic studies to measure the yields and kinetics of hemoglobin, plasma protein, and DNA adducts following oral administration of radiolabeled 1-nitropyrene or 1,6-dinitropyrene. These studies were primarily descriptive in nature. Second were measurements designed to characterize the nature of the adducts detected after in vivo treatment. Finally, there was a series of in vitro experiments in which biomolecules were treated with known nitropyrene metabolites and the products were compared with those found following in vivo administration of the parent compounds.

Hemoglobin Adducts

In the first series of studies, experiments were performed to study the basic characteristics of the adduct formed following nitropyrene ingestion. Hemoglobin adducts were found in rats after a single oral administration of either 1-nitropyrene or 1,6-dinitropyrene. Adduct yield was proportional to 1-nitropyrene dose. The constant of proportionality, 0.08% ± 0.05%, remained steady over a 10,000-fold dose range. Dinitropyrene adducts were formed with similar efficiency.

Once formed, the 1-nitropyrene–hemoglobin adducts were eliminated from blood according to first-order kinetics with a half-life of nearly 14 days which is faster than the normal rate of erythrocyte decay in rats but is comparable to the life span of other hemoglobin adducts (Green et al. 1984). The clearance of [^{3}H]1-nitropyrene was said to be "faster" than that of unmodified rat erythrocytes on the basis of the observation that [^{3}H]1-nitropyrene followed first-order clearance kinetics with a half-life of approximately 14 days, whereas, according to the literature, erythrocytes turn over completely in 60 days with zero-order kinetics. Clearance curves with those parameters would actually cross; neither could be truly considered "faster." For example, at 30 days half of the erythrocytes would have been eliminated while [^{3}H]1-nitropyrene would have fallen to 22% of its original value; however, at 60 days the erythrocytes would be entirely cleared while 5% of [^{3}H]1-nitropyrene would remain. Which one clears "faster" in this case is difficult to say, and the biological import is ambiguous. The approximately 14-day half-life for clearance of [^{3}H]1-nitropyrene was derived from linear regression analysis of the clearance curve on a semilogarithmic plot (Figure 3). These data do not follow a particularly straight line. A more thorough statistical analysis would have included a test for linearity and a standard error indicating the precision of the approximately 14-day slope.

To determine the effect of continuous carcinogen administration, 1-nitropyrene was administered to rats via gavage three times a week for five weeks. As expected, 1-nitropyrene–hemoglobin adduct levels increased with each treatment. However, once treatment was completed the decline in hemoglobin adduct concentration was unexpectedly rapid. The authors' suggestion that this was due to a difference between chronic and acute regimens is not convincing. The dose-response data in Figure 3 and Figure 5 of the Investigators' Report were said to be linear over 5 orders of magnitude. However, the statistical *p*-values cited in reference to these figures are not proper evidence of linearity; they indicate nonzero slope, having assumed linearity. The lowest point in Figure 3 was precisely determined but was out of line with the others points, and it may have exerted undue influence on the slope of the line.

Several experiments were carried out to determine the nature of the 1-nitropyrene– and 1,6-dinitropyrene–hemoglobin adducts detected in animal tissues. An unexpected finding was that virtually all the adducts detected in hemoglobin were bound to the heme moiety, not to the globin portion. Other investigators have reported that treatment with chemicals, such as 4-aminobiphenyl (Green et al. 1984), leads to adduct formation exclusively in the globin moiety. Dr. El-Bayoumy's group found two chromatographically distinct 1-nitropyrene–heme adducts but were unable to determine their structure. In preparation for future mass spectroscopic studies, the heme adducts were methylated and zinc complexes were purified.

In a supplementary study Dr. El-Bayoumy examined the binding of 2-nitrofluorene to rat hemoglobin following ingestion and found it to be 0.06% ± 0.02% of the administered dose, a value comparable to that found with 1-nitropyrene. In contrast to the results with 1-nitropyrene, the 2-nitrofluorene did not form adducts with the heme moiety of hemoglobin.

Plasma Albumin Adducts

Because of the complexity of heme adduct chemistry, the investigators chose an alternative approach to the problem of developing a nitro-PAH protein dosimeter: namely, to study plasma albumin adducts rather than hemoglobin adducts. This approach was reasonable because albumin is abundant in body fluids; moreover, unlike hemoglobin, albumin is not sequestered in the red cell. Two chromatographically distinct 1-nitropyrene–albumin adducts were detected in rat plasma. The clearance curves for two [^{3}H]1-nitropyrene–albumin adducts (Figure 7 in the Investigators' Report) were said to have the same 60-hour half-life, comparable to the half-life of normal albumin. However, half-lives were obtained by linear regression on a semilogarithmic scale, although the linearity of the data is questionable. No standard error for the 60-hour estimate was provided, which could have served as a basis for comparing the two curves. Paired comparison of time points by analysis of variance would have been a more appropriate way of establishing that the clearance curves of the two adducts were parallel.

DNA Adducts

1-Nitropyrene–DNA adducts were also detected in rat tissues following ingestion of the parent compound. The DNA adducts found in mammary tissue were separated using thin-layer chromatography. Several spots were detected; however, most of these spots could not be identified. A minor product eluted with *N*-(deoxyguanosin-8-yl)-1-aminopyrene. This adduct has previously been detected in *Salmonella typhimurium* and in mammalian cell systems, and its formation is thought to proceed through a nitroreduction pathway. The complexity of the adduct pattern following 1-nitropyrene administration was thought to be due to the formation of many ring-oxidized metabolites.

The DNA adduct results differ from those of Dr. Beland and coworkers, who found *N*-(deoxyguanosine-8-yl)-1-amino-8-nitropyrene in mammary tissue of 1-nitropyrene-treated rats (Smith et al. 1990; Beland 1991). Dr. Beland ascribed the presence of this adduct to contamination of his 1-nitro-

pyrene preparation with 1,8-dinitropyrene. *N*-(deoxyguanosin-8-yl)-1-aminopyrene, the adduct identified in Dr. El-Bayoumy's work was a minor, barely detectable product in Dr. Beland's studies. Mass spectroscopic measurements of Dr. Beland's 1-nitropyrene preparation indicated a 1,8-dinitropyrene contaminant level of no more than 0.15%. Thus, in Dr. Beland's system, dinitropyrene adduct formation was at least 100 times more efficient than mononitropyrene adduct formation. The preparation used by Dr. El-Bayoumy had been purified on silica gel to a purity of more than 99.9%. No dinitropyrene was detected.

Some of the discrepancies between the Beland and El-Bayoumy studies most likely arise from differences in experimental procedures, particularly in the animal treatment procedures. Beland and coworkers administered 1-nitropyrene subcutaneously to newborn Sprague-Dawley female rats once a week for eight weeks. Dr. El-Bayoumy treated F344 rats once via gavage. A second difference in the two sets of experiments was the presence of several adducts other than *N*-(deoxyguanosin-8-yl)-1-aminopyrene in DNA samples analyzed by Dr. El-Bayoumy following 1-nitropyrene administration. No evidence of these additional adducts was found in Dr. Beland's studies. Once again, the difference may have been due to differences in treatment procedures.

The two sets of experiments cannot be directly compared because of the differences in experimental procedure and the lack of data on absolute yield. However, it should be emphasized that oral administration, as performed by Dr. El-Bayoumy, subjects 1-nitropyrene to an environment very different from that encountered after subcutaneous administration. Orally administered agents are metabolized by normal intestinal flora and must pass through the liver before reaching target tissues, while material delivered subcutaneously may reach target tissues directly through the blood. Resolution of the discrepant findings will require systematic experiments in which rats are treated with 1-nitropyrene via several routes and quantitative measurements of adduct yield are compared.

The DNA adduct pattern in liver following 1,6-dinitropyrene administration was not as complicated as that found following 1-nitropyrene administration, possibly because the metabolism of 1,6-dinitropyrene in rats is considerably simpler than that of 1-nitropyrene. The pattern consisted of a single adduct, whose chromatographic mobility was consistent with that of *N*-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene. In view of the fact that the ^{32}P-postlabeling pattern obtained with 1,6-dinitropyrene was much simpler, the investigator suggested that 1,6-dinitropyrene–DNA adducts would be better biomarkers than 1-nitropyrene–DNA adducts. Whether, indeed, this would be feasible in the case of human exposures is problematic in view of the fact that dinitropyrenes are only 1% as abundant in environmental emissions as 1-nitropyrene.

Adduct Formation in vitro

The investigators conducted in vitro studies of known nitropyrene metabolites to determine which metabolites play a role in the formation of adducts found in vivo. An important issue addressed in these studies was the role of the nitro group in adduct formation. The structural studies implicated the nitro group in some protein and DNA Adducts. However, the 1-acetylamino-*x*,*y*-dihydroxypyrene associated with albumin adducts appeared to originate from ring-oxidation and nitroreduction of 1-NP.

Dr. El-Bayoumy excluded 1-nitrosopyrene and 1-nitropyrene-4,5-oxide as major contributors to hemoglobin adduct formation because they were found to react primarily with the globin portion of the molecule, which was not the subunit that formed an adduct with 1-nitropyrene in vivo. This conclusion may have been premature because it excludes the possibility that different in vivo and in vitro reaction rates may account for the difference in hemoglobin products under the two conditions. 1-Nitrosopyrene and 1-nitropyrene-4,5-oxide both reacted efficiently with albumin, which suggests that they play a role in albumin adduct formation.

In vitro reactions between DNA and several known 1-nitropyrene metabolites (1-nitropyrene-4,5-oxide, 1-nitropyrene-*x*-oxide, and trans-1-nitropyrene-4,5-dihydrodiol) produced chromatographically complex adduct patterns. Some of the adducts were similar in chromatographic behavior to those in DNA taken from rats treated with 1-nitropyrene.

In summary, Dr. El-Bayoumy's work suggests that hemoglobin and albumin adducts may be useful biomarkers of human 1-nitropyrene exposure. In rats, hemoglobin adducts have a 14-day half-life. If the stability of 1-nitropyrene–hemoglobin adducts proves to be similar in humans, the level of 1-nitropyrene–hemoglobin adducts should reflect exposure during the preceding month. The albumin adduct life span in rats was similar to that of unmodified albumin. If a similar correspondence is found in humans, albumin adducts would have a half-life of 20 to 25 days in humans. Measuring both adducts may provide an analytical approach to monitoring human exposure.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

HEI's objective in funding this study was to facilitate the development of useful biomonitoring techniques for measuring exposure to diesel engine exhaust. Dr. El-Bayoumy

clearly demonstrated that hemoglobin and albumin adducts can be useful measures of 1-nitropyrene exposure. However, before these findings can be applied to human studies, further work is needed to identify the hemoglobin- and albumin-bound 1-nitropyrene adducts. The nature of the proposed phenolic 1-acetylaminopyrene-4,5-oxide intermediate, which was proposed to bind to serum albumin, also needs to be confirmed. The next step is to develop sensitive, specific, and inexpensive assays for these adducts. Several approaches are suggested by Dr. El-Bayoumy's work.

One approach is to use standard analytical chemistry methods to quantify DNA and protein adducts. As outlined in his original proposal, Dr. El-Bayoumy intends to use gas chromatography with mass spectrometry as an analytical tool for identifying of hemoglobin adducts. Because of the special characteristics of the heme moiety, he intends to methylate the adducts and determine their structure. If successful, the technique has the potential of being a rapid method to screen populations for recent nitropyrene exposure. Alternatively, it may be possible to generate adduct-specific antibodies and quantify the protein adducts using standard immunoassay techniques. Adduct-specific immunoassays have been developed for several chemicals of environmental interest (Groopman et al. 1992). Dr. El-Bayoumy is currently developing an immunoassay for one of the 1-nitropyrene–albumin adducts.

Dr. El-Bayoumy suggests that once the hemoglobin and albumin adducts are identified, they could be used to monitor human exposure. However, even if the nature of the adducts in the rat heme and albumin is established, this does not automatically lead to a biomarker of human exposure. It has now been well established that the oxidative metabolism of 1-nitropyrene is species- and tissue-specific and reflects the pharmacological status of the animals. This is because the oxidative metabolism of 1-nitropyrene can be carried out by a variety of P-450 isozymes (Howard et al. 1988, 1990). Thus, unlike the adduct obtained by nitroreduction, the specificity of the oxidative metabolism of 1-nitropyrene in humans and the presence of the same adducts as in the rat will have to be established.

Developing sensitive methods for measuring DNA adducts will be more problematic than developing protein adduct assays. Although quite sensitive, the ^{32}P-postlabeling method has low specificity. In addition, adducts unrelated to specific chemical exposures are often detected in animal studies. These products make interpretation of the gas chromatogram extremely difficult. Finally, the method is highly labor intensive and extremely sensitive to slight variations in procedure. Until the methods for quantifying DNA adducts become highly specific and technically simple, it is unlikely that 1-nitropyrene–DNA adducts will be as useful for molecular epidemiology as will protein adducts.

CONCLUSIONS

The overall goal of this study was to develop methods that could ultimately be applied to detect and quantify nitropyrene adducts in biomolecules of exposed individuals. The experiments described in Dr. El-Bayoumy's report indicate that stable adducts were formed in hemoglobin, in plasma albumin, and in white blood cell DNA of F344 rats following nitropyrene ingestion. Surprisingly, 1-nitropyrene formed an adduct with the heme subunit rather than the globin portion. These results differ from those obtained with other PAHs of hemoglobin, which bind to the globin portion.

Dr. El-Bayoumy's results suggest that measuring 1-nitropyrene–protein adducts may be a practical way to measure recent nitro-PAH exposure. This work provides a good foundation for the development of quantitative assays of diesel emissions exposure. However, before nitropyrene adducts of either DNA or proteins can be used as biomarkers in epidemiological studies, more work is needed to determine the nature of the adducts and to develop sensitive, specific, and inexpensive assays.

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