RESEARCH REPORT

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Respiratory Epithelial Penetration and Clearance of Particle-Borne Benzo[*a*]pyrene

Per Gerde, Bruce A Muggenburg, Margot Lundborg, Yohannes Tesfaigzi, and Alan R Dahl

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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 matter) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 200 projects at institutions in North America and Europe and has published over 100 research reports.

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HEI STATEMENT

Synopsis of Research Report 101

Penetration of Lung Lining and Clearance of Particles Containing Benzo[*a*]pyrene

Diesel exhaust is a mixture of gases and soot. Soot consists of carbon particles with bound inorganic salts, metals, and more than 450 organic compounds. The organic compounds include genotoxic polynuclear aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene (B*a*P), that cause cancer in laboratory animals. Soot inhalation is believed to be a possible contributor to lung cancer risk in occupationally exposed humans because it is readily inhaled and deposits in the lungs. Some researchers believe that PAHs must be released from soot and become bioavailable before they exert genotoxicity. However, the fate of the PAHs and their role in the toxicity of diesel exhaust are not well understood.

For the current study, Dr Alan Dahl and colleagues of the Lovelace Respiratory Research Institute in Albuquerque, New Mexico, planned to expose dog trachea to B*a*P bound to model soot particles and to determine whether it became bioavailable and reacted with the tracheal epithelium. When Dr Dahl left the Lovelace Institutes, Dr Per Gerde became Principal Investigator on this project and added exposures of peripheral lung alveolar region to the study design.

APPROACH

The investigators removed most of the organic compounds from diesel exhaust particles and bound radiolabeled B*a*P to them as a surrogate for all PAHs. They exposed the lower respiratory tract of three dogs to the particles and measured the levels of particle-bound B*a*P and free B*a*P released from particles in the peripheral region of the lungs. After approximately six months, they exposed only the trachea to the particle-bound B*a*P for similar measurements and isolated peripheral lung tissue to measure the long-term stability of B*a*P on the particles.

RESULTS AND INTERPRETATION

Soon after lung exposure, free B*a*P was detected in blood but its concentration quickly dropped, indicating that most of the bioavailable B*a*P had been released. Metabolites of B*a*P later appeared in blood, and some radioactive material became bound to peripheral lung tissue. Six months later, much of the B*a*P remained particle-bound in peripheral lung tissue and lymph nodes and was considered unavailable for genotoxic reactions. After tracheal exposures, some B*a*P became bioavailable and was metabolized. Therefore, both exposures in this study indicate that some particle-bound B*a*P becomes bioavailable and has the potential to exert genotoxic effects.

Diesel exhaust particles may not react the same as the model particles used in this study. The model particles contain B*a*P as a single surrogate for a complex mixture of PAHs and other organic molecules normally found on diesel exhaust particles. Release of the other organic constituents from normal particles would likely affect the rate and extent of B*a*P release. Further, the binding strength between B*a*P and the model particles may differ from that in diesel exhaust particles. In addition, the B*a*P concentration on the model particles was much greater than that calculated to be present on diesel soot particles. This concentration would be expected to influence its release rate. The investigators recognize these concerns but defend their study design as critical to address their aims. The interesting and provocative results of this pilot study must be interpreted carefully. The importance of genotoxic PAHs in the diesel exhaust remains to be confirmed.

This Statement, prepared by the Health Effects Institute, summarizes a research project sponsored by HEI from September 1997 to November 1999 and conducted by Dr Per Gerde of the Lovelace Respiratory Research Institutes, Albuquerque NM. The following Research Report contains both the detailed Investigators' Report and a Critique on the study prepared by the Institute's Health Review Committee.

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HEALTH EFFECTS INSTITUTE

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Research Report 101

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HEI STATEMENT

This Statement is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary.

PREFACE

INVESTIGATORS' REPORT

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The Report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in selecting or managing the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.

CRITIQUE Health Review Committee

The Critique about the Investigators' Report is prepared by the HEI Health Review Committee and Staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss remaining uncertainties and implications of the findings for public health.

RELATED HEI PUBLICATIONS

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PREFACE

In 1994, HEI initiated a research program to investigate the complex issues associated with the health effects of exposure to particulate matter (PM)* in the air. This program was developed in response to growing concern about the possible public health significance of reported associations between daily fluctuations in levels of PM and changes in daily morbidity and mortality in time-series epidemiology studies. These results were questioned for a variety of reasons, including the lack of support from experimental studies and the lack of a mechanism to explain how such effects would occur. To address these issues, HEI undertook two research initiatives in 1994: (1) the Particle Epidemiology Evaluation Project (Samet et al 1995, 1997), which evaluated six of the time-series epidemiology studies that had reported effects of PM on mortality; and (2) a program of toxicology and epidemiology studies (funded from RFA 94-2, "Particulate Air Pollution and Daily Mortality: Identification of Populations at Risk and Underlying Mechanisms"), which aimed to understand better how PM might cause toxicity and what factors might affect susceptibility. All of the studies from RFA 94-2 have been completed and published (Checkoway et al 2000; Dockery et al 1999; Godleski et al 2000; Goldberg et al 2000; Gordon et al 2000; Lippmann et al 2000; Oberdorster et al 2000; Wichmann et al 2000).

In 1996, HEI issued RFA 96-1, "Mechanisms of Particle Toxicity: Fate and Bioreactivity of Particle-Associated Compounds," seeking studies that would improve our understanding of toxicologically relevant characteristics of ambient particles by investigating the biologic role of particle constituents (such as their bioavailability and fate after being inhaled) and how they interact with cell components in different species. In all, HEI has issued five requests for research on PM and funded 34 studies or reanalyses over the last six years.

This Preface provides general regulatory and scientific background information relevant to studies funded from RFA 96-1, including the study by Per Gerde described in the accompanying Investigators' Report, Review Committee Critique, and HEI Statement. This is one of three studies from RFA 96-1 that were completed in 2000; the remaining two are scheduled for completion in 2001. The *HEI Program Summary: Research on Particulate Matter* (Health Effects Institute 1999) provides information on all PM studies funded since 1996.

* A list of abbreviations and other terms appears after the Investigators' Report.

BACKGROUND

Particulate matter is the term used to describe a complex mixture of anthropogenic and naturally occurring airborne particles. The size, chemical composition, and other physical and biologic properties of PM depend on the sources of the particles and the changes the particles undergo in the atmosphere. In urban environments, these particles derive mainly from combustion, including mobile sources such as motor vehicles and stationary sources such as power plants. The most commonly used descriptor of particle size is *aerodynamic diameter*. Based on this parameter, ambient particles tend to fall into three size classes (often defined as modes): ultrafine or nuclei mode (particles less than 0.1 μm in diameter); fine or accumulation mode (particles between 0.1 and 2.5 μm in diameter); and coarse (particles larger than 2.5 μm in diameter). Fine and ultrafine particles are dominated by emissions from combustion processes while coarse particles are mostly generated by mechanical processes from a variety of noncombustion sources. Generally, the ultrafine and fine fractions are composed of carbonaceous material, metals, organic compounds, and sulfate, nitrate and ammonium ions. The coarse fraction is generated mechanically and consists of insoluble minerals and biologic aerosols with smaller contributions from primary and secondary aerosols and sea salts (US Environmental Protection Agency 1996).

A number of early epidemiology studies indicated that human exposure to high concentrations of PM, such as London fog, had deleterious effects (such as increased number of deaths), particularly in children, the elderly, and those with cardiopulmonary conditions (Firket 1931; Ciocco and Thompson 1961; Logan 1953; Gore and Shaddick 1968). Because of this apparent relation to increased mortality, the US Environmental Protection Agency [EPA] has regulated the levels of ambient PM since 1971, when the Clean Air Act was first promulgated. This act authorized the EPA to set National Ambient Air Quality Standards (NAAQSs) for a number of potentially harmful air pollutants (including PM) in order to protect the health of the population, particularly those thought to be sensitive.

The first NAAQSs for PM were based on controlling total suspended particles up to 40 μm in diameter. In 1978, the standard was revised to regulate inhalable particles, or particles that can deposit in the respiratory tract and therefore have greater potential for causing adverse health effects. These particles have an aerodynamic diameter of 10 μm or

less (PM_{10}). More recent epidemiology studies, published in the early 1990s, indicate a relatively consistent association between small short-term increases in PM levels and increases in both mortality and morbidity from respiratory and cardiovascular diseases (reviewed by the Committee of the Environmental and Occupational Health Assembly, American Thoracic Society [Bascom et al 1996]).

Some studies also suggested that long-term exposure to low PM levels is associated with adverse effects (Dockery et al 1993; Pope et al 1995). These latter studies also pointed to a possible role of fine particles (less than 2.5 μm in aerodynamic diameter [$PM_{2.5}$]). In 1997, the EPA considered the evidence for the effects of fine particles sufficient to promulgate fine particle standards while retaining the PM_{10} standards (US Environmental Protection Agency 1997). The next review of the PM NAAQSs is scheduled to be completed by 2002.

RESEARCH PROGRAM FROM RFA 96-1

The wealth of epidemiology data published in the early 1990s suggested an association between PM and health effects, but aspects of these findings were not well understood. Problems involved uncertainties in exposure estimates, confounding by weather or other factors, the role of copollutants, and mechanisms by which particles may cause effects. Moreover, although the epidemiology findings were consistent across different communities exposed to distinct mixes and levels of pollutants, they were not well supported by either human chamber studies or animal inhalation studies aimed at delineating pathologic changes that might result in death. Failure of the experimental studies to provide support for the epidemiology findings was attributed to insufficient statistical power, use of particles not representative of ambient particles, or use of animals not representative of the individuals susceptible to increased mortality.

By the mid 1990s, researchers recognized that the research required to advance our understanding of the association between particle exposure and daily mortality found in epidemiology studies needed to focus on identifying (1) susceptible populations, (2) mechanisms by which particles may lead to increased mortality, and (3) characteristics of the particles responsible for the effects. It was recognized that both epidemiology and experimental studies would be required.

The HEI program initiated in 1994 aimed at addressing all of these research needs. Under RFA 96-1, HEI funded studies focused on the role of components of PM in toxicity. These studies used particles derived from mobile sources, from other well-defined combustion processes, or from ambient air to test hypotheses related to particle attributes and PM toxicity. Animal studies evaluated the role of peroxides associated with fine particles on toxicity in the rat lung (Debra Laskin of the Environmental and Occupational Health Sciences Institute) and the relative toxicity of ultrafine particles (iron and cerium) inhaled alone or in combination with soot (carbon) in neonatal and adult rats (Kent Pinkerton of the University of California, Davis). An in vitro study examined the role of particle-bound transition metals in PM toxicity (Ann Aust of Utah State University), and a clinical study compared the inflammatory response in endobronchial biopsy samples from normal and asthmatic humans exposed to concentrated ambient particles or diesel exhaust (Stephen Holgate of Southampton General Hospital, United Kingdom). The study presented in this Research Report by Per Gerde examined how particle binding affects the bioavailability of benzo[*a*]pyrene delivered to the lung.

CONTINUING RESEARCH

Many of the key questions identified in the early 1990s are still relevant and research continues to address them. Research strategies have evolved, however, as results from previous studies have provided insights into the most promising animal models and endpoints for evaluation. In addition, advances in exposure assessment and statistical methods have pointed to new approaches for conducting epidemiology studies. Under RFA 98-1, *Characterization of Exposure to and Health Effects of Particulate Matter*, HEI supported four exposure assessment studies, two epidemiology studies, two human controlled-exposure studies, and six animal studies of susceptibility to and toxicity of particulate matter.

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INVESTIGATORS' REPORT

Respiratory Epithelial Penetration and Clearance of Particle-Borne Benzo[*a*]pyrene

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ABSTRACT

Exposure to diesel exhaust is a suspected risk factor for human lung cancer. The carbonaceous core of the soot particles found in diesel exhaust and the condensed organic compounds adsorbed (or bound) onto the surface of the particles are both possible contributors to this suspected risk. The extent and rate at which organic procarcinogens desorb from soot particles in the lungs after environmental and workplace exposures and the degree of metabolic activation in the lungs are also not known.

We explored the relationship between a model polynuclear aromatic hydrocarbon (PAH)* and a typical carrier particle by measuring the rate of release, extent of release, and metabolic fate of benzo[*a*]pyrene (B*a*P) bound onto the carbonaceous core of diesel soot after bolus aerosol exposures of the dog's peripheral lung and trachea. Exogenous B*a*P was bound onto preextracted diesel soot at a surface concentration corresponding to 25% of a monomolecular layer. After deposition in the alveolar region, a fraction of B*a*P was rapidly desorbed from the soot and quickly absorbed into the circulating blood. Release rates then decreased drastically. When the B*a*P coating reached approximately 16% of a monolayer, it was not bioavailable and remained on the particles after 5.6 months in the lung. The bioavailability of B*a*P on particles retained in lymph nodes was markedly higher, however: after 5.6 months the surface coating of B*a*P was reduced to 10% of a monolayer. Fractions of B*a*P that remained bound to the soot surface during this 5.6 months had a low reactivity—nearly 30% of the radioactive compounds extracted from recovered soot particles were still B*a*P, the parent compound. In contrast, the rapidly released fraction of B*a*P, which was quickly absorbed through the alveolar epithelium after inhalation, appeared mostly unmetabolized in the circulation, along with low concentrations of phase I and phase II B*a*P metabolites. Within approximately 1 hour, however, this rapidly absorbed fraction of B*a*P was metabolized, most likely in the liver, with the metabolite spectrum being dominated by conjugated phase II metabolites. The fraction of B*a*P desorbed from particles deposited on the epithelium of the conducting airways was absorbed by the epithelium but slowly penetrated the capillary bed. The absorbed B*a*P was rapidly metabolized in the airway epithelium, as indicated by the influx of tritiated water (^3H_2O) from the lungs into the circulation.

The results suggest that the dosimetry of inhaled, highly lipophilic B*a*P during typical exposures is bimodal. The larger fraction of bioavailable B*a*P deposited in the alveolar region was absorbed mostly unaltered into the blood through the alveolar type I cells and was metabolized systemically. A smaller fraction of bioavailable B*a*P was deposited on the airway mucosa and rapidly metabolized, most likely in the airway epithelium. The substrate levels of B*a*P in the epithelium of the conducting airways exceeded the systemic levels by up to two orders of magnitude. This dramatic site-of-entry to systemic duality in the dosimetry of inhaled B*a*P is likely to be similar in most mammalian species and should be considered in risk assessment models for PAHs in humans.

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

This Investigators' Report is one part of Health Effects Institute Research Report 101, which also includes a Preface, a Critique by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr Per Gerde, Karolinska Institute, Institute of Environmental Medicine, Box 210, Stockholm SE-171-77, Sweden.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R828112 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

INTRODUCTION

Diesel soot particles are important contributors to the pollution of some workplace atmospheres as well as to urban air. Particles are involved in the increased daily mortality in the general population during episodes of higher air particulate levels in larger cities (Pope et al 1995), and

exposure to diesel soot is suspected of increasing the risk of lung cancer in humans (Mauderly 1994; Nauss 1995). With a relative risk ratio usually less than 2, the association is disputed, however, mainly because of possible confounding effects of smoking (Muscat 1996). Nevertheless, because of the large populations at risk of exposure, the association warrants further investigation.

Diesel exhaust particles consist of an aggregated core of ultrafine carbonaceous particles surrounded by a condensate of different hydrocarbons (Amann and Siegla 1982). The condensate contains a low concentration of PAHs (Heinrich et al 1995), some of which are known to be carcinogenic. However, the mechanism by which diesel exhaust may induce lung cancer remains unclear. Diesel exhaust is a pulmonary carcinogen in rats chronically exposed by inhalation (Heinrich et al 1995; Nikula et al 1995), but so are carbon black and titanium dioxide particles at similar exposure levels. A lung cancer incidence once thought to be caused by the content of carcinogens bound to the soot surface (Kotin et al 1955) in rats correlates more closely with an increased level of inflammation of the rat lung and build up of particle deposits (Oberdörster 1995). Other rodent species, such as hamsters and mice, are less sensitive (Mauderly et al 1996). Also, particles are retained differently in the lungs of monkeys than in rats and tend to induce more inflammation in rat lungs than in monkey lungs (Nikula et al 1997a,b). It therefore remains unclear whether the tumor response of the rat lung at high tissue burdens of solid particles is a reaction unique to that species and whether the phenomenon is similar to lung cancer risk in humans (ILSI Risk Science Institute 2000). Thus, the PAHs in the organic coat of the diesel soot may have a role in carcinogenesis, albeit with critical contradictions that must be resolved.

The levels of extractable PAHs on diesel soot are quite low in comparison to their levels on other air pollution aerosols such as coal tar pitch, for which lung cancer risk has been assessed with greater certainty (Heinrich et al 1994). Vostal (1983) has even questioned whether the carcinogenic PAHs on diesel soot are at all bioavailable in the lungs. Whereas PAHs can be easily extracted from the soot particles with organic solvents such as toluene or dichloromethane, results are dubious when liquids simulating the lung lining layer are used (Buddingh et al 1981; King et al 1981). The most common model solution for the liquid lung lining has been dispersions of surfactant liposomes in saline. Usually low or undetectable amounts of PAHs have been released from the diesel soot into this model solution. Yet, elevated levels of DNA adducts of PAHs in white blood cells have been observed in humans after exposure to diesel exhaust (Hemminki et al 1994; Sabro Nielsen et al 1996). According to other reports, exposure to carrier aerosols with much higher levels of organic extractable PAHs, such as tar pitch, has not led to elevated levels of DNA adducts in white blood cells (Kuljukka et al 1998; Carstensen et al 1999).

Different methods of deriving a dose measure for exposure to particle-associated PAHs have given conflicting results, so we sought to answer three critical questions regarding the toxicity of particle-associated PAHs: (1) Do PAHs have to be released from their carrier aerosols in order to become toxic to surrounding tissues? (2) If so, how do we quantitate the potential toxic level of PAHs on such particles? What is the extracting capacity of organic solvents in vitro compared with fluids lining the lung in vivo? (3) Once released from particles, how are PAHs absorbed in the lung, and are there differences in the metabolic fate of PAHs deposited in the two major regions of the lungs?

We chose to address these problems using a single-component surrogate for PAHs bound to the organic-denuded core particles of diesel soot. Our aim was to understand the exposure and dosimetry of PAHs bound to particles, in order to comprehend the link between early events, such as exposure, deposition, bioavailability, and metabolic activation; and later events, such as enzyme induction, adduction to DNA, and mutation patterns. We have not directly addressed the issue of whether inhaled PAHs on diesel soot are a human lung carcinogen and have not excluded the possibility that particles may contribute to lung cancer risk by means other than acting as carriers of genotoxic PAHs. B*a*P is a natural first choice for a PAH surrogate. B*a*P is a proven carcinogen in several animal models (Mehlman et al 1997), and mutations in lung tumors of smokers have a pattern of hot spots at the same codons as those of tumors induced in mice with B*a*P (Denissenko et al 1996). The dog was chosen as a model for the present study because its lungs are large enough to study regional deposition in the conducting airways and in the peripheral lung, and dogs may develop airway epithelial changes from exposure to tobacco smoke similar to those seen in human smokers (Park et al 1977).

SPECIFIC AIMS

The overall aim of this study was to investigate critical aspects of the particle association of B*a*P bound to diesel soot denuded of organic compounds. These aspects included bioavailability, rates of absorption, metabolic activation, and DNA adduction in the respiratory tract of the dog.

There were three specific objectives:

1. To measure (a) the alveolar absorption of diesel soot–bound B*a*P after deposition of an aerosol bolus in the dog's peripheral lung; (b) the metabolic turnover of diesel soot–bound B*a*P after alveolar absorption; and (c) the retention of B*a*P on diesel soot particles recovered from lung tissues after 6 months.
2. To measure the rate of desorption of B*a*P from diesel soot in vitro into 1-*n*-octanol.
3. To measure (a) the airway absorption of diesel soot–bound B*a*P after deposition of an aerosol bolus in the dog's occluded trachea; (b) the metabolite pattern of B*a*P in the tracheal mucosa after tracheal exposure; and (c) the DNA adducts in the epithelium and subepithelium, and in systemically exposed heart tissues of the tracheally exposed dog.

METHODS AND STUDY DESIGN

EXPERIMENTAL DESIGN

Three dogs were exposed to diesel soot–bound B*a*P in two consecutive sets of exposures. In the first set, dogs were exposed to a single breath bolus of aerosolized diesel soot that deposited on the airways and in the alveolar or peripheral region of the lung. Clearance of B*a*P from the lung following this deposition pattern was monitored by repeatedly sampling blood from both sides of the systemic circulation for 1 hour.

In a second set of exposures, at 154, 168, and 190 days after the alveolar exposures, respectively, the same dogs were exposed only in the airspace of the temporarily occluded trachea to a bolus of the same soot-bound B*a*P. Clearance of B*a*P to blood was monitored by repeatedly sampling blood from the azygous vein, which drains much of the tracheal mucosa, and from blood entering and leaving the lungs. Blood was sampled for 3 hours after the exposure and then the dogs were killed. The distribution of B*a*P and its metabolites in the tissues was determined.

PREPARATION OF PARTICLES

Collection of Diesel Soot

Diesel soot was collected from a three-cylinder, 3.8 L tractor engine (Bolinder-Munktell, Model 1113 TR) working at 80% of its rated 41.2 kW output at the Swedish Engine Test Center, Uppsala. The engine was run at 1,600 rpm on diesel fuel (Swedish environment class MK 3), and the exhaust was diluted 11-fold with air before precipitation on a Tepcon electrostatic filter (Model 2200, ActAir, Cardiff, United Kingdom) at 44°C. The entire exhaust flow of 1,600 kg/hr was passed through the filter. About 40 g of soot was scraped from the Teflon-coated electrodes and stored in the dark at –20°C.

Preparation of the B*a*P-Coated Diesel Soot

The collected diesel soot was preextracted twice with toluene in a Soxhlet apparatus for 6 hours, then dried at 130°C for 70 hours. The specific surface area of the organic-denuded soot (± SD) (Brunauer-Emmett-Teller method) (Adamson 1982) was 137 ± 1 m^2/g (n = 3).

Two identical batches of B*a*P-coated soot were prepared, one with unlabeled B*a*P for size determination of the aerosol and one with tritium-labeled B*a*P (Amersham, TRK 662, 463.3 dpm/pg uniformly labeled, batches: 0.8 X SP2 + 0.2 X B99A, 98% purity) for the exposures.

B*a*P from stock solution was evaporated to dryness under a stream of nitrogen and dissolved again in methanol. Denuded diesel soot (10.2 mg) and the B*a*P (150 ng) in 4 mL methanol were added to a melt-seal vial. The diesel and methanol suspension was gently agitated for 2 hours and evaporated to dryness under a stream of nitrogen. The vial was melt-sealed under a reduced nitrogen atmosphere and heated to 240°C for 2 hours to allow the B*a*P to bind evenly over the soot surface. After cooling, the cake of soot was finely ground with a spatula before further use. No crystals of B*a*P were visible in the soot as observed in a fluorescence microscope. A triplicate sample of the soot was extracted in a Soxhlet apparatus with toluene for 24 hours to determine the reextractable fraction of B*a*P on the soot. The extracted samples of soot were combusted to determine the remaining ^{3}H-activity on the particles. The produced batch of ^{3}H-B*a*P-coated soot was used throughout the study and contained 0.0145 ± 0.0001 μg B*a*P/μg soot, corresponding to a 25% coating of B*a*P on the soot surface (100 A^2/molecule [1A = 1 Å = 1 × 10^{-10}]). The level of surface coating was chosen from preliminary experiments to give an about equal distribution between firmly bound and easily extractable B*a*P. This distribution would allow both fractions to be quantitated in the different model systems used.

Extraction of Diesel Soot in Octanol In Vitro

To simulate the extent and rate at which soot-bound hydrocarbons are released in a lung lining layer fluid, the desorption of B*a*P from the labeled soot was measured in 1-octanol. A phospholipid and saline suspension would have seemed a more realistic choice, but extraction of highly lipophilic B*a*P from the soot surface into the highly dispersed lipid phase across gaps of water where solubility

is low creates a high resistance to mass transfer. This resistance outside the particles can give an artifactually low rate of release that can be easily misinterpreted as low or no desorption of B*a*P from soot surfaces. In the lungs, the surfactant layers and cell membranes are more nearly continuous, creating less resistance to mass transfer in the immediate vicinity of the particles. A better simulant of such a medium is a continuous organic phase where B*a*P has a similar chemical potential as in phospholipid membranes—hence, the choice of 1-octanol.

The experiment was conducted in a vertical, cylindrical glass reactor 27 mm in diameter with four low vertical glass baffles. The solution was stirred at 400 rpm with a two-bladed impeller (15-mm diameter and 12-mm blade heights). 1-Octanol pro analysi (17 mL) was added to the reactor and adjusted to 37°C. About 120 μg of the B*a*P-coated soot was added to the octanol, and the mixture was stirred immediately. After 20 seconds, the stirring was briefly stopped, and 0.5 mL suspension was quickly removed and centrifuged at 13,000*g* (Microcentaur, MSE, United Kingdom) for 8 minutes. From the clear supernatant, three 50 μL samples were transferred to scintillation vials for counting using Ultima Gold scintillation fluid (Packard Instruments, Meriden CT). The sampling procedure was repeated at 12, 24, 35, 46, and 58 minutes, 1 hour 10 minutes, 1 hour 30 minutes, and 2, 3, 4, 5, 6, 24, and 48 hours after addition of the soot. The time of separation of particles from the supernatant was counted from 1 minute after the sample was removed from the reactor.

Samples of the supernatant were observed with transmission electron microscopy to estimate the amount of soot that remained suspended in the solution after centrifugation. This fraction was negligible in comparison with the sedimented fraction of soot.

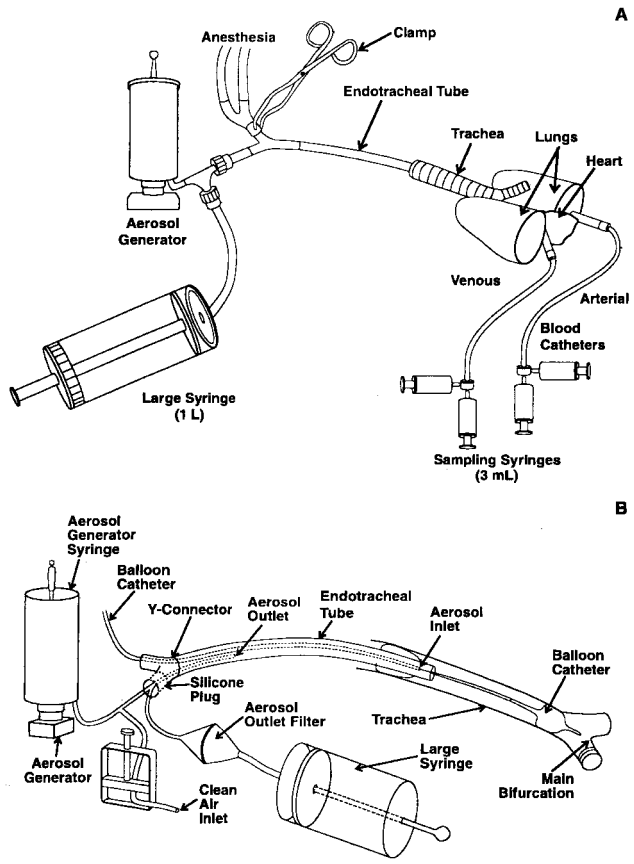

Figure 1. Exposure apparatus. (A) The dog's alveolar region was exposed in a single breath to an aerosol bolus of organic-denuded diesel soot with bound B*a*P. Bloodborne clearance was monitored by repeatedly sampling blood from both sides of the systemic circulation for 1 hour after the exposure. (B) An occluded section of the dog's trachea was exposed to the resuspended bolus of B*a*P bound onto diesel soot. Bloodborne clearance was monitored by repeatedly sampling blood from the azygous vein, which drains the tracheal mucosa, and from both sides of the systemic circulation.

Generation of Reaerosolized Diesel Soot

Single-breath boluses of aerosolized diesel soot were generated by means of a novel patented method (Figure 1A) (Gerde 1999). The aerosol generator consisted of a pressure chamber, a spherical powder chamber, and a fast-releasing valve. The pressure chamber was connected to the powder chamber via the valve, and the powder chamber had a narrow conduit leading to ambient pressure in the aerosol generator syringe. Nitrogen (220 mL) compressed to 80 atm was loaded into the pressure chamber and soot was loaded into the powder chamber. When the valve was released, the soot was explosively suspended and pressurized by nitrogen, equilibrating the pressure between the pressure and powder chambers. While in suspension, the soot agglomerates ejected through the narrow conduit into the aerosol generator syringe. Passage of the small orifice in the ejecting conduit allowed an explosive, yet controlled decompression of only a fraction of the suspension at a time, generating a micron-size aerosol. At the same time, the plunger of the syringe was pushed up by the volume of decompressed carrier nitrogen. After decompression, the aerosol was ready for exposure.

The average mass mean aerodynamic diameter (± SD) of the administered diesel soot, as determined repeatedly with the unlabeled soot, was 1.3 ± 0.2 μm (n = 5). The aerosol for the alveolar exposures was generated and passed through a set of tubings identical to the one used during the exposures. The aerosol was sized in a quartz crystal cascade impactor (QCM Cascade Impactor System, Model PC-2, California Instruments, Sierra Madre CA). Transmission electron microscopy revealed the reaerosolized soot to be rather densely packed aggregates of soot

spherules (Figure 2). Diameters of individual spherules typically ranged from 0.01 to 0.04 μm (Amann and Siegla 1982). Assuming a density of 2 g/mL of the spherules indicates that most of the surface area was external surface with little intraspherular porosity. The dimensions of the intraaggregate porosity of the soot was likely to be large enough not to contribute significantly to overall mass transfer resistance of bound B*a*P from micron-size particles to surrounding lung medium (Gerde and Scholander 1989). Delayed release of bound B*a*P was, thus, likely to be caused by the kinetics of surface desorption.

The dose of diesel soot deposited during the alveolar exposures was determined using a total filter (Millipore AA 1.2 μm, 25 mm) at the end of the endotracheal tube in the exposure set up. An aerosol bolus of the radiolabeled B*a*P-diesel soot was forced through the filter at the same flow rate used for the exposures. The amount of B*a*P on the filter was determined by total combustion and measurement of radioactivity by liquid scintillation counting (LSC). Converted to amount of soot, the deposition (± SD) was 36 ± 20 μg (n = 6). Tests for particle deposition were inserted into the exposure series. To reduce the risk of tritium contamination, the aerosol generator was cleaned between experiments by repeatedly ejecting gas without loading particles and then catching loosened particles on a total filter.

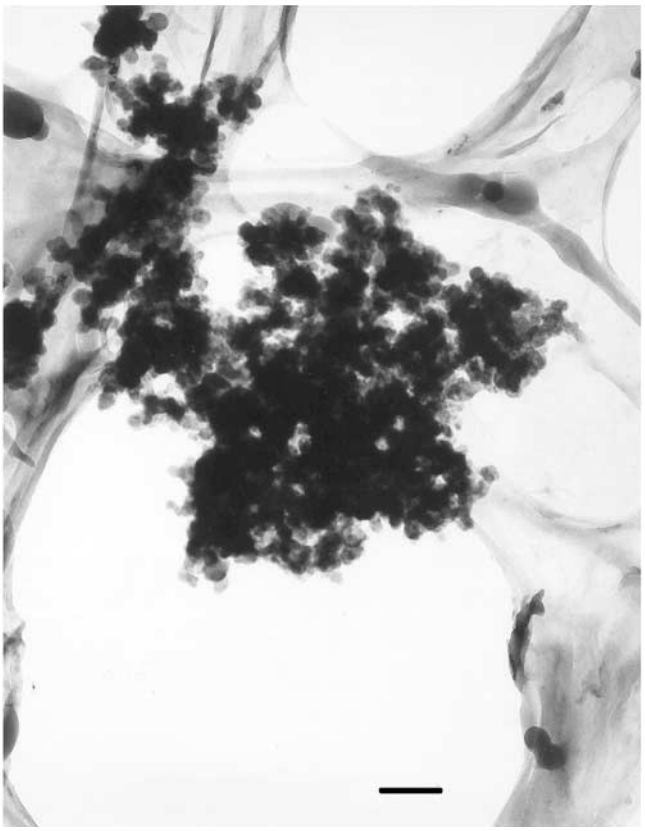

Figure 2. Transmission electron micrograph of aerosolized diesel soot caught on a Millipore filter. The length of the bar below is 0.136 μm.

To measure deposition during the tracheal exposures, the aerosol generator was connected to a series of tubing similar to that used during the exposures, but with a steel tube of similar dimension used to simulate the trachea. To simulate the stickiness of the airway liquid lining, the steel tube was coated with a layer of ultrasonic gel (Parker Laboratories, Fairfield NJ). The aerosol generator was triggered, and the diesel soot aerosol was passed through the occluded steel tube with similar flow rates and residence times as used during the exposures. Deposition in the trachea was estimated from that measured in the similarly dimensioned steel tube. The steel tubes were ultrasonically extracted with methanol, and the extract was transferred and evaporated to dryness on a filter paper. The level of radioactivity in the filter paper was determined by total combustion and LSC. Using this procedure, deposition (± SD) of soot in the trachea was estimated to be 0.7 ± 0.3 μg (n = 6).

ANIMAL EXPERIMENTS

Animals

Three dogs (Marshall Farms, North Rose NY) were used in this study. The dogs weighed 8.0 ± 0.9 kg. Food was withheld for 18 hours prior to the procedures. Each dog was given 0.2 mL of acepromazine subcutaneously. Anesthesia was induced 15 minutes later with isoflurane using a face mask.

Alveolar Exposures

When light surgical anesthesia was achieved, a tube was placed in the trachea, and the level of surgical anesthesia was maintained using isoflurane inhaled via the tube. Dogs were prepared for aseptic procedures. After surgical cut down over the femoral artery and vein, two catheters filled with heparinized saline were positioned: one in the posterior vena cava close to the right heart and one in the thoracic aorta. The anesthetized dog was connected to the exposure apparatus (see Figure 1A). Immediately before exposure, the dog was hyperventilated for 3 minutes to obtain an equally long period of apnea, and the line to the anesthesia machine was closed. With its exit tube closed, the aerosol generator was triggered, and about 220 mL of aerosol was generated. As soon as the generator syringe was filled, the aerosol bolus was gently pushed into the apneic dog within 4 to 5 seconds. The aerosol bolus was immediately followed by 90 mL of clean air from the large syringe to push the aerosol out into the alveolar region.

The aerosol was allowed to precipitate in the inflated lungs for about 2 minutes while the dog was still apneic. Next, the dog was ventilated several times through a total filter for a short period to remove the remaining suspended aerosol and then reconnected to the anesthesia machine.

Blood sampling began at the moment the clean air was injected after the aerosol bolus. Blood (approximately 1 mL/sample) was sampled from both sides of the systemic circulation for 1 hour. Immediately after sampling, approximately 0.2 mL of each blood sample was transferred to 2-mL paper thimbles (Packard Instruments) for determination of total radioactivity. The samples were immediately frozen at –80°C, as were the remaining fractions of the blood intended for metabolite analysis.

After the blood-sampling period, the catheters were removed, the blood vessels repaired, and the surgical incisions closed. The dogs were carefully observed during recovery. At 5.6 months after the alveolar exposures, the occluded trachea was exposed to the same preparation of diesel soot with bound B*a*P and the dogs were killed. The four tracheobronchial lymph nodes and some peripheral lung tissue were sampled for analysis of diesel soot remaining from the alveolar exposures. These tissues were stored at –80°C until processing.

Tracheal Exposures

The dogs were prepared for aseptic surgical procedures. When light surgical anesthesia was achieved, an endotracheal tube was placed in the trachea, and surgical anesthesia was maintained using isoflurane via the tube. After surgical cut down over the femoral artery and vein, two catheters filled with heparinized saline were positioned: one in the thoracic aorta and one in the posterior vena cava at the entrance to the right atrium. The right chest was entered through the fourth intercostal space. Apical and cardiac lung lobes were pushed posteriorly, and the azygous vein was located. The vein was dissected free for several centimeters just before its junction with the anterior vena cava, and a catheter filled with heparinized saline was passed into the vein lumen in the dissected area to collect blood periodically.

The anesthetized dog was connected to the exposure apparatus (Figure 1B). The endotracheal tube had two tubes inside. One was the aerosol inlet tube, which was connected to the aerosol generator syringe. This tube extended to the cuff end of the tracheal catheter. The other tube was used as the aerosol outlet from the trachea. This tube extended some 50 mm into the endotracheal tube and was connected to a 37-mm glass fiber filter outside the tracheal tube. Both the aerosol inlet and outlet exited through a sealed branch of a Y-connector. The other branch was connected to the anesthesia machine (Figure 1B).

The anesthetized, intubated dog was hyperventilated for 3 to 4 minutes to provide equal amounts of apneic time. When the dog was in apnea, the anesthesia machine was disconnected, and a balloon catheter (27-mm maximal balloon diameter) (Meditech, Boston Scientific Corporation, Watertown MA) was inserted and advanced to the proximal end of trachea at the carina. The balloon was inflated to occlude the tracheal lumen, and the outlet around the balloon catheter was sealed with a dental dam and hose clamp. This procedure occluded a length of about 80 mm of the trachea.

With the aerosol inlet to the trachea closed, the aerosol generator was triggered and the plunger pushed up about 220 mL. Immediately thereafter, the aerosol inlet was opened, and the generated aerosol was slowly pushed into the occluded trachea and on through the aerosol outlet filter. When the plunger of the aerosol syringe reached the bottom, the aerosol was left to settle in the trachea throughout the period in apnea or about 2 minutes. Toward the end of this period, the side branch of the aerosol inlet tube was opened to ambient air, and approximately 1 L of air was pulled from this inlet through the trachea and the aerosol outlet filter, using a large syringe downstream of the outlet filter. The balloon catheter was deflated and removed, and the dog was reconnected to the anesthesia machine.

Immediately after the exposure, 2-mL samples of blood were repeatedly taken from the azygous vein and both sides of the systemic circulation over a 3-hour period. Immediately after samples were drawn, approximately 0.5 mL of blood was transferred to combustion paper cups for determination of total radioactivity. The rest of the blood in each syringe was frozen on dry ice and stored at –80°C until analysis. At the end of the 3-hour sampling period, the isoflurane level was increased to provide deep surgical anesthesia, and the dog was killed by exsanguination through a heart puncture. Immediately after death, the trachea, extrapulmonary bronchi, and lungs were excised intact from the thoracic cavity.

The tracheal catheter was left in place until immediately before microdissection to limit transport of deposited diesel soot from the exposed section of the trachea by mucociliary clearance. Using a reported airway dissection technique (Plopper et al 1983), the trachea and bronchial segments exposed to the aerosol bolus were microdissected into centimeter-long segments starting from the larynx, proceeding through the left main bronchus through the fourth generation, then through the right main bronchus through the fifth generation (Figure 3). Two airway segments, 4 cm long, were also collected to measure DNA adducts in airway epithelium and subepithelium. One segment had been directly

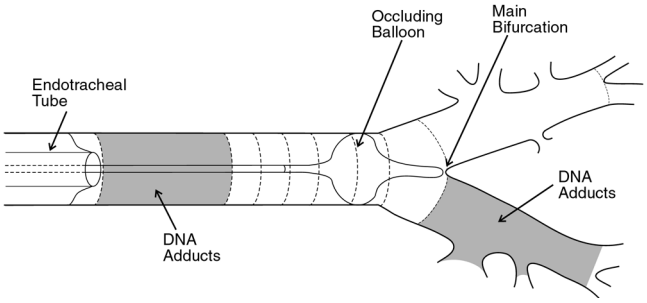

Figure 3. Dissection pattern of upper bronchial tree after exposure of temporarily occluded trachea to aerosol bolus of B*a*P bound to organic-denuded diesel soot.

exposed to the B*a*P-diesel aerosol and located in the trachea. The other one, not directly exposed, was located in the right main bronchus. The mucous layer of all airway segments was rinsed with saline and immediately frozen. The airway sections intended for DNA adduct analysis were immediately immersed in 0.1% Pronase solution in Dulbecco Modified Eagle Medium and incubated at 37°C for 1 hour to remove epithelial cells (Thomassen et al 1986). All the other segments were frozen and stored at –80°C.

After incubation, the airway epithelial cells inside the segments were rinsed into petri dishes, collected, and centrifuged. The epithelial cells and the denuded airway segments were then frozen and stored at –80°C. Airway sections to be used for separation of B*a*P into metabolites and parent compound were stored under argon. The four tracheobronchial lymph nodes were dissected free and frozen separately for recovery of diesel soot deposited during the previous alveolar exposures. All tissues were stored at –80°C until further processing.

Radiochemical Analysis and Metabolite Fractionation

Before further analysis, the samples were dried by vacuum distillation with collection of water for LSC. ^3H_2O is an important indicator of the intensity of the metabolic turnover of radiolabeled B*a*P. When dry, the B*a*P-related tritium in blood and tissue samples was measured by total combustion and LSC of the ^3H_2O generated in the combustion (Gerde et al 1998). In between every other to every fifth sample, a volume of distilled water was sampled through the combustion cycle to control carryover of radioactivity from one sample to the next. At the specific activity of the radiolabeled B*a*P used, a detection limit set to counts 50% above background (approximately 40 disintegrations per minute [dpm]) corresponded to approximately 0.2 fmol of B*a*P-equivalent.

The metabolite patterns in blood and tissues were analyzed as previously described (Scott et al 1998). The dried blood samples were resuspended in saline and extracted five times with ethyl acetate with periodic centrifugation, and the organic fraction was removed. The organic fraction contained parent compound and its lipophilic phase I metabolites. The aqueous fraction was centrifuged, and the supernatant and separated pellet were combusted separately. Water-soluble radioactivity was from the conjugated phase II metabolites, and the radioactivity of the separated pellet gave the covalently bound fraction of B*a*P. To improve uptake of B*a*P and its lipophilic metabolites into the high-pressure liquid chromatography (HPLC) elution fluid from the pooled organic fraction, the lipids of this solution were saponified with calcium oxide and reextracted with ethyl acetate. After centrifugation and washing of the precipitate with ethyl acetate, the organic extract was dried and dissolved again in the HPLC elution fluid (water and methanol). The chromatograms were used to separate B*a*P from its phase I metabolites. Figure 4 shows an overview of the metabolite pattern of B*a*P.

Recovery of Diesel Soot from Tissues

Soot was recovered by a modified procedure of Sun and colleagues (1989) to analyze the content and nature of B*a*P still bound to diesel soot retained in tissues. The tracheobronchial lymph nodes and approximately 1 g of peripheral lung tissue from each dog were homogenized with a Tissuemizer in 10 mL of distilled water. Homogenate (5 mL) was layered on top of 15 mL 25% sucrose solution in a 38 mL centrifuge tube (polycarbonate, 1 × 3.5 inches). The tubes were centrifuged for 2 hours at 100,000*g* (Beckman L8-60M).

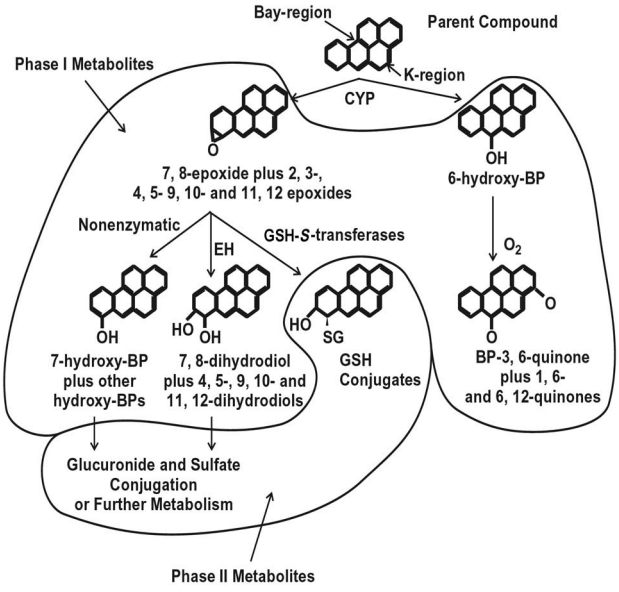

Figure 4. Overview of the metabolite pattern of B*a*P.

After centrifugation, the upper tissue homogenate layer plus some of the sucrose layer were transferred and air dried for analysis of radioactivity by total combustion. Two 1-mL samples were taken from the sucrose layer and counted directly after adding 20 mL of Ultima Gold. The rest of the sucrose layer was carefully removed. The precipitated particles were wiped up with small puffs of cotton and placed on filter paper to air dry. When dry, the cotton balls were folded into the filter paper to form a package that was secured with a paper clip. The dry cotton-filter paper package was placed in a Soxhlet apparatus and refluxed for 24 hours. Aliquots of the toluene solution were counted, and the total amount of extracted radioactivity was calculated. Samples of the toluene extract were evaporated to dryness, then dissolved again in the elution fluid for HPLC. Chromatograms of the toluene extracts contained substantial amounts of water-soluble radioactivity that eluted with the void volume. Because of the unknown nature of the B*a*P-equivalent radioactivity recovered from the particles, it was divided into B*a*P parent, organic-extractable and water-soluble B*a*P-equivalent radioactivity.

The filter paper extracted by the Soxhlet apparatus was air dried for 24 hours, then combusted. For each sample the total amount of radioactivity in the toluene extract (M_{TE}) was compared with that in the combusted filter paper containing the extracted particles. We assumed that the fraction of total radioactivity not extracted with toluene in a Soxhlet apparatus was the same whether the particles had resided in the lungs or not. This allowed the amount of soot recovered from the tissues to be calculated, as well as the total amount of radioactivity (M_{TOT}) expected to have been associated with this soot before the exposure, assuming homogeneous distribution. By comparing M_{TOT} with M_{TE}, the amount of B*a*P recovered in the toluene extract and with the amount remaining on the extracted particles (M_{EP}) as measured by combustion, the fractional bioavailability (BA) of soot-bound B*a*P in the tissues was calculated according to:

$$BA = 1 - [(M_{TE} + M_{EP})/M_{TOT}].$$

DNA Adducts

The levels of tritiated DNA adducts of B*a*P were measured in tracheal epithelium, in epithelial-denuded tracheal tissue, and in similar samples of the mainstem bronchi unexposed to the B*a*P-diesel soot aerosol (Figure 3). Epithelial cells (about 20 to 50 mg/sample) and the weighed frozen airway section (approximately 500 mg) from the denuded tracheal tissue were incubated in 300 µL of proteinase K solution (20 mg/mL) at 50°C over night while shaking. DNA was extracted from cells and tissues. Approximately 500 mg of tissue as 3-mL homogenates were used for the two subepithelial samples from each dog. For the two epithelial cell samples, a scaled-down procedure was used. The residues from each extraction step were saved for quantitation of radioactivity. After the phenol extraction, the residual unbound radioactivity was measured by extraction with five equal volumes of diethyl ether (Mitchell 1985). The radioactivity in the ether fractions was counted after evaporation of the ether in scintillation vials. The ether fraction contained background radioactivity. About one third of the length of the epithelial-denuded airway segment was cut transversely. The mucosa down to the cartilage and the noncartilaginous stretch of the circumference were dissected for extraction of DNA. The remaining two thirds of the airway section was combusted for determination of total radioactivity together with all fractions generated during the DNA extractions. The total retained radioactivity of airway sections intended for the DNA extractions could thus be determined.

After quantitation of DNA by ultraviolet spectrophotometry, each liquid DNA sample was soaked into combustion-aid cotton in combustion cups and dried on the vacuum line. DNA-bound tritium was then determined by combustion of the paper cups. Between each DNA sample, samples with distilled water were combusted to determine a baseline of background radioactivity. ^3H_2O dried from the DNA samples was also assayed by LSC. Three samples of heart tissue were taken from the dogs for determination of tritiated DNA adduct levels in tissues exposed to B*a*P only via the systemic circulation.

In measuring DNA adducts of tritiated toxicants, some adducts may be false positives derived from a direct tritium exchange between either the labeled toxicant and extracted DNA, or ^3H_2O and extracted DNA. With the enormous excess of unlabeled water in all tissues, a direct exchange between labeled water and DNA would seem unlikely. Regarding B*a*P itself, a direct nonmetabolic exchange of tritium between parent compound and DNA would be possible, but not so likely. Incubating tritiated B*a*P (^{3}H-B*a*P) with biomolecules such as phosphatidylcholine from egg yolk for hours maintained a high purity of the B*a*P and gave undetectable levels of ^3H_2O (unpublished observation, Gerde and associates 1995). Tritiated DNA adducts of B*a*P from mouse liver generated by intraperitoneal injection have been used as reference samples (Phillips and Castegnaro 1999).

STATISTICAL METHODS AND DATA ANALYSIS

Data are presented as means and standard deviations. Due to small sample sizes, no further statistical analyses were performed.

RESULTS

ALVEOLAR EXPOSURE

After the bolus of denuded diesel soot with bound B*a*P was deposited in the peripheral lung, desorbing B*a*P-equivalent appeared rapidly in the systemic circulation (Figure 5). The concentration in the blood peaked at 2.1 ± 0.6 minutes in all three dogs. However, dog C had approximately a fourfold higher systemic level of B*a*P in the blood than the other two, most likely because of a greater deposited amount of diesel soot (Figure 5, dog C). Clearance of B*a*P-equivalent from the lungs was estimated by trapezoid integration of the net increase in blood concentration of B*a*P upon passage of the lungs ($C_{OUT} - C_{IN}$) multiplied with the cardiac output Q (1.42 L/min).

For each dog, the fraction of bioavailable B*a*P-equivalent retained in the lungs, *F*, was calculated from clearance according to:

$$F = 1 - \frac{\int_0^t (C_{OUT} - C_{IN}) Q \, dt}{\int_0^T (C_{OUT} - C_{IN}) Q \, dt}$$

where *t* is time, *dt* is time increment, and *T* is the duration of the period of a net influx of B*a*P-equivalent into the circulation. Whereas cumulative clearance was about tenfold higher in dog C than in dogs A and B (Table 1), the fractional retention of B*a*P-equivalent, as determined by the integration, was similar in all three dogs. The first half-time of absorption (± SD) was 4.3 ± 0.8 (n = 3) minutes (Figure 6). The net influx into the circulation (the difference between B*a*P concentration in blood from the left and right heart), peaked (± SD) at 1.8 ± 0.8 minutes (n = 3). For dogs A and B, approximately 30% of the estimated deposited amount of B*a*P-equivalent was cleared to the blood during the early period when a net influx from the lungs to the blood could be measured (Table 1). This is similar to the fraction of B*a*P

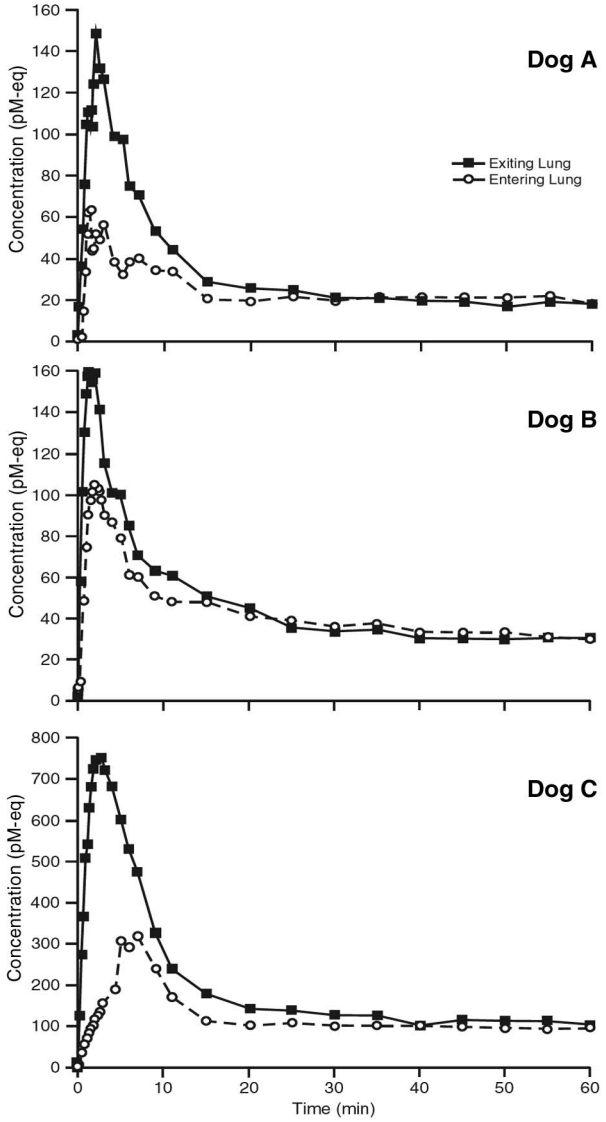

Figure 5. Concentration of B*a*P-equivalent activity in blood in dogs A through C as function of time after inhalation exposure of alveolar region to aerosol bolus of B*a*P bound to organic-denuded diesel soot. Note that the concentration scale of the panel for dog C is different from those of A and B.

Table 1. Clearance of B*a*P-Equivalent to Blood and Retention of Soot Particles in Lung Tissues Compared with Estimated Amounts Deposited in Alveolar Region[a]

Parameter	Dog A	Dog B	Dog C
Cumulative clearance of B*a*P-equivalent in blood within ~30 min of exposure (ng)	192	113	1770
Cumulative clearance of B*a*P-equivalent in blood (fraction of deposited)	0.37	0.22	3.4
Soot concentration in lung at 5.6 months after exposure (µg/g)	0.39	0.55	0.96
Estimated total amount of soot deposited in lung (µg)	23	32	56
Soot recovered/soot deposited in lung	0.64	0.89	1.56

[a] Blood clearance was calculated by integrating the increase in concentration of B*a*P-equivalent systemic blood upon passage of the lungs multiplied by the cardiac output (see Figure 5 and the Alveolar Exposure section under Results).

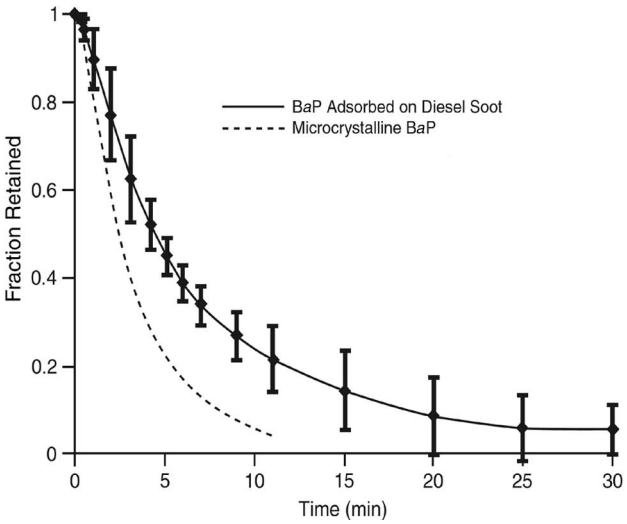

Figure 6. Fractional retention in the dogs' lungs as a function of time of the readily bioavailable BaP-eq bound to denuded diesel soot. The dashed curve shows the corresponding fractional retention of microcrystalline BaP (data from Gerde et al 1993b). Cumulative clearance was determined by integrating the net increase in concentration of BaP-eq in systemic blood upon passage of the lungs times the cardiac output. In each dog fractional retention of bioavailable BaP-eq on the soot was calculated by taking one minus cumulative clearance divided by total clearance. Error bars show SD, n = 3.

released from the soot particles during extraction in 1-octanol in vitro (Figure 7). Note that the release in Figure 6 is related only to the readily available fraction of B*a*P, whereas the release in Figure 7 is related to the total amount of B*a*P on the soot. Initially, soot-bound B*a*P was quickly released in 1-octanol. Within minutes the concentration on the soot decreased from the initial 25% to approximately 18% surface coating, but in the next 48 hours the concentration only dropped to about 16% surface coating. At this point, 36% of the original amount of B*a*P on the soot was released. The in vitro extraction thus showed that desorption from the particles is most intense at the moment of deposition and that the release rate then decreases with the inverse of time after deposition. At 24 hours after deposition in 1-octanol, the release rate of B*a*P dropped to approximately 1/10,000th of the initial release rate (Figure 8).

^3H_2O, indicating metabolism, appeared rapidly in the blood with a net influx from the lungs in all three dogs during the first minutes after the exposure. The level of ^3H_2O in the systemic blood increased rapidly up to 40 minutes after the exposure, then leveled off (Figure 9). In all three dogs, an early net influx of ^3H_2O from the lungs indicated a rapid onset of metabolism in airway mucosa. In dog A, this net influx of ^3H_2O from the lungs persisted throughout the 1-hour blood sampling period. In contrast, only a small fraction of the B*a*P-equivalent activity entering the blood during the rapid initial pulse was in the form of metabolites—phase I and phase II metabolites in about equal amounts (Figures 10 and 11). The HPLC chromatogram of Figure 11B shows separation of the organic-extractable fraction of B*a*P-equivalent (B*a*P parent and phase I metabolites) in blood exiting the lungs of one dog at 2.2 minutes after exposure. The rapid initial pulse of B*a*P decreased quickly toward a much lower and steadier level of radioactivity that tapered off with a much longer half-time. Within 40 minutes of the exposure, the slowly declining fraction of radioactivity in the blood became dominated by phase II metabolites and bound radioactivity (Figure 10).

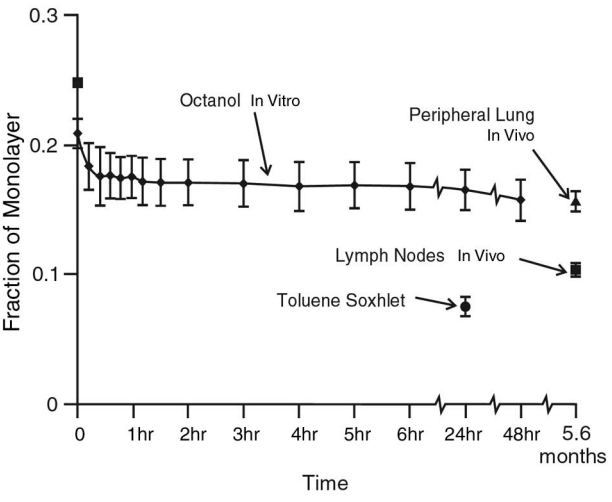

Figure 7. Release of B*a*P from diesel soot in well-stirred solution of 1-octanol at 37°C, in toluene during Soxhlet extraction, and in tissues after 5.6 months retention in vivo. Surface concentration as a function of time is given as a fraction of a monomolecular layer of B*a*P bound to the soot surface. The initial surface concentration on the soot was 25% of a monomolecular layer. Error bars show SD, $n = 4$ in vitro, $n = 3$ in vivo.

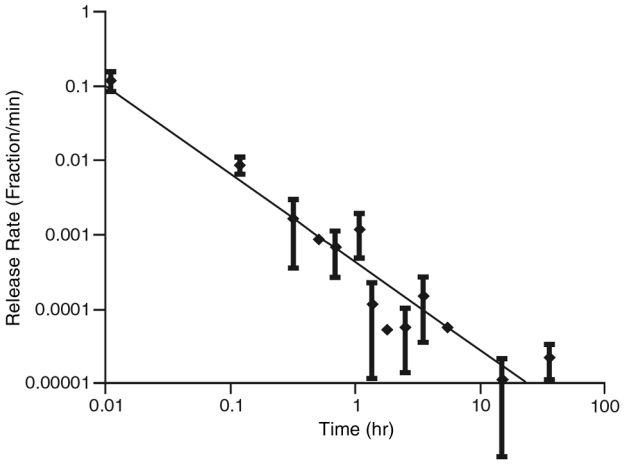

Figure 8. Release rate of soot-bound B*a*P into 1-octanol as a function of time, given as fraction of total. The fitted decay function is $f(x) = [4.239 \times 10^{-4}]/x^{1.191}$ and $r^2 = 0.911$. Note that both axes are in log scale.

Diesel soot was recovered by ultracentrifugation from tissues of the peripheral lung and lymph nodes after 5.6 ± 0.6 (n = 3) months retention time. Results show that 37 ± 3% (n = 3) and 59 ± 2% (n = 3) of the originally bound BaP did not remain on the soot in peripheral lung and lymph nodes, respectively (Figure 7). During centrifugation of peripheral lung tissue and lymph nodes, respectively, 99 ± 0.5 and 91 ± 12% (n = 3) of the radioactivity was recovered from below the sucrose layer (ie, from the particles). The rest was in the tissue homogenate-containing layer on top. This suggests that most of the retained radioactivity came from the particle-associated BaP rather than tissue-bound BaP. For dogs A and B, the amount of soot recovered from the lung tissues corresponded to a long-term retention of soot in the lungs of approximately 77% (Table 1), which is close to the measured long-term retention of inert particles in the dog's lung (Snipes 1989). For dog C the amount recovered exceeded the amount deposited, as estimated from filter tests. Analysis of BaP-equivalent extracted from the recovered soot showed that about one third remained as parent compound on the particles (Table 2) with nearly half being lipophilic metabolites and decay products of BaP (Figure 12).

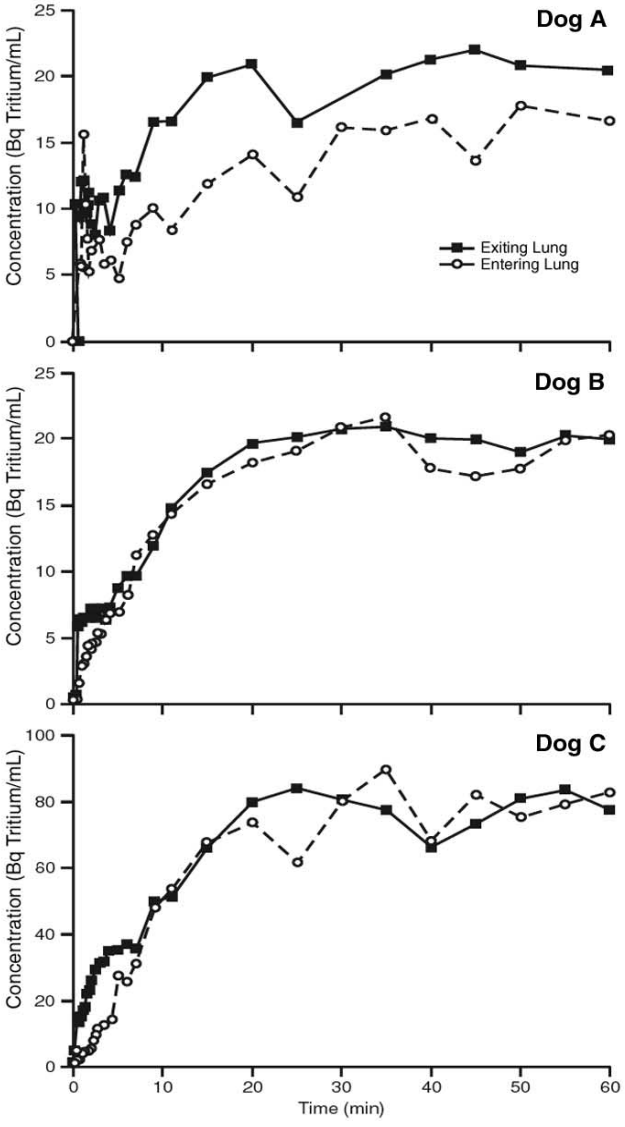

Figure 9. Concentration of ^3H_2O in blood as function of time in dogs A through C after exposure of alveolar region to aerosol bolus of denuded diesel soot with bound BaP. Note that the concentration scale of the panel for dog C is different from those of dogs A and B.

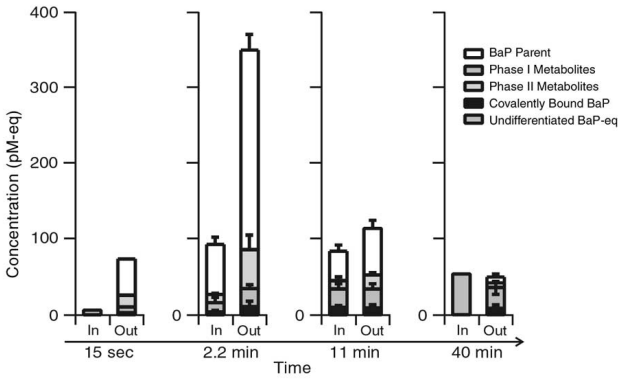

Figure 10. Metabolite pattern of BaP in systemic blood at different time points after deposition of aerosol bolus in alveolar region of denuded diesel soot with bound BaP. Each pair of bars shows, respectively, the average concentration of different metabolite categories in blood entering (IN) and exiting (OUT) the lungs at that particular time. Error bars show SD (n = 3) of the metabolite categories given as fraction of total radioactivity, not of absolute concentration.

TRACHEAL EXPOSURE

Even 5.6 months after alveolar exposures, significant amounts of radioactivity were retained in lung parenchyma, tracheobronchial lymph nodes, and blood (blood samples prior to tracheal exposures contained elevated levels of ^3H_2O and BaP-equivalent activity) (Figures 13 and 14). After the tracheal exposures, the concentration of BaP-equivalent activity in blood increased measurably in dogs A and C, but not in dog B. ^3H_2O increased in the blood of all three dogs after the tracheal exposure more rapidly than did the BaP-equivalent, which is consistent with the higher mobility of water in tissues.

After the blood-sampling period, high levels of radioactivity were found in the mucous lavage fluid and tissues of the occluded segment of trachea in all three dogs. The concentrations tended to increase toward the proximal end of the occluded trachea, the direction of mucociliary transport (Figure 15). Of the total BaP-equivalent activity retained in the exposed tracheal section, 67 ± 18% (n = 3)

was in the mucous lavage fluid and the rest in the tissue. We assumed that most of the radioactivity in the tissues was released from the diesel soot, whereas the dominating fraction of radioactivity in the mucous lavage fluid was still soot-associated. This is consistent with the measured fraction released into 1-octanol in vitro after a similar period of time.

Fractionation of the radioactivity in tracheal tissues into metabolites indicated that substantial fractions of B*a*P had been metabolized into phase I and phase II metabolites

Table 2. B*a*P-Associated Radioactivity Bound to Soot in Respiratory Tract Tissues at 5.6 Months After In Vivo Exposure of Alveolar Region[a]

Tissue	Water Soluble	Organic Extractable	Parent B*a*P
Peripheral lung	16 ± 6	50 ± 7	34 ± 13
Lymph nodes	22 ± 3	49 ± 16	29 ± 17

[a] Values are means ± SD in %; $n = 3$.

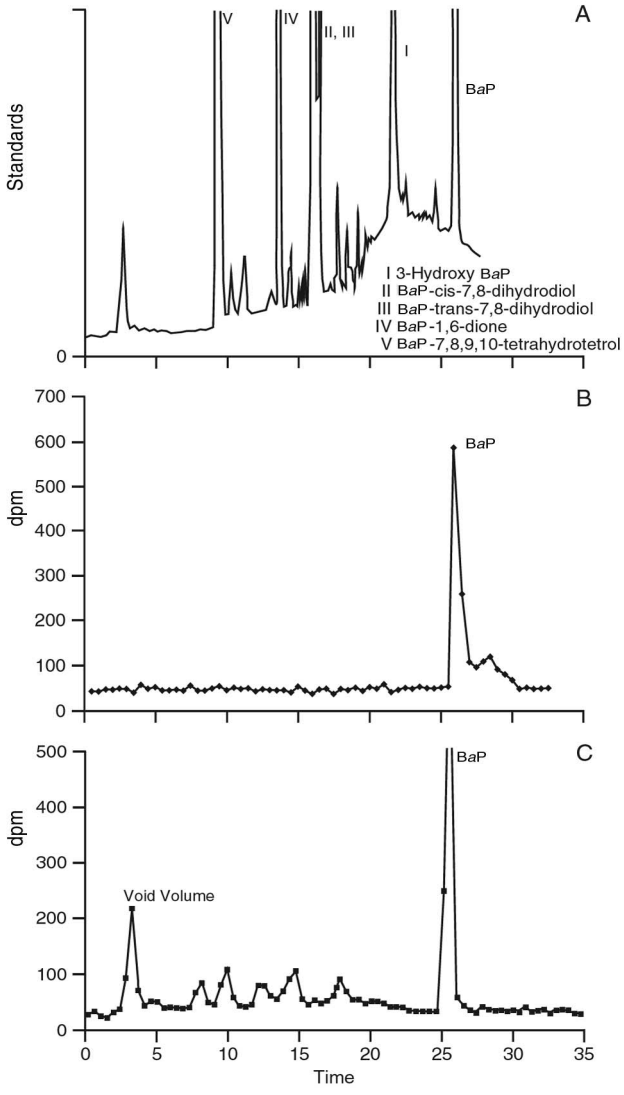

Figure 11. Composition of organically extractable B*a*P-equivalent activity after short-term retention as solute in blood and lung tissues. HPLC chromatograms. (A) B*a*P and organic extractable metabolites (standards). (B) Blood exiting the lungs at 2.2 minutes after inhalation of an aerosol bolus of B*a*P bound to denuded diesel soot. The gross metabolite pattern is shown in Figure 10. (C) Tracheal mucosa at 3 hours after exposure of the temporarily occluded trachea to an aerosol bolus of B*a*P bound to denuded diesel soot. The gross metabolite pattern is shown in Figure 16.

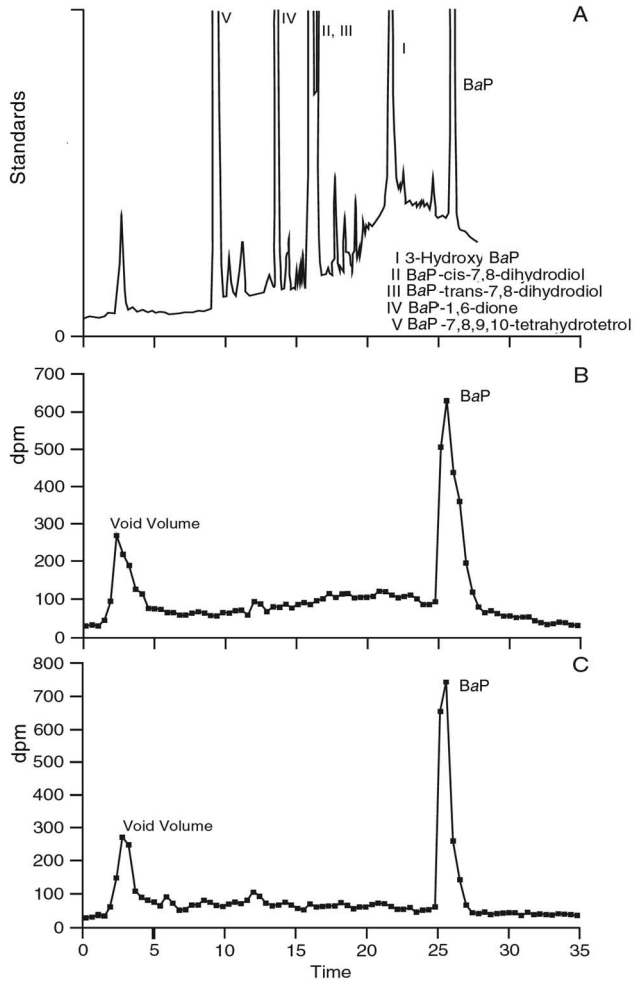

Figure 12. Composition of B*a*P-equivalent activity extracted from soot surfaces after long-term retention in lung tissues. HPLC chromatograms. (A) Extracted B*a*P and metabolites or decay products (standards). (B) Toluene extract of diesel soot recovered from the lung parenchyma 5.6 months after the bolus inhalation of B*a*P bound to denuded diesel soot. (C) Toluene extract of diesel soot recovered from the tracheobronchial lymph nodes 5.6 months after the bolus inhalation of B*a*P bound to denuded diesel soot.

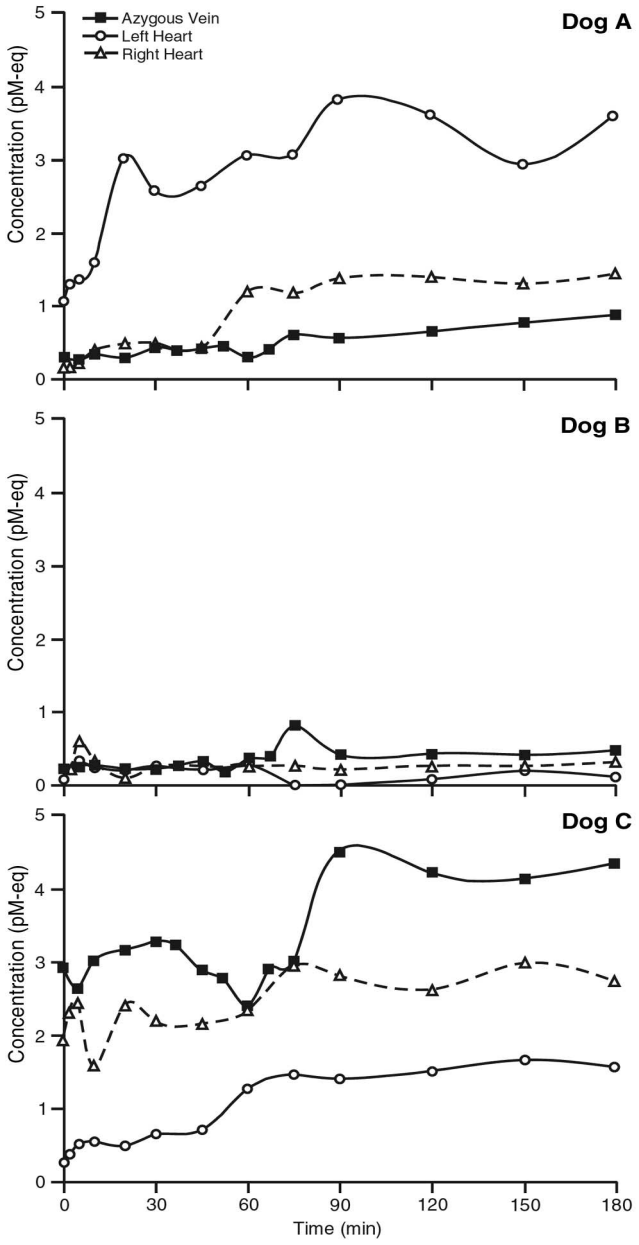

Figure 13. Concentration of B*a*P-equivalent in blood as function of time in dogs A through C after exposure of temporarily occluded trachea to aerosol bolus of B*a*P bound to denuded diesel soot.

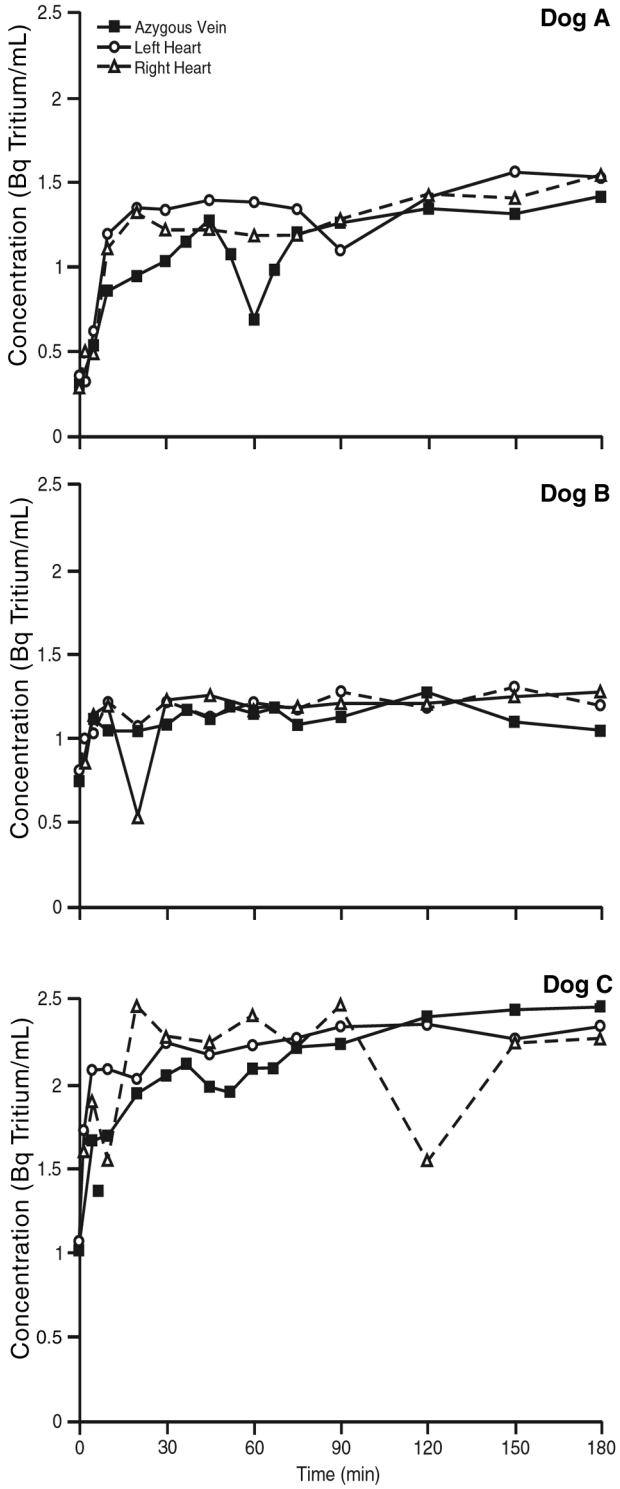

Figure 14. Concentration of $^{3}H_{2}O$ in blood as a function of time in dogs A through C after exposure of temporarily occluded trachea to aerosol bolus of B*a*P bound to denuded diesel soot.

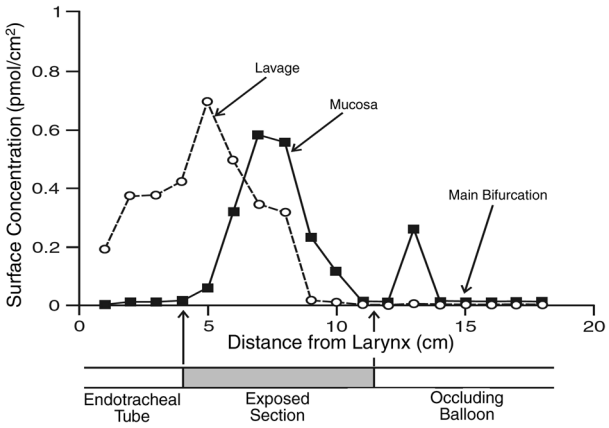

Figure 15. Surface concentration of B*a*P-equivalent activity in mucous lavage fluid and tracheal mucosa as a function of position along the upper bronchial tree at 3 hours after exposure of temporarily occluded trachea to aerosol bolus of denuded diesel soot with bound B*a*P.

(Figures 11C and 16). A large fraction was also generally bound to the tissue.

The analysis of tritiated DNA adducts was inconclusive regarding potential increased DNA damage as a result of local metabolism in the epithelium. The concentrations in all tissues were in the range of 2.2 to 27 adducts per 10^8 nucleotides, except in the lung tissues of dog A, where the DNA adducts in the lung tissues were below the detection limit of 0.5 (Table 3). The low levels in the tracheal tissues of dog A were not a result of smaller amounts of DNA extracted from this dog. In the two dogs where adducts could be detected in lung tissues, the epithelium and subepithelium were not notably different. In addition, levels of DNA adducts were similar in heart tissues from all three dogs—including the one with undetected levels in the lung, and levels in the heart were the same or even higher than those of the lung.

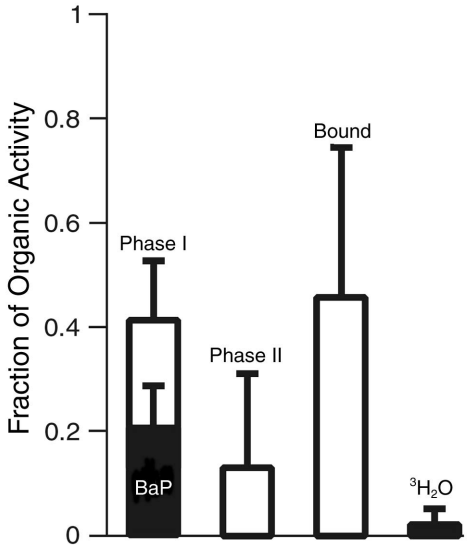

Figure 16. Distribution of B*a*P among its major metabolite groups in tracheal mucosa at 3 hours after exposure of temporarily occluded trachea to aerosol bolus of denuded diesel soot with bound B*a*P.

Table 3. Tritiated DNA Adducts of B*a*P at 3 Hours After Tracheal Exposure[a]

	Dog A	Dog B	Dog C
Tracheal epithelium	< 0.5	2.7	21
Tracheal subepithelium	< 0.5	2.2	27
Bronchial epithelium	< 0.5	9.0	18
Bronchial subepithelium	< 0.5	4.7	21
Heart tissue	16	23	17

[a] Values are adducts/10^8 nucleotides. The tracheal epithelium was directly exposed to the B*a*P-carrying diesel aerosol, whereas the bronchi and heart were exposed via the circulation.

DISCUSSION AND CONCLUSIONS

Single-breath exposures to aerosol boluses have not been used extensively in toxicology studies. Compared with prolonged inhalation exposures, however, bolus exposures allow study of rapid components of kinetics, including the return of systemic flow of soluble toxicants to the lungs. During inhalation exposures to an aggregated micron-size aerosol, resistance to mass transfer of sparingly soluble toxicants within such aggregates is likely to be negligible, whereas this component can quickly become dominant in larger aggregates of adhesive particles formed during instillation exposures of liquid dispersions (Gerde et al 1991). The kinetics of instillation exposures, especially for hydrophobic solutes on particles, is therefore likely to be distorted by spotty deposition and disruption of mucous layers.

In the alveolar exposure series, exposure of dog C exceeded by several fold the amount delivered in the eight other tests and exposures of that series. Whereas weighing errors of this magnitude are unlikely, dislodging of particles trapped in the pressurizing channels of the aerosol generator during previous use is a likely explanation. Modification of the aerosol generator and automation of the exposure procedure have removed this source of error and can be expected to improve repeatability.

Despite problems of providing repeatable exposures, the data derived from a single-component PAH bound to denuded soot may clarify the timing of events leading to

the target dose of soot-associated PAHs in human lungs after low-level inhalation exposures.

TOXICITY OF B*a*P

Of the radioactivity recovered from the soot surface after 5.6 months retention in lung tissues, nearly one third was B*a*P parent compound (Table 2). When presented as a solute to the tracheal mucosa (Gerde et al 1997) or when released from particles to the mucosa (Figure 16), parent B*a*P was reduced to similar fractions within a mere 3-hour period—approximately a 1,000-fold difference in reactivity. The data clearly suggest that a particle-bound fraction of B*a*P in the lungs is essentially nonreactive and must be released in order to participate in toxicity to the surrounding lung tissues. The molecular size of the active pocket of the P450 enzyme should require previous desorption of B*a*P from the soot surface before metabolism occurs. With the short range of the sorptive forces in liquid media—once desorbed and before being metabolized—the metabolic fate of the B*a*P molecule should be independent of any history of particle association (Gerde et al 1991). The fraction of metabolites or decay products of B*a*P bound to the soot surface after 5.6 months retention in tissues should be taken as a indication of stability rather than reactivity. While metabolites of B*a*P previously desorbed and metabolized in tissues may have readsorbed on the soot surface, some B*a*P may also have been chemically degraded on the soot surface—particularly in the acidic and oxidative environment of macrophage phagolysosomes (Lundborg et al 1992).

We find it likely that the observed relationships between particle-bound B*a*P and low reactivity and between free B*a*P and high reactivity in the lungs also hold for a collection of genotoxic PAHs on native soot at much lower concentrations. PAHs must be released from the soot in order to induce genotoxicity to the surrounding tissues, and the bioavailability of particle-associated PAHs is thus a critical factor in determining the dose of such agents to the lungs. This does not exclude the possibility that carrier particles of PAHs may enhance lung cancer risk through a nonspecific particle-induced inflammatory process. In the latter case, however, because of the minute fraction of the particle surface sites likely to be occupied by firmly bound PAHs, bound genotoxic PAHs should not be factors of importance.

The confidence at which the above conclusion can be drawn depends on the metabolite fractionating scheme and on the level of contamination of the separated particles with tissues in the sucrose centrifugation. For this pivotal conclusion, we feel the confidence is quite high.

BIOAVAILABILITY OF B*a*P ON PARTICLES IN VIVO AND IN VITRO

The bioavailability of carcinogenic PAHs on soot depends on the strength of solvation of PAHs in the lung lining fluid relative to the sorptive forces of the PAHs on the soot surface. The strength of adsorption of PAHs on soot is very high when coatings on the soot surface are low but decreases rapidly when surface concentrations on the soot increase. During combustion in diesel engines, ultrafine soot particles are generated, and in the gas phase, PAHs are formed. Subsequently, when the exhaust rapidly cools outside of the combustion zone, PAHs are bound, first by adsorption and then by condensation, to the soot particles (Burtscher et al 1998). PAHs are strongly adsorbed at the limited number of high-energy sites, which cover only a fraction of the soot surface (Burtscher and Schmidt-Ott 1986). Once these surfaces are occupied, adsorption proceeds at sites of decreasing binding energies. Because the strength of adsorption or binding to particle surfaces is likely to quickly decrease with increased surface coating (Natusch and Tomkins 1978), a limited increase in binding energies (or rather the activation energy of desorption) dramatically increases the residence time of the adsorbed PAH molecules on the soot surfaces (Adamson 1982). Over a limited span of binding energies, residence times on the soot surface proceed from being much shorter than the characteristic times of transport processes of PAHs in the surrounding tissues to much longer than these times (Gerde et al 1991). B*a*P adsorbed or bound to denuded diesel soot seems to follow this theoretical behavior of PAHs bound to soot, both in vivo and in vitro.

The major fraction of bioavailable B*a*P in this study was released from the soot within minutes of deposition, both in the alveolar region and in 1-octanol in vitro (Figures 6 and 7). For dogs A and B, the cumulative amount cleared from the alveolar region to the blood during the first half hour after exposure was similar to the fraction released in 1-octanol (Table 1; Figure 7).

After the tracheal exposures, blood concentrations were too low and variable to quantitate the release from the soot. However, the bioavailability of B*a*P on tracheally deposited soot was similar to that in the alveolar region as indicated by the distribution of B*a*P-equivalent between the tissues of the tracheal mucosa and the lavaged mucous lining layer of that airway segment. The 67% retention of radioactivity in the lavage fluid with the rest in the underlying mucosa gave a crude indication of the distribution between mostly particle-associated radioactivity in the lavage fluid and soluble B*a*P-equivalent in the tissues below. When B*a*P was sprayed as a solute onto the tracheal mucosa of the dog, only 4% of the B*a*P-equivalent remained in the lavage fluid

after 3 hours (Gerde et al 1997). Similar short-term bioavailability in alveoli and bronchi is a logical consequence considering that the surfactant is likely to be the prime agent of extraction for particle-associated PAHs in the alveolar region, as well as in the mucous lining layer of the conducting airways (Ueda et al 1984; Pettenazzo et al 1988; Girod et al 1991).

For long-term bioavailability, diesel soot recovered from the peripheral lung after 5.6 months had a 16% surface coating of B*a*P, which is about the same as the fraction of B*a*P remaining on the soot after a 48-hour extraction in 1-octanol (Figure 7). This suggests that, as is the case in 1-octanol in vitro, the release of B*a*P from the soot continues to decrease over time and becomes very slow soon after deposition in the lungs. However, soot recovered from the tracheobronchial lymph nodes after 5.6 months had significantly less retained B*a*P on the surfaces; only 10% of the monolayer remained. This surprising result implies that the ultimate bioavailability of carcinogens adsorbed to particles depends on the microenvironment in which the particles have resided in the lungs, and a single liquid in vitro cannot simulate release of particle-associated hydrocarbons in all respiratory tract tissues. A higher bioavailability of B*a*P in tracheobronchial lymph nodes may result from a large fraction of soot particles translocated there with pulmonary macrophages (Harmsen et al 1985).

Toluene, the strong organic solvent used as reference, removed the most B*a*P, but not even Soxhlet extraction for 24 hours decreased B*a*P below a surface coating of 7% of a monomolecular layer on the soot surface (Figure 7). In addition, the denuded soot is likely to contain some native bound organics that were not removed during the preextraction procedure. In measuring the intense short-term bioavailability, extraction in 1-octanol seems to give a better estimate of the readily bioavailable fraction of B*a*P on soot particles residing in the lungs and airways than does toluene extraction. With long-term bioavailability, however, release of B*a*P-equivalent is distinctly different between lung parenchyma and lymph nodes.

With our model particles, we have found that the release of B*a*P from the denuded diesel soot differs distinctively between different solvent systems and tissues in vivo. This finding does not indicate whether native carcinogenic PAHs on diesel soot are released in the lungs, but clearly solvents can be chosen better to assess whether this happens. The entire content of potentially carcinogenic PAHs extracted from typical samples of diesel soot can be bound to surface sites having energies that allow desorption in toluene or other strong solvents but not in tissues in vivo. In such a case, using PAHs extractable in toluene or dichloromethane as a measure of dose will overestimate the genotoxic risk. With the unknown distribution of sorptive energies of native PAHs on diesel soot, and with bioavailability difficult to measure in vivo, the genotoxic dose after inhalation of diesel exhaust is hard to quantitate. Our results indicate, however, that extraction methods in vitro using less potent solvents, such as 1-*n*-octanol, can be developed to better predict bioavailability in vivo.

The methods presented for determining bioavailability in vivo and in vitro are novel and need further validation. Short-term bioavailability in the alveolar region was estimated from clearance to blood for the two dogs in which the lung concentration of soot was consistent with the filter estimates of lung deposition. The tracheal bioavailability estimate assumed that radioactivity in the mucosa was mostly in soluble form, whereas radioactivity in the lavage fluid was mostly particle associated. In comparison with previous absorption studies using dissolved B*a*P, the estimate seems reasonable. Sources of error are the incomplete lavage of particles from the airway lumen and uptake of significant fractions of soot particles into airway epithelial tissues.

With long-term bioavailability, we assumed that the fraction of firmly adsorbed B*a*P not removed with Soxhlet extraction in toluene remained on the surface regardless of whether the particles had resided in tissues. Therefore, the nonextractable fraction of B*a*P could be used as an indicator of the amount of soot particles in a sample, which in turn allowed us to calculate an approximate bioavailability of B*a*P on the soot. One possible error would be if over time in vivo, the radiolabeled B*a*P migrated from the high energy sites, particularly with chemically reacted substance. This is difficult to control, but the low standard deviation between samples from two types of tissues in three different dogs indicates a distinctive limit of release.

ABSORPTION AND METABOLIC FATE OF B*a*P AFTER RELEASE FROM PARTICLES

Most of the bioavailable B*a*P on the soot was released within minutes of deposition in the lung (Figure 7). Subsequent absorption in the epithelium is a serial, independent process, governed primarily by the type of epithelium on which the soot particle was deposited. While B*a*P was adsorbed onto the denuded soot at a sufficiently high surface concentration to allow quantitation, this may be the first time the kinetics of release, absorption, and metabolism of a PAH in the lungs have been measured after low-level deposition of an aerosol bolus. The average density of deposition of soluble B*a*P in the alveolar region was estimated to be approximately 4 ng/m^2, assuming a 30% bioavailability of the B*a*P deposited over 40 m^2 lung surface (Weibel and Gil 1977). Similarly for the trachea, where the occluded section had a surface area of approximately

45 cm^2, the density of deposition was estimated to be approximately 700 ng/m^2. Both numbers are within the order of magnitude of estimates for deposition of total PAHs in humans after smoking a single cigarette (Gerde et al 1997).

B*a*P was rapidly absorbed when deposited on the alveolar type I epithelium in a process not likely to be different from absorption of instantaneously dissolving microcrystals of B*a*P (Gerde et al 1993a,b). The slightly faster rate of absorption of crystalline B*a*P compared to soot-adsorbed B*a*P could reflect the difference between a likely instantaneous dissolution of B*a*P crystals in the lungs and the continually slowing desorption rate of B*a*P from the soot surfaces (Figure 6). The initial appearance of B*a*P in the blood after alveolar exposures is much slower than can be expected for less lipophilic, small molecular weight solutes. The delay before peak concentration is attained in the blood is a critical indication that resistance is caused primarily by slow diffusion through the air–blood barrier and not by slow dissolution or desorption from particles. This behavior of highly lipophilic diffusants in tissues can be predicted by a mathematical model (Gerde et al 1993 a,b).

The delayed culmination followed by a first-order decline in the concentration is a strong indication that penetration occurs mostly through the thinnest barrier available—the air–blood barrier of the type I cells. With a general square relationship between characteristic time of penetration and barrier thickness (Crank 1975), absorption already in the bronchiolar epithelium should be considerably slower, and tracheal absorption of B*a*P in the dog is known to be much slower (Gerde et al 1997). Thus, the metabolic pattern of B*a*P entering the circulation during early clearance after the alveolar region exposures (Figure 10) primarily reflected influence by passage through the alveolar type I epithelium, despite the likely deposition of soot also on bronchial and bronchiolar epithelium. More mobile ^3H_2O, though, may well have quickly penetrated the thicker bronchial and bronchiolar sections of the air–blood barrier during the same exposures (Figure 9).

The intense pulse of B*a*P entering the systemic circulation through the alveolar type I cells was mostly unmetabolized. This fraction of B*a*P was quickly taken up by distal compartments and seemed to be intensely metabolized. A low level of about equal amounts of phase I and phase II metabolites appeared in the circulation during this process (Figure 10). Within 40 minutes of the exposure, the phase II conjugates dominated the metabolite spectrum of B*a*P in the blood. This suggests that the metabolism of B*a*P proceeds quickly toward only detoxified phase II metabolites circulating in the blood. With its large surface area and short distance of diffusion, the alveolar type I epithelium represents a major entry portal for passage of unmetabolized B*a*P into the systemic circulation.

When released on the thicker bronchial and bronchiolar epithelium, the absorption of B*a*P was much slower than the initial release from the soot particles. Absorption in the airways proceeded as slowly and with similar levels of metabolic activation as was seen with B*a*P sprayed as a solute onto the airway walls (Gerde et al 1997). Slow absorption leads to high tissue concentrations of B*a*P in the airway epithelium as substrates for the local activating enzymes of the mucosa. Of the soluble radioactivity retained in the tracheal mucosa 3 hours after the exposure, only 20% was parent compound with the rest being bound and unbound metabolites of B*a*P (Figures 11C and 16). This metabolite pattern together with the appearance of ^3H_2O after the deposition suggest that the metabolic turnover in airways of deposited and rapidly desorbed B*a*P is over within hours of a low-level exposure.

Based on our low-level exposures of dogs to aerosol boluses of B*a*P bound to denuded diesel soot, a general scheme of the dosimetry of similar-sized PAHs can be outlined (Figure 17). Driven primarily by physicochemical and biochemical mechanisms, the presented dosimetry should be similar with equally lipophilic PAHs such as

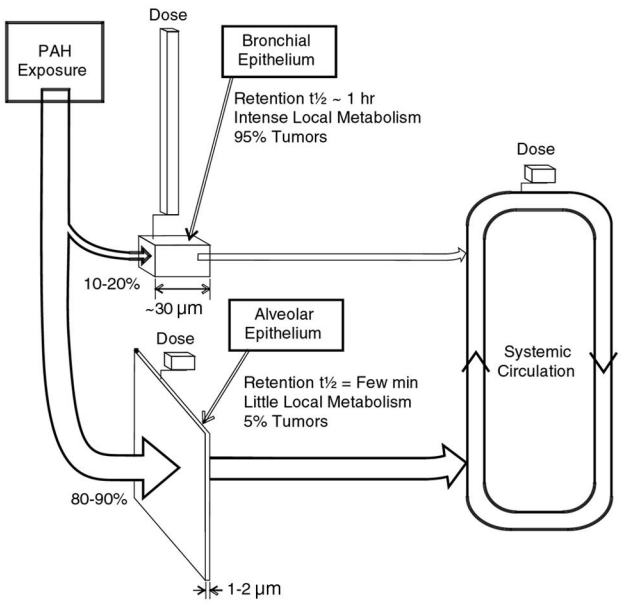

Figure 17. The hypothetical fate of inhaled bioavailable fraction of PAH in the lungs. A larger fraction, typically 80% to 90% of the carrier aerosol, is deposited in the alveolar region. Desorbing PAHs are rapidly absorbed into the circulating blood, with little influence of local metabolism, while the site-of-entry concentration is briefly elevated. A smaller fraction of 10% to 20% of the inhaled bioavailable PAHs is deposited, slowly absorbed, and intensely metabolized in the bronchial and bronchiolar epithelium at a greatly elevated site-of-entry concentration.

7,12-dimethylbenz[*a*]anthracene and dibenzo[*a*,*l*]pyrene. After release, the bioavailable fraction of PAHs has a marked biphasic dosimetry in the lungs. During exposures to typical carrier aerosols, some 80% of the readily bioavailable fraction of PAHs is likely to be deposited on the alveolar type I epithelium (Yeh et al 1996) and to become systemic rapidly. Subsequently, this fraction of PAHs is absorbed by metabolizing tissues primarily in the liver (Mollière et al 1987) but likely also in other tissues such as the white blood cells (Thompson et al 1989; Fung et al 1999).

Only approximately 20% of the desorbed PAHs is likely to be absorbed into the metabolizing epithelium of the tracheobronchial and bronchiolar region (Yeh et al 1996). Possibly, some absorption into alveolar type II cells may also occur. Because of slow penetration of lipophilic PAHs into the capillary bed below the entrance epithelium of the conducting airways, however, the concentration in directly exposed airway cells will be much higher than in cells exposed via the systemic circulation (Figure 17). Assuming that the 20% tracheobronchial deposition occurs evenly over a 30 μm thick epithelium covering 1 m^2 of the bronchial tree, the initial concentration in these cells will be approximately 40-fold higher than the 80% fraction of PAHs likely to be absorbed in the alveolar region into the 5 L of circulating blood of a human. After rapid distribution of the systemic fraction into about 20 kg of richly perfused tissues, this difference has grown to approximately 200 times. In addition, any increased density of deposition of carrier particles at the airway bifurcations (Martonen 1992; Balásházy et al 1999) could increase this difference even further.

Thus, in the case of realistic exposures to solid core particles with bound PAHs, critical exposures of surrounding tissues are likely to be dominated by the pulse of PAHs desorbed shortly after deposition on the airway lining layer. Intense metabolism began immediately after deposition in our animals, and if the appearance of ^3H_2O in the blood is used as an indicator of metabolism, the metabolism was most active within 1 hour of deposition of the carrier aerosol in the lungs (Figures 9 and 14).

Levels of tritiated DNA adducts did not mirror the dosimetry of B*a*P and its metabolites, however, where an elevated adduct level in the highly exposed tracheal epithelium compared with subepithelium and heart tissues was expected. Instead, adduct levels in the epithelium were generally lower than in the subepithelium and heart tissue (Table 3). Albeit surprising, the results are not substantially different from adduct measurements in humans: These results have demonstrated a rather weak dose-response relationship in individuals exposed to vastly different levels of PAHs (Sabro Nielsen et al 1996; Kuljukka et al 1998; van Delft et al 1998; Carstensen et al 1999), as well as no obvious differences in adduct levels between directly exposed lung tissues and adduct levels in systemically exposed white blood cells (Phillips et al 1990; Rojas et al 1998). While the poor correlation between toxicant dose and the DNA adduct levels is surprising, the presented method of mapping the mechanisms of dosimetry of PAHs at low exposure levels may help to explain these discrepancies.

ACKNOWLEDGMENTS

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OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Gerde P, Muggenburg BA, Lundborg M, Dahl AR. 2001. The rapid alveolar absorption of diesel soot-bound benzo[(*a*)]pyrene: Bioavailability, metabolism, and dosimetry of an inhaled particle-borne carcinogen. Carcinogenesis (in press).

ABBREVIATIONS AND OTHER TERMS

B*a*P	benzo[*a*]pyrene
dpm	disintegrations per minute
HPLC	high-pressure liquid chromatography
^{3}H-B*a*P	tritiated B*a*P
^3H_2O	tritiated water
LSC	liquid scintillation counting
NAAQS	National Ambient Air Quality Standard
PAH	polynuclear aromatic hydrocarbon
PM	particulate matter
$PM_{2.5}$	particles with an aerodynamic diameter of 2.5 μm or less
PM_{10}	particles with an aerodynamic diameter of 10 μm or less

In response to RFA 96-1, *Mechanisms of Particle Toxicity: Fate and Bioreactivity of Particle-Associated Compounds* (discussed in the Preface to this Research Report), Dr Alan Dahl of the Lovelace Respiratory Research Institute in Albuquerque, New Mexico, submitted an application entitled "Epithelial Penetration and Clearance of Particle-Borne Compounds." Dahl and coworkers planned to determine whether inhaled benzo[*a*]pyrene (B*a*P),* bound to particles in diesel engine exhaust or to iron (ferric oxide) particles, reaches the tracheal epithelium where it can damage tissue. External reviewers thought that it was important to study the dose of particle-bound compounds to the airways and that the particles selected for study were relevant. The HEI Research Committee concurred and approved the study for funding.†

In September 1998, Dr Dahl left the Lovelace Institutes and Dr Per Gerde, who provided the diesel exhaust particles with bound B*a*P, became Principal Investigator for this component of the study. He added exposures of peripheral lung alveolar tissue to the study design. The component that was to investigate the effects of B*a*P-bound iron was eliminated because the investigators concluded that similar work with other metals had been done earlier and the expectation of new information was low.

SCIENTIFIC BACKGROUND

Diesel exhaust is a complex mixture of gases and soot. The soot consists of carbon particles with bound inorganic salts, metals, and more than 450 organic compounds (International Agency for Research on Cancer 1989). The organic compounds include polynuclear aromatic hydrocarbons (PAHs) and their derivatives. Some of the PAHs in diesel soot, such as B*a*P and nitropyrenes, are genotoxic and cause cancer in laboratory animals. Diesel soot particles are readily inhaled because of their submicron aerodynamic diameter and deposit in the lungs. Research on diesel exhaust over the past twenty years focused almost exclusively on its potential contribution to human lung cancer risk (reviewed by Mauderly 2000). Several organizations have reviewed the relevant science, including the epidemiology and experimental studies of diesel exhaust and lung cancer, and have classified (or proposed to classify) the exhaust mixture (or the particulate component of the mixture) as a potential, probable, or definite human carcinogen (National Institute of Occupational Safety and Health 1988; International Agency for Research on Cancer 1989; World Health Organization 1996; California EPA 1998; US Environmental Protection Agency 1999; reviewed by Health Effects Institute 1999). The general consensus based on experimental studies is that long-term exposure to high levels of diesel exhaust is carcinogenic in rats and that the epidemiologic data consistently identify diesel exhaust as a potential or probable human carcinogen (Health Effects Institute 1999; US Environmental Protection Agency 1999).

Researchers believe that exposure to diesel exhaust may also cause health effects unrelated to lung cancer, but we know even less about these noncancer effects of diesel exhaust than we do about its carcinogenicity. Epidemiology research on respiratory symptoms and function among miners, dock workers, and others was conducted in the United States and Europe during the 1980s. The findings were inconclusive, but taken together with toxicologic evidence, they implicate occupational exposure in both acute and chronic respiratory disease (Cohen and Higgins 1995; Watson and Green 1995; Cohen and Nikula 1999; reviewed in Health Effects Institute 1999). Mauderly (2000) reviewed the current status of research on noncancer effects of diesel exhaust. Because the study by Gerde and colleagues focused on diesel exhaust and lung cancer, the remainder of this Background concentrates on the possible carcinogenicity of diesel exhaust.

Concern for the carcinogenicity of diesel soot initially focused on its content of genotoxic PAHs (Mauderly 2000). Researchers believe that these compounds must be removed from the carbon core in vivo in order to become available for biologic reactions. Early studies indicated that proteins or other non-DNA molecules would bind and inactivate the potentially mutagenic compounds and that biological fluids released little mutagenic activity from soot (Brooks et al 1981; King et al 1981; Li 1981). Bond and coworkers (1984) reported, however, that alveolar macrophages in culture metabolized organic compounds bound to diesel soot added to the culture medium and released organic metabolites into the medium, indicating

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

This document has not been reviewed by public or private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsement by them should be inferred.

† Dr Dahl's study began in September 1997. Total expenditures were $321,300. A draft Final Report, prepared by Dr Gerde, was received in November 1999. A revised Final Report was accepted for publication by the HEI Review Committee in June 2000. During the review process, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Critique. This Critique is intended to aid the sponsors of HEI and the public by highlighting the strengths and limitations of the study and by placing the Report into scientific perspective.

that soot-bound compounds became bioavailable. Sun and colleagues (1984) and Bond and coworkers (1986) provided additional evidence for the bioavailability of inhaled PAHs. They administered soot-bound B*a*P or 1-nitropyrene to rats by inhalation and reported that each compound was released from soot, metabolized, and either bound to pulmonary tissues or excreted. Although these studies indicate that soot-bound PAHs became bioavailable, Dasenbrock and coworkers (1996) reported that lung tumor rates for rats exposed to 15 weekly applications of a preparation of diesel soot particles whose B*a*P content was approximately 1,000 times that of normal particles were not significantly different from control rats.

Rats exposed to diesel exhaust for long periods of time develop lung tumors (Heinrich et al 1986; Iwai et al 1986; Brightwell et al 1989) by a mechanism that does not appear to involve genotoxic PAHs (Mauderly et al 1994; Heinrich et al 1995; Nikula et al 1995). In the latter three studies, prolonged exposure of rats to high levels of diesel exhaust or to carbon black or titanium dioxide particles (which have an extremely low content of bound organic molecules) produced nearly identical lung tumor responses. In the study by Mauderly and colleagues (1994), tumors were associated with impaired clearance of inhaled material from the lung. This led to a progressive accumulation of particles and damage to the surrounding tissues, most likely due to the observed inflammation and hyperplasia. Thus, while studies in rats suggest that long-term inhalation of high diesel exhaust levels causes lung tumors by a particle-driven inflammatory process, release of bound PAHs in other species may also cause tumors, especially after long-term, low-level exposure where excess accumulation of particles does not occur. Currently, the role of PAHs in the carcinogenicity of diesel exhaust is uncertain. As Mauderly (2000) states, "while it remains plausible that at least a portion of the soot-borne organic material is released in the lung and is available to exert genotoxicity, the extent and rapidity of the bioavailability of the organic fraction and the relationship between these compounds and adduction of DNA and mutagenicity in vivo remain subjects of debate." The present study by Gerde and colleagues builds on earlier studies of the bioavailability of particle-bound PAHs (Bond et al, 1984, 1986; Sun et al 1984) by determining the kinetics of release, absorption, and metabolism of B*a*P, as a surrogate PAH, after low-level deposition in the lungs.

STUDY GOALS AND DESIGN

The overall aim of the study was to evaluate the in vivo and in vitro properties of B*a*P bound to diesel exhaust particles from which the native organic chemicals were removed. (The denuded diesel exhaust particles with bound B*a*P are called B*a*P-bound denuded particles in this Critique.) A major focus of the study was to measure the release (bioavailability) and metabolic fate of particle-bound B*a*P in the lungs.

The study had three specific objectives:

1. Expose dogs to aerosolized particles that deposit in the airways and in the peripheral (alveolar) region of the lung and measure: alveolar absorption of B*a*P; metabolic turnover of particle-bound B*a*P; B*a*P retained on particles recovered from lung tissues months after exposure.
2. Measure the rate of desorption of B*a*P from denuded particles by 1-n-octanol in vitro.
3. Expose only the trachea to the same particles and measure: airway absorption of particle-bound B*a*P; metabolite pattern of B*a*P in the tracheal mucosa; and level of DNA adducts in the tracheal epithelium, tracheal subepithelium, and heart tissue.

Gerde and coworkers collected particles from the exhaust produced by a diesel tractor engine, removed the organic molecules from the particle surface by extraction with toluene, added the denuded particles to a solution of tritiated B*a*P (^{3}H-B*a*P), and used the radioactively labeled particles for animal exposures. They prepared an identical batch of particles with nonradioactive B*a*P to determine particle size.

The investigators twice exposed three dogs to the labeled denuded particles whose average mass median aerodynamic diameter was 1.3 ± 0.2 µm. In the first exposure, they used a sophisticated new method of particle administration (developed by Gerde and described in the Investigators' Report) to deposit a single bolus of 36 ± 20 µg labeled denuded particles in the peripheral region of the lung (termed *alveolar exposures* in the report). Gerde and colleagues monitored clearance of ^{3}H-B*a*P and its metabolites from the lung for one hour by sampling blood from both sides of the systemic circulation. They measured the total blood radioactivity, termed *BaP-equivalents* because the precise nature of the radioactive materials was unknown, and they identified soluble B*a*P and its phase I and phase II metabolites by high pressure liquid chroma-tography. (Phase I metabolites are potentially toxic forms produced during B*a*P metabolism; phase II metabolites are detoxified forms of phase I metabolites.) Another metabolite of ^{3}H-B*a*P is tritiated water (^3H_2O). The investigators measured the levels of ^3H_2O in blood by removing water by vacuum distillation and determining its radioactivity.

The second exposure of the three dogs took place 154, 168, or 190 days after the alveolar exposures. The investigators temporarily occluded each dog's trachea to block airflow to the peripheral airways. They used the same new method of particle administration to expose the trachea to an aerosolized bolus of 0.7 ± 0.3 µg of ^{3}H-B*a*P-bound denuded particles (termed *tracheal exposures* in the Investigators' report). They monitored clearance of labeled B*a*P and its metabolites from the trachea to the blood for three hours by sampling the azygous vein and the blood entering and leaving the lungs. As with the alveolar exposures, the investigators measured the levels of ^3H_2O and B*a*P-equivalents in blood. After the three-hour sampling period, they killed the dogs and isolated tissue from peripheral lung, trachea, and bronchi to determine:

1. the amount of B*a*P released from B*a*P denuded particles compared with the amount of B*a*P remaining bound to denuded particles (including that which remained after the earlier alveolar exposures);
2. the levels of B*a*P and its metabolites in tracheal tissue; and
3. the levels of B*a*P-DNA adducts in tracheal tissue by measuring DNA-bound tritium. (The investigators also measured DNA adducts in heart tissue, which was exposed to B*a*P via the blood.) Mucous lavage was performed by rinsing the tracheal tissues with saline to compare the levels of particle-bound and tissue-bound B*a*P.

Gerde and coworkers estimated the extent to which and rate at which soot-bound PAHs might be released into lung fluids by extracting the radioactive particles with an organic solvent, 1-n-octanol at 37°C and then measuring radioactivity in the octanol for up to 48 hours.

This study design uses model particles with B*a*P as a single surrogate for the complex mixture of PAHs and other organic molecules normally found on the particles produced by engine combustion of diesel fuel. Three concerns were not addressed by this design:

1. Other organic constituents released from normal diesel exhaust particles containing bound B*a*P would likely affect the rate and extent of B*a*P release.
2. The strength of binding between B*a*P and denuded diesel particles may differ from that of normal diesel particles.
3. The B*a*P concentration on the model particles was much greater than on diesel soot particles (Heinrich et al 1995). This density would most likely influence its release rate.

In support of their model, the authors propose that questions such as the relation between reactivity of free PAHs versus particle-bound PAHs are better probed using a model containing a single component. The authors' preliminary experiments indicated that the B*a*P concentration chosen for the present study would allow them to quantify the radioactivity in both the firmly bound and released B*a*P.

RESULTS AND INTERPRETATION

The experimental procedures showed meticulous design and conduct and demonstrated exceptional surgical and technical skills. Although the investigators defend the composition of their model particles as critical to address their aims, the results must be considered in light of the concerns with the model particles discussed earlier in this Critique.

ALVEOLAR EXPOSURES

B*a*P-equivalents (total radioactivity) appeared rapidly in blood, reaching a peak 2.1 minutes after exposure to particle-bound B*a*P. In two dogs, approximately 30% of the estimated amount of B*a*P deposited in the lungs was cleared to the blood during this early period when a net influx of radioactivity from the lungs to the blood could be measured. The results with the third dog were substantially different; therefore, the new method of aerosol generation may require further refining to increase its reproducibility. In each dog, the parent compound, ^{3}H-B*a*P, accounted for most of the blood radioactivity at this time, with small amounts of phase I and phase II metabolites present in approximately equal concentrations. Forty minutes after exposure, phase II metabolites predominated, along with tissue-bound radioactivity. These results suggest that some of the particle-bound B*a*P deposited in the alveolar region was released as free B*a*P, passed through the alveolar epithelium to the blood, and was metabolized systematically (most likely in the liver).

In vitro extraction with octanol revealed that the concentration of B*a*P bound to denuded particles decreased rapidly within minutes and then dropped only slightly over the next 48 hours. Because the rapid release of B*a*P by octanol paralleled the appearance of B*a*P-equivalents in blood in vivo, the investigators propose that the major fraction of bioavailable B*a*P is released minutes after deposition in the lung. These findings are provocative and indicate that B*a*P may become bioavailable. The other organic compounds found on normal particles may influence the degree of B*a*P release, however; therefore, it is

uncertain how these experimental findings may be extrapolated to inhalation of normal particles.

^3H_2O, a product of B*a*P metabolism, appeared in the blood within minutes after exposure. Its concentration increased rapidly up to 40 minutes after exposure and then plateaued. Because the catalytic site of the enzymes that metabolize B*a*P is smaller than B*a*P-bound denuded particles, it is likely that B*a*P had to be released from denuded particles before it could be metabolized; therefore, the appearance of ^3H_2O is another sign that B*a*P became bioavailable.

Five to six months after alveolar exposure, more B*a*P was retained on denuded particles isolated from peripheral lung tissue than on denuded particles isolated from tracheobronchial lymph nodes; therefore, the investigators proposed that the bioavailability of particle-bound carcinogens may depend on the lung microenvironment. The significant amount of radioactivity that remained associated with denuded particles suggests that this fraction of B*a*P remained firmly bound to denuded particles in lung tissues for months. This bound B*a*P is likely to have little genotoxic capability.

TRACHEAL EXPOSURES

The concentration of B*a*P-equivalents in blood was too low to quantify; however, the concentration of ^3H_2O increased, indicating that a fraction of B*a*P became available for metabolism. The low level of B*a*P-equivalents in blood may have reflected their slower passage through the bronchiolar epithelium, which is thicker than the alveolar epithelium. In addition, water passes through epithelial tissue faster than B*a*P metabolites. The investigators identified phase I and II metabolites in tracheal tissue homogenates and tissue-bound radioactivity, other indications that a portion of the particle-bound B*a*P became bioavailable. They detected high levels of B*a*P-equivalents in the mucous lavage fluid and mucosal tissue from the occluded segment of the trachea. The lavage fluid and tissue radioactivity tended to increase toward the proximal end of the trachea, the direction of mucociliary transport that clears particles from the lung. Almost 70% of the B*a*P-equivalents in the exposed trachea were in the mucous lavage fluid and the rest in the tissue. The authors believe that mucous lavage radioactivity represents B*a*P-bound denuded particles and that radioactivity in the tissue represents free B*a*P released from denuded particles. They interpret the finding of bound B*a*P as being similar to the situation in the alveolar region, where significant amounts of radioactivity remained firmly associated with denuded particles. DNA adduct levels, a potential indicator of epithelial cell DNA damage, were low and the results were inconclusive.

Overall, results of the alveolar and tracheal exposures indicate that a portion of the B*a*P bound to the model particles became bioavailable in the lung. Nevertheless, the composition and surface characteristics of the model particles limit extrapolation of the data to the consequences of inhaling normal diesel particles.

CONCLUSIONS

Results of this study indicate that a portion of B*a*P bound to denuded particles became rapidly bioavailable in the lungs and had the potential to be genotoxic. A large fraction remained bound to particles, both immediately after exposure and five to six months later, and was unlikely to be genotoxic. However, these provocative and interesting results were obtained using model particles with radioactive B*a*P replacing the multitude of organic chemicals produced on normal particles during engine combustion of diesel fuel. The rate and extent of B*a*P bioavailability would be expected to be influenced by the other organic components of normal diesel particles and it is uncertain how closely the strength of B*a*P binding to the model particles resembled that of normal particles. In addition, the concentration of B*a*P on the model particles was much higher than that calculated to be present on normal particles and would also be expected to influence its release rate. The investigators recognize these concerns but defend the composition of their model particles as critical to address their aims. Nevertheless, the degree to which the results of this small pilot study in three dogs can be extrapolated to inhaling normal diesel particles is limited, and the importance of PAHs in the potential toxicity of diesel exhaust remains to be confirmed.

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