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Markers of Exposure to Diesel Exhaust in Railroad Workers

Marc B. Schenker, Steven J. Samuels, Norman Y. Kado, S. Katharine Hammond, Thomas J. Smith, Susan R. Woskie

Division of Occupational and Environmental Medicine, Department of Internal Medicine, Center for Occupational and Environmental Health, and Department of Environmental Toxicology, University of California, Davis, CA; California Air Resources Board, Sacramento, CA; and Department of Family and Community Medicine, Environmental Health Sciences Program, University of Massachusetts Medical School, Worcester, MA

Includes the Commentary by the Institute's Health Review Committee

Research Report Number 33

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Printed at Capital City Press, Montpelier, VT.
Library of Congress Catalogue No. for the HEI Research Report Series: WA 754 R432.

♻ The paper in this publication meets the minimum requirements of the ANSI Standard Z39.48-1984 (Permanence of Paper) effective with Report Number 21, December 1988, and with Report Numbers 25 and 26 excepted. Reports 1 through 20, 25, and 26 are printed on acid-free coated paper.

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HEALTH REVIEW COMMITTEE'S COMMENTARY Health Effects Institute

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ABSTRACT

Diesel exhaust is a known mutagen and a potential human carcinogen. Recent epidemiological studies have demonstrated a small increase in the risk of lung cancer from diesel exhaust exposure. However, many epidemiological studies have used crude estimates of exposure, and even accurate measures of exposure may not be accurate estimates of the internal dose received. Measurement of diesel exhaust exposure also has been limited by the absence of a standard marker. This study was undertaken to evaluate the usefulness of urinary mutagenicity as a biological marker of diesel exhaust exposure in the workplace. We measured the exposure of individual railroad workers to diesel exhaust by using personal air samples taken over two consecutive work shifts. Nicotine in the samples was measured to adjust the respirable particle concentrations for active and passive cigarette smoking. Urine samples were collected at the end of the study work shifts and were analyzed for markers of cigarette smoking (nicotine, cotinine) and for mutagenicity, using a sensitive microsuspension assay (micro preincubation assay; *Salmonella* strain TA98 with or without S9 enzyme). The number of cigarettes smoked on the study shift was recorded, and subjects completed a questionnaire at the end of the second day on personal habits and exposures at home and work. Multiple regression analyses were used to analyze independent determinants of urinary mutagenicity, including a generalized least-squares analysis that divided residual variation into between- and within-person components.

Eighty-seven subjects completed 151 two-day protocols; an additional four subjects provided usable data for a single day (n = 306 samples). Respirable particle concentration was not a good marker of diesel exhaust exposure when contamination by environmental tobacco smoke existed in the work location, but respirable particle concentration that was adjusted for environmental tobacco smoke correlated with a priori assessments of diesel exhaust exposure by job grouping. Phenanthrene concentration, as a potential marker, was measured in a subset of personal samples, and correlated with known diesel exhaust exposure by job grouping. A constant ratio of phenanthrene to respirable particles in area samples from diesel exhaust-exposed work locations suggested that phenanthrene is promising as a marker for diesel exhaust. Mutagenic activity was also measured from extracts of respirable particles in a few personal filter samples, and this technique may be useful for further investigation in epidemiological studies.

Among smokers, a dose-response relationship was observed between urinary mutagenicity and the number of cigarettes smoked on the study day. After cigarette smoking was controlled for, no association was present between diesel exhaust exposure and urinary mutagenicity. Among nonsmokers, detectable concentrations of mutagens were present in the urine, but no association could be found between markers of diesel exhaust or environmental tobacco smoke and urinary mutagenicity. Self-reported ingestion of certain foods (cabbage, Brussels sprouts, and fish) was independently associated with lower urinary mutagenicity (using the *Salmonella* tester strain TA98 with metabolic activation by S9 enzyme).

Several factors may have contributed to the absence of an association between diesel exhaust exposure and urinary mutagenicity. These include the lower-than-expected workplace exposures to diesel exhaust; the sensitivity of the urine mutagenicity assay; inexact diesel exhaust exposure estimates despite the use of markers more accurate than respirable particle concentrations; the unknown kinetics of diesel exhaust absorption, metabolism, and excretion, and the limited ability to study them in the field; and the limited statistical power of the study to observe very low levels of urinary mutagens. We conclude that, at the levels of exposure to diesel exhaust or environmental tobacco smoke in this study, the mutagens associated with these levels of exposure were undetectable in the urine. The use of urinary mutagenicity for epidemiologic studies would require higher exposure levels or greater sensitivity. Our study provides no basis on which to draw conclusions about the cancer risks associated with diesel exhaust exposure.

INTRODUCTION

In the late 1970s, the known mutagenicity of diesel exhaust and the projected increase in diesel engine use in the United States raised concerns about the increased risk of

[1] Correspondence may be addressed to Dr. Marc B. Schenker, Division Chief and Associate Professor, Division of Occupational and Environmental Medicine, University of California, Davis, CA 95616.

cancer from diesel exhaust exposure (Pepelko et al. 1979; National Research Council 1981). Epidemiological studies of lung cancer among workers with occupational diesel exhaust exposure had previously shown conflicting results, but the studies were often limited by small sample size, inadequate duration of exposure, or limited follow-up (Schenker and Speizer 1979; Schenker 1980). Several new epidemiological studies were begun to evaluate the effects of long-term exposure to diesel exhaust (Howe et al. 1983; Hall and Wynder 1984; Schenker et al. 1984; Wong et al. 1985), and the health effects of diesel exhaust exposure have been reviewed (Ishinishi et al. 1986; McClellan 1987).

A major weakness in the epidemiological studies of respiratory disease due to environmental agents, including the early studies of diesel exhaust-exposed workers, has been limited or inaccurate determination of exposure or dose (Axelson 1985). Many occupational epidemiology studies use indices as crude as employment in a certain industry as indicators of exposure. Some studies use job classification or work area, but these are not always based on exposure measurements. The use of inaccurate exposure measures in epidemiological studies may reduce the study sensitivity, and may thus bias the results toward the null hypothesis of no association.

Estimates of dose may also be affected by many factors that cause individual differences in reactions to similar exposures. These include age, gender, genotype, diet, smoking, nonoccupational exposures, and drug use. Because of these factors, even accurate measurements of exposure may not be accurate estimates of the internal dose received by an individual.

Individual differences in reactions to environmental agents may be estimated better by measurements of dose in biological specimens. Biological measurements may also validate exposure measurements made by personal exposure sampling. Measurements of occupational or environmental exposures in biological specimens may be of specific chemicals or their metabolites, or of effects caused by the exposure. Examples of the latter include mutagenicity and chromosomal changes. Mutagenic activity in the urine as a result of exogenous exposure was first observed among cigarette smokers by Yamasaki and Ames (1977); subsequently it has been associated with occupational exposures to other known or suspected carcinogens (Hemminki et al. 1977; Everson 1986). Measurement of urinary mutagenicity is convenient for occupational studies because of its low cost and the ease of sample collection, and it is particularly useful for complex mixtures, such as diesel exhaust, because it is not specific for a single chemical species.

An important consideration in studies of biological markers for diesel exhaust is control for the effects of cigarette smoking, a known cause of mutagen excretion in the urine. Control for smoking in epidemiological studies traditionally has been done by questionnaire, but newer studies have used more specific biochemical markers such as cotinine (a metabolite of nicotine) in the blood or urine (Benowitz et al. 1986). These methods were used in this study to measure the dose of cigarette smoke independently. Cigarette smoke from active and passive (environmental) smoking may also contribute to measured respirable particle concentrations. It is important, therefore, to have an independent determination of cigarette smoke to adjust the measurements of diesel exhaust exposure that use respirable particles, and to evaluate the independent determinants of urinary mutagenicity.

EXPOSURE ASSESSMENT

Characterization of environmental or occupational exposures for studies of human health effects is very difficult because exposure conditions are highly variable. Three dimensions of exposure must be determined for each subject: composition, concentration, and time profile. The problem is further complicated by the need to evaluate three possible routes of entry into the body: inhalation, skin absorption, and ingestion. Air concentrations vary widely with location or work activity and are generally not stable over time.

Ideally, an exposure assessment strategy should have two components: (1) determination of daily exposure immediately prior to urine sampling; and (2) estimation of average exposure over the previous several months. For our study, the first requirement was fulfilled by measuring the exposure of each subject on two consecutive days and by collecting urine samples at the end of each day. For the second requirement, it was not practical to measure each subject's daily personal exposure over several months, but we could group men with similar job descriptions and examine the variability of urinary mutagenicity relative to exposure differences within and between groups.

Variability of Exposure

Occupational exposure to air contaminants generally is substantially variable across companies, industrial operations, jobs, work areas, individuals, and time. Variability is seen in both the composition and the concentration of contaminants. Our previous work with railroads has demonstrated clearly that exposure to diesel exhaust is not an exception (Woskie et al. 1988a,b). Thus, there is a range of exposures against which the variability of urinary mutagens can be compared.

Concentrations of air contaminants have been found to vary by orders of magnitude over days, hours, or even minutes. The reasons for this extreme variability are many: source emissions vary in strength and composition over time; passage of contaminants from the source to the exposed individual permits random transport processes, such as turbulent mixing, to dilute them with other materials (for example, with uncontaminated air); and in an occupational setting, the exposed individual's behavior may affect either emissions or the transport processes. Thus, it is necessary to use statistical sampling strategies to characterize exposures.

Our earlier work among railroad workers confirmed the highly variable exposures within job categories (Woskie et al. 1988b). For example, 95 percent of the respirable particle concentrations for electricians were in the interval between 35 and 905 $\mu g/m^3$. Thus, personal exposure in this setting is highly variable, and to estimate the relation between diesel exhaust exposure and urine mutagenicity, exposure for an individual should be measured on the work shift preceding urine sampling.

Complex Composition of Diesel Exhaust

Diesel exhaust is a complex mixture of gases, vapors, and aerosol, and the vapors and particles continuously interact. The main constituents are carbonaceous soot particles and hundreds of organic compounds: unburned fuel hydrocarbons, oxygenated compounds, and a wide variety of pyrolysis products, including polycyclic aromatic compounds (Hammond et al. 1985). Immediately after emission, the hot particles rapidly lose vapors, but as emissions cool, the volatile materials may condense. Inorganic material is also present: sulfur dioxide and sulfuric acid aerosol from sulfur in the fuel; nitrogen oxides (nitric oxide and nitrogen dioxide) that are formed during combustion; and other inorganic material from fuel additives and other sources. As this mixture is diluted by diffusion and turbulent mixing in the atmosphere, it interacts with other air contaminants and additional compounds may be formed (for example, by oxidation). The composition of diesel exhaust, therefore, is not fixed, but varies depending on the fuel composition, the engine type and operating conditions, how long after emission it is analyzed, and what else is in the air. As a result, it is not possible to measure diesel exhaust directly.

Use of Markers for Air Contaminants

One solution to the problem of how to quantify exposure is to identify and measure "markers." A marker is a single compound or indicator whose concentration has been shown to vary proportionately with the agent of interest. A good example is the use of airborne nicotine as a marker for cigarette smoke, which is a very complex mixture of thousands of compounds. Different brands of cigarettes contain various levels of nicotine (Federal Trade Commission Report 1985) in mainstream smoke, the smoke that is drawn through the cigarette and into the lungs. Increases in puff volume and duration may produce large increases in mainstream smoke yields of nicotine and tar, but only small variations in the ratio of nicotine to total particulate matter (Schlotzhauer and Chortyk 1983). Reducing nicotine in mainstream smoke is achieved by filtering the smoke, increasing ventilation, and changing the design of the cigarette in other ways (Schlotzhauer and Chortyk 1983).

Sidestream smoke, which is emitted from the cigarette directly into the air, is the major component of environmental tobacco smoke. Experiments have shown that the burning end of the cigarette emits a roughly constant amount of nicotine (Hammond et al. 1987). Little variability in the nicotine levels of sidestream smoke is found among different brands of cigarettes, regardless of degree of ventilation (Rickert et al. 1984). Because sidestream smoke generally contains a constant fraction of nicotine, airborne nicotine can be used as a marker for airborne cigarette smoke. As part of this study, we developed a method to collect and analyze nicotine and to use nicotine as a marker for exposure to environmental tobacco smoke. Chamber studies and diesel-free subgroups were used to estimate the relationship between nicotine and respirable particles.

A similar approach was used with diesel smoke, and several markers were proposed. Because diesel exhaust particles are submicron-sized, the mass of respirable particles in the air can be a marker of diesel exhaust if exhaust is the only, or at least the predominant, source of respirable particles in an area. Because cigarette smoke is a common source of respirable particles in some work areas, an estimate of the amount of cigarette particles should be subtracted before the respirable particles are used as a marker. Even after this correction is made, there still may be other important sources of particles in an area, so another, more specific, marker was sought. In our previous samples of exhaust from railroad diesel engines, we found phenanthrene to be the polycyclic aromatic hydrocarbon (PAH[2]) present in the highest concentration (Hammond et al. 1985). This finding was consistent with other reports in the literature that diesel engines seem to produce more phenanthrene than do spark-type engines or other combustion sources (Jensen and Hites 1983; Behymer and Hites 1984). Phenanthrene was selected, therefore, as another possible marker for diesel exhaust. Methods were developed to collect and

[2] A list of abbreviations appears at the end of this report for your reference.

analyze phenanthrene on personal samplers as part of this research.

MUTAGENICITY ASSAY

Investigators have estimated exposure to complex mixtures by determining mutagenic activity in physiologic fluids (Legator et al. 1976; Yamasaki and Ames 1977; Falck et al. 1980; Dolara et al. 1981; Baker et al. 1982; Sorsa et al. 1985). Urine has been the most widely used physiologic fluid because it is convenient to collect, is noninvasively acquired, and is one of the major routes of excretion for mutagens. There have been several short-term tests used for this purpose (Vainio 1985), but the most widely used and validated is the *Salmonella* microsome assay (Ames et al. 1975).

In studies in which the *Salmonella* test was used for detecting mutagenic activity in physiologic fluids (mostly urine), samples collected from control individuals who were not exposed to high concentrations of complex mutagenic mixtures generally had low or undetectable mutagenic activity (Yamasaki and Ames 1977; Falck et al. 1980; Aeschbacher and Chappuis 1981; Kriebel et al. 1985). Numerous studies have been conducted on the mutagenicity of cigarette smokers' urine, but very few on nonsmokers' or passive smokers' urine (Bos et al. 1983; Sorsa et al. 1985). Studies of urinary mutagenicity from passive smoking have often not accounted for such important covariates as urine dilution (Bos et al. 1983). Methods of detecting mutagenic activity in human physiological fluids have been reviewed by Everson (1986) and Venitt (1988).

With respect to diesel particulate matter, Belisario and coworkers (1984) reported on the bioavailability of the mutagens adsorbed to diesel particles. They administered diesel particles to rats by intraperitoneal injection and found mutagenic activity in the rats' urine 24 hours later. The dose of particulate matter required to double the mutagenic response (using *Salmonella* strain TA98 with metabolic activation by S9) over that of control rats was approximately 100 to 200 mg/kg of body weight. Urine was tested directly, without a concentration step. The authors also reported that passing urine through XAD-2 resin resulted in about 60 percent recovery of the mutagens added. Higher recoveries, of 70 to 90 percent of mutagenic activity, were obtained by passing urine in succession through XAD-2 and XAD-7 resins.

Belisario and colleagues (1985) further evaluated these procedures for extracting mutagenic metabolites from the urine of rats administered diesel particulate matter. Although they reported that direct extraction with dichloromethane was the most efficient method, the volumes of urine they extracted, which had relatively high concentrations of diesel-associated mutagens, were small. Liquid extraction becomes impractical for concentrating larger volumes of urine as well as for processing larger numbers of urine samples, and the use of solid adsorbents, such as XAD-2, becomes more appealing. Since XAD has been the adsorbent used in studies measuring mutagenic activity in the urine of cigarette smokers, and the data base for using the resin method of extraction is larger than for other methods, we considered its use in the current study. One factor that makes the XAD method of extraction favorable is that it traps mutagens in urine, but differentially does not trap histidine, which can cause the histidine prototrophs of *Salmonella* to revert. If present, these "pseudorevertants" can increase the background mutagenic activity.

To detect the low levels of mutagenic activity in the urine of nonsmokers, an assay with greater sensitivity than the standard plate incorporation test of the *Salmonella* microsome assay would be helpful. Numerous modifications of the Ames test procedure have been reported to increase its sensitivity to selected compounds (Gletten et al. 1975; Green et al. 1977; Malling 1977; Yahagi et al. 1977; Andrews et al. 1978; Mitchell 1978). For example, Falck and coworkers (1980) have used a fluctuation test to detect low levels of mutagen in the urine of rubber workers, but these results are not directly comparable with the existing data base of mutagens tested in the standard plate incorporation test.

We have previously reported a simple (microsuspension) modification of the *Salmonella* assay that is more sensitive than the standard plate incorporation test, as judged by absolute amounts of mutagen added for a specific mutagenic response (Kado et al. 1983, 1985). The modification consists of adding 10 times more bacteria (approximately 10^9 per incubation tube) and 5 to 10 times less metabolic enzyme, compared to the plate incorporation method. The total mixture volume is approximately 0.2 mL, and the mixture is incubated for 90 minutes before it is poured, according to the standard protocol. Results from this procedure (expressed as revertants per plate) can be compared to the data base existing for mutagens and complex mixtures compiled from the standard plate assays. This modification has been validated with a number of standard mutagens and with cigarette smokers' urine, air particulate matter from community air, and diesel exhaust. The range for increased sensitivity, based on absolute amounts of mutagen required for a specific mutagenic response, has been about 10 to 50 times that of the standard plate incorporation assay of Ames and associates (1975). This is based on the slopes of the linear portion of the dose-response curve for each compound or complex mixture. The amount of metabolic activation with S9

required for a specific response is about 5 to 10 times less than for the plate incorporation test.

Major reasons for the increase in sensitivity of the microsuspension assay include the larger number and higher concentration of bacteria used to provide more "targets," use of preincubation (optimum is approximately 90 minutes for a variety of compounds and complex mixtures), and the small volume of incubation mixture used (0.2 mL). These parameters were investigated using diesel extracts from light- and heavy-duty diesel engines (described below in the Mutagenicity section).

In this investigation, we used the *Salmonella* microsuspension assay for monitoring absorbed doses of mutagens in humans, particularly among individuals exposed to diesel exhaust and cigarette smoke. We also examined some of the mutagenic characteristics of the assay, using diesel exhaust extracts from airborne particulate matter collected at the railroad facilities and extracts from particles collected from heavy-duty diesel engines.

EXPOSURE AND THE KINETICS OF MUTAGEN EXCRETION

The ideal approach for exposure assessment in an epidemiological study is to determine each subject's personal exposure on each day that is relevant to the effect being studied. In this study, inhalation of diesel exhaust, which contains mutagens, is presumed to result in proportionate increases in urinary excretion of mutagens. Some railroad personnel work in areas where significant amounts of diesel exhaust are present, for example, machinists and electricians in engine repair shops. Mutagenic compounds deposited in these workers' lungs are likely to be excreted over a period of time that depends on the material's absorption, biotransformation, and elimination kinetics. Because the chemical composition of diesel exhaust is very complex, and the identities of many of the mutagens are uncertain, it was unclear what the kinetic relationship would be between the deposition of the mutagens and their appearance in the urine. Thus, the choice of time period for exposure characterization and urine collection was also difficult. Very little information is available concerning the kinetics of mutagen excretion in humans, especially regarding exposure to complex mixtures. Yamasaki and Ames (1977) reported that urine collected in the morning from cigarette smokers had less mutagenic activity than urine collected the previous evening, suggesting that mutagens were excreted or detoxified in a period of hours. This observation has been confirmed by others (Recio et al. 1982; Kobayashi and Hayatsu 1984).

We have reported on the kinetics of mutagen excretion in the urine of cigarette smokers (Kado et al. 1985). The mutagen excretion patterns after smoking a single cigarette suggest that the mutagens, as detected by the *Salmonella* microsuspension assay, are absorbed rapidly (in three to five hours) and are eliminated from the body following first-order kinetics. The calculated rate constant is approximately 0.1/hour, and the half-life is approximately seven hours.

Because both cigarette smoke and diesel exhaust are derived from combustion processes, we assumed for this study that mutagens from diesel exhaust would follow a similar pattern of elimination. However, the physical and chemical makeup of diesel particles is different from that of cigarette smoke, which might result in differences in the absorption, as well as the elimination, of the associated mutagens. The chemical differences are exemplified by the mutagenic activity of the two types of particles. Extracts from diesel particles have considerable mutagenic activity without metabolic activation from adding S9 (TA98 – S9), which almost matches the activity with metabolic activation by S9 (TA98 + S9) (Claxton 1981; Schuetzle 1983). Extracts of environmental tobacco smoke have much less activity without S9 than with S9 (Lofroth and Lazardis 1986; Claxton et al. 1989). If mutagens from diesel exhaust were eliminated in a pattern similar to cigarette smoke mutagens, urinary mutagenic activity could be assumed to be highest immediately after an eight-hour period of exposure. However, the total activity represents elimination of chemicals with different elimination rates, and if there is a significant fraction of materials with long elimination half-lives, then during months of exposure there may be a buildup of slow-clearing diesel mutagens in the urine. This would reduce the apparent relation of total mutagenicity to daily exposure, but theoretically would be detectable because there would be a higher level of urinary mutagenicity among diesel exhaust-exposed workers than among unexposed workers.

SPECIFIC AIMS

The specific aims of this project were as follows:

1. To refine and validate methods for estimating diesel exhaust exposure in epidemiological studies. The standard marker for diesel exhaust exposure, personal exposure to respirable particles, was to be compared to more specific markers of diesel exposure, such as phenanthrene concentration. Methods were also to be refined to measure exposure from other sources of respirable particles, notably environmental tobacco smoke.
2. To refine and validate laboratory methods for using uri-

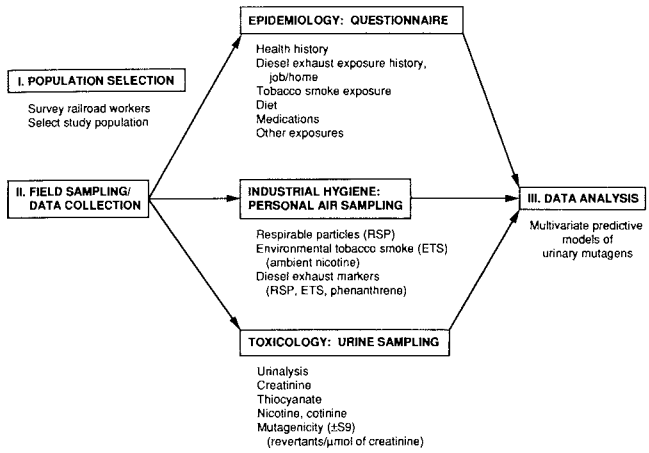


Figure 1. Outline of study design.

nary mutagenicity to assess exposure to diesel exhaust particles, which contain known mutagens and potential carcinogens.

3. To conduct a field survey of railroad workers, with subjects recruited from among diesel exhaust-exposed and unexposed workers. Occupational exposure to diesel exhaust was to be characterized by questionnaire and by personal samples of airborne contaminants on two consecutive work shifts. Urine samples were to be collected at the end of each work shift for measurement of urinary mutagenicity. Nondiesel sources of respirable particles or urinary mutagens were also to be evaluated.
4. To analyze the concentration of urinary mutagens from study samples (specific aim 3) for specific determinants, including diesel exhaust exposure, cigarette smoking, nonoccupational exposures, and any interactions among these factors.

METHODS

EPIDEMIOLOGY

All data collection was performed through a series of field visits to a single railroad facility in New England (Figure 1). Previous surveys of this railroad had provided data on all current employees, including job categories, usual work activities, and cigarette smoking (Garshick et al. 1987). Six field visits were completed between June 1984 and April 1985 (Table 1). Most sampling was done during the winter months because our previous work had shown that diesel exhaust exposures among railroad workers were highest in the winter (Woskie et al. 1988b). The study team for each visit consisted of an epidemiologist, an industrial hygienist, and at least two assistants.

Subjects for each visit were selected on the basis of job category and work location. All subjects were male; no women were employed in the job categories surveyed. An attempt was made to distribute job groupings across the separate visits, but this was not always possible for logistic reasons. For example, it was necessary to monitor the personal sampling pumps on each subject frequently throughout the entire work shift, and this could be done only in work locations that were geographically close to each other.

After obtaining informed consent, several separate sources of data were collected from each subject over a two-day sampling period. An overview of the data collection is presented below and in Figure 1. Details about the handling and analyses of specific samples are covered in subsequent sections.

Day 1

- Subjects recruited and informed consent forms signed (sometimes done on preceding day).

Table 1. Number of Study Subjects and Samples by Visit and Job Group[a]

		Job Group[b]					
Visit	Date	Braker	Carman	Clerk	Engineer	Shop Worker	Total[b]
1	Jun '84	4/7	0/0	7/14	1/2	0/0	12/23
2	Nov '84	0/0	11/22	4/8	0/0	0/0	15/30
3	Dec '84	3/6	0/0	4/8	2/4	19/38	28/56
4	Jan '85	1/4	0/0	1/2	0/0	18/58	20/64
5	Feb '85	10/18	7/13	4/8	5/10	3/6	29/55
6	Apr '85	1/2	0/0	9/20	0/0	17/56	27/78
Total		19/37	18/35	29/60	8/16	57/158	131/306

[a] Some subjects provided samples on more than one visit. Those individuals are counted separately for each visit in this table. The total number of subjects was 87.

[b] Numerator = number of subjects. Denominator = number of samples.

- Personal sampling pumps and filter cassettes attached at beginning of shift. Industrial hygiene log begun of daily work activity, work area, temperature, and field conditions.
- Sampling pumps and samples collected at end of shift and industrial hygiene log completed on work activities, cigarettes smoked, work location, and sample quality during the personal sampling.
- Spot urine collected from all subjects.
- Data sheet on biological specimen collection completed.

Day 2

- Personal sampling pumps and filters attached at beginning of shift. Industrial hygiene log begun.
- Pumps and samples collected at end of shift and industrial hygiene log completed.
- Spot urine collected from subjects.
- Health survey questionnaire completed by subjects on medical history and diesel exposure, smoking, nonworkplace exposures, and diet over preceding two days.

The questionnaire included specific questions on the perception of exposure to diesel exhaust on the study days, number of workers who were smoking cigarettes around the subject, use of or exposure to potential nonoccupational mutagens during the preceding week, diet, and specific questions on smoking habits (see Appendix A). The smoking questions included the brand of cigarette smoked and the depth of smoke inhalation.

INDUSTRIAL HYGIENE

General Approach

Personal exposures to diesel exhaust and other air contaminants were measured by personal sampling. Air was drawn from a location near the subject's breathing zone. Since a constant flow rate of air was used, the sample represents a time-weighted average of the air concentration present during the sampling period. Full-shift samples were collected whenever possible, thus ranging in duration from 7 to 10 hours.

When this project began, no suitable methods were available to measure personal exposure to environmental tobacco smoke or to diesel exhaust, or to measure the two markers chosen for these contaminant mixtures, nicotine and phenanthrene, respectively. Therefore, in the early phase of the project, exposure assessment focused on developing methods to collect these materials efficiently, desorb them from the collectors, and accurately analyze them. Validation studies were conducted in the laboratory, in an environmental exposure chamber, and in the field. Details of the validation of the method developed to measure personal exposure to nicotine as a marker for environmental tobacco smoke have been published (Hammond et al. 1987).

Phenanthrene, like many lower molecular weight PAHs, is not collected efficiently by filters used to collect airborne particulate matter. These low-vapor-pressure compounds volatilize from the filter during sampling; therefore, many high-volume collection schemes for area sampling use downstream sorbents, such as XAD-2 resin or polyurethane foam (You and Bidleman 1984). Small tubes of XAD-2 resin have also been used for personal sampling, but they have two disadvantages. First, they increase the pressure drop of the sampling train substantially, and many personal sampling pumps are not able to maintain constant flow rate throughout the day. Second, XAD-2 resin must be Soxhlet-extracted, which is time-consuming and expensive for each sample; therefore, sampling with resin is inappropriate for epidemiological studies in which hundreds of samples are collected. Polyurethane foam can be extracted ultrasonically, which substantially reduces analysis time and cost, and the open-cell structure of polyurethane foam introduces very little back pressure during air sampling. Polyurethane foam has been used for high-volume sampling, but not for personal sampling. Therefore, we chose to develop a method of personal sampling for PAHs that employs both a filter and a series of cylindrical polyurethane foam plugs. The sampling pump drew air through three collectors arranged in series: a high-efficiency filter to collect the respirable particles that pass through a 10-mm nylon cyclone preseparator (50 percent cutoff at 3.5 μm, based on the American Council of Governmental Industrial Hygiene [1968] criteria); a bisulfate-treated filter to capture nicotine vapor; and a series of three 2.4-cm-long plugs of polyurethane foam to capture phenanthrene and other organic vapors (Figure 2). Laboratory tests have shown each of these devices to be efficient collectors of the designated materials, and have demonstrated that the upstream devices do not remove any of the materials destined for lower collectors; for example, the treated filter for nicotine does not remove phenanthrene.

Quality Control

Laboratory. Reagent and laboratory blanks were analyzed concurrently to detect contamination or instrument drift. Calibration standards were used to set the instrumental response. Service contracts were used to maintain equipment in good operating condition. In addition, each method had specific quality control measures.

Field. The equipment was calibrated before each use, and the calibration was rechecked at the end of each day. Field blanks (sample media assembled into sampling trains, but not used) were obtained to detect contamination of sam-

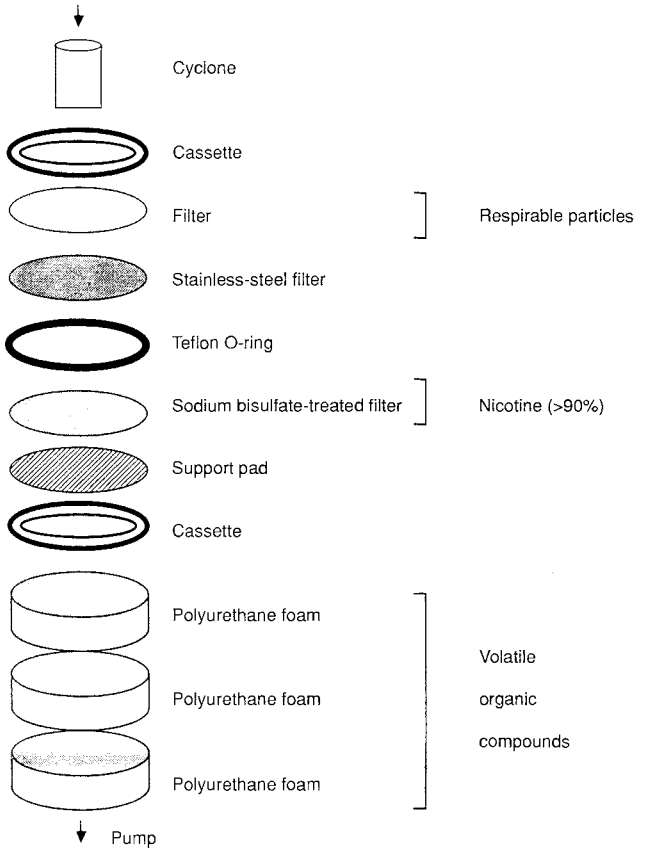


Figure 2. Schematic of sampling train for personal samples.

ples. All equipment was checked for constant flow and for battery capability before each field trip and was serviced as needed.

Respirable Particles

Respirable particles were collected on a 37-mm Teflon-coated fiber filter (Emfab TX40 HI20-WW, Pallflex Corp., Putnam, CT). Each filter was placed in a polystyrene cassette (Millipore Corp., Bedford, MA) on a stainless-steel backup screen (MSA Corp., Pittsburg, PA) that had been precleaned by soaking in 1:1 (v/v) nitric acid:water for one hour, then rinsed with deionized distilled water, methanol, and dichloromethane. A Teflon washer (37 mm outer diameter × 32 mm inner diameter × 0.5 mm thick) was used to separate the stainless-steel backup screen from the treated filter used to collect nicotine. The washer was precleaned by ultrasonication with detergent and water, then rinsed with deionized distilled water, dichloromethane, and methanol. The mass of particles collected on the first filter in the sampling train was determined gravimetrically by weighing the filter before and after sampling. All weighing was done in a room with controlled temperature (70° ± 5°F) and relative humidity (50 ± 10 percent).

Quality control was maintained by periodically weighing a control filter, and checking zero and a 10-mg tare weight. The control filter monitored effects caused by humidity changes, and the zero and the 10-mg weight monitored the balance's zero and calibration, which may drift. The zero was checked after sets of 10 filters. If the zero changed by more than ± 5 μg, the balance was recalibrated and the previous 10 filters were reweighed. The control filter and tare weights were weighed at the beginning and end of a set of 20 filters. If the control filter weight changed by more than ± 7 μg, the balance was recalibrated and the previous 20 filters were reweighed. If the tare weight changed by more than ± 3 percent, the balance was recalibrated.

The air flow, drawn by a small pump through each personal sampling train, was calibrated (± 5 percent) in the field with a rotometer that had been calibrated with a bubble flow meter (primary standard) before and after each use. Collected air volumes that required corrections due to differences (of more than 10 percent) in barometric pressure or in temperature were corrected during data entry and analysis.

Timed samples were collected as breathing zone personal samples. Each sample was collected over a single work shift (7 to 10 hours). The following information was collected on each sample:

1. Identifier number
2. Interstate Commerce Commission (ICC) job code
3. Sample type
4. Pump number
5. Flow rate
6. Sample number
7. Start and stop times
8. Worksite (facility type)
9. Work location (specific)
10. Shift
11. Date
12. Meteorologic data (outdoor temperature, barometric pressure)
13. Indoor temperature and humidity (measured)
14. Location of sample (indoor or outdoor)
15. Number of cigarettes smoked while subject wore sampler (subject counted cigarettes in pack before and after shift)

After sampling, filters were removed from cassettes and placed in tin storage canisters (Freund Co., Chicago, IL)

with Teflon liners. The storage canisters and liners were precleaned by the same procedure as the Teflon washers, described above. The filters were stored with dry ice during the field trip and transferred to a freezer (−20°C) on return to the laboratory. The filters were reweighed after 18 to 24 hours of conditioning in the humidity-controlled weighing room.

Nicotine

Nicotine vapor is alkaline, so it was collected by treating a filter with an acid, sodium bisulfate, that binds with the nicotine to form a nonvolatile salt. Teflon-coated glass-fiber filters (Emfab TX40 HI20-WW, Pallflex Corp.) were soaked with 4 percent aqueous sodium bisulfate (reagent grade) in a watch glass, and then allowed to air dry. This treatment left 7 to 10 mg of sodium bisulfate on the filters. The coated filter was mounted in a 37-mm cassette on a cellulose support pad, under the Teflon washer and downstream from the untreated filter that collected the airborne particles (Figure 2).

Loss of nicotine vapor was checked by placing a second treated filter after the primary nicotine collector as a backup. To measure the amount of nicotine vapor lost to the backup, the collection cassette was exposed to high concentrations of ambient environmental tobacco smoke in a 34-m^3 environmental chamber (Hammond et al. 1987). These experiments were conducted with four people actively smoking cigarettes in the chamber, resulting in an aged mixture of sidestream and exhaled mainstream smoke, that is, the environmental tobacco smoke found in rooms with smokers present. Air was drawn through the collection unit for one to seven hours. All of the backup filters contained less than 1 percent of the amount of nicotine collected on the primary filter.

The treated filters were stored on dry ice in the field, and in freezers at −20°C in the laboratory, prior to analysis. These samples were analyzed within four to six months of sample collection. Tests with filters spiked with known amounts of nicotine confirmed that the samples were stable under these conditions for at least six months.

Nicotine in these samples was determined by aqueous desorption and gas chromatography. Sample filters were vortexed in 2 mL of water with 100 µL of ethanol; then 2 mL of 10 N sodium hydroxide was added to form the free base, and the mixture was vortexed again. The free base was extracted by liquid-liquid extraction with 250 µL of ammoniated heptane (gaseous ammonia was bubbled through the heptane for 30 seconds). An aliquot of the heptane was immediately removed for gas chromatographic analysis (6-ft by ⅛-in stainless-steel column packed with Chromosorb W coated with 10 percent Apiezon L that contained 3 percent potassium hydroxide; isothermal separation at 170°C; nitrogen-selective detector).

Laboratory tests determined that the liquid-liquid extraction of nicotine into the heptane was virtually complete. Recovery of known amounts of nicotine spiked onto clean filters was used to determine the overall extraction efficiency. A pure water extraction medium recovered an average of 75 percent, but was highly variable. Addition of a small amount of ethanol led to an average recovery of 98 ± 2 percent for 0.5-µg spikes, which represents the lower end of expected amounts of material that would be collected in areas with limited cigarette smoke contamination. The limit of detection for field sampling at 1.7 Lpm for eight hours is an average concentration of 0.2 µg/m^3 nicotine, based on gas chromatographic limits of quantification and linearity.

Phenanthrene

Phenanthrene was collected by adsorption on small, cylindrical plugs (2.4 cm thick × 37 mm in diameter) of polyurethane open-cell foam. One-inch-thick sheets of polyurethane foam were purchased from Flexible Foam Products (Spencerville, OH). The polyurethane foam was cleaned before use by extracting it according to the same procedure that was used for analysis: sets of about 20 polyurethane foam plugs were extracted with heptane containing 5 percent dichloromethane by ultrasonication for 15 minutes; plugs were removed from the solution, the excess liquid was squeezed out with forceps, and the process was repeated with fresh solution. After squeezing out excess solution, the plugs were air-dried in a hood. The sample collector was assembled from three plugs in series within a cassette extender (7.5 cm long × 37 mm in diameter). The extender was connected to the base of the filter cassette for personal sampling (Figure 2). Before and after sampling, the extenders containing polyurethane foam plugs were stored in Ziploc plastic bags. Immediately after sampling, the extenders were stored on dry ice until they were returned to the lab and stored in the freezer at −20°C.

In addition to sampling with the 37-mm-diameter plugs, a small set of high-volume samples was collected from the railroad repair shop with three plugs (7.2 cm long × 76 mm in diameter) in series, using a General Metals High-Volume Sampler (Model 2000) with a 20.4- × 25.4-cm Teflon-coated glass-fiber filter.

Laboratory and field experiments were used to determine the optimal extraction procedure and to evaluate the collection efficiency. Plugs were spiked with 50 µg or 0.2 µg of phenanthrene and repeatedly extracted. The fraction of ma-

terial recovered in each extraction was determined. The efficiency of collection by polyurethane foam plugs was compared with that of XAD-2 resin by adding known amounts of standard solutions of six PAHs—naphthalene, fluorene, phenanthrene, pyrene, chrysene, and benzo[*a*]pyrene (B*a*P)—to 15 filters. Five spiked filters were stored in the freezer until analysis; five were assembled into sampling trains with XAD-2 tubes downstream, and five were assembled into sampling trains with polyurethane foam plugs downstream. Clean air was drawn through each of the 10 sampling trains for eight hours at 1.7 Lpm. The filters, XAD-2 resin samples, and polyurethane foam plugs were each extracted by their own protocols (filters ultrasonically with dichloromethane; XAD-2 resin by Soxhlet extraction with dichloromethane; polyurethane foam plugs ultrasonically in a solution of 5 percent dichloromethane in heptane). Front and back sections of the XAD-2 resin were extracted and analyzed separately, and each of the three polyurethane foam sections was extracted and analyzed separately. The amount of PAHs on each of these collection devices was then determined by gas chromatography on a 15-m DB-5 fused silica column, operated at 30 mL of nitrogen/minute, with a temperature program of 75°C initial temperature to 235°C final temperature, at a rate of 4°C per minute, using a flame ionization detector.

Collectors also were placed in diesel locomotive repair shops to determine how airborne phenanthrene is partitioned across three or four plugs run in series. Virtually all the phenanthrene was found on the first plug, and trace amounts were on the third plug. Therefore, a sampling train containing three plugs was chosen for field work, and all three plugs normally would be analyzed together although, if desired, they could be analyzed separately. The three polyurethane foam plugs from each sample were extracted by ultrasonication twice in fresh solutions of 5 percent dichloromethane in heptane; these extraction solutions were combined and concentrated under a stream of nitrogen to 500 μL.

The extract solutions were analyzed by a two-stage liquid chromatographic analysis, including a class fractionation step followed by an analytical separation. All liquid chromatography was performed on a Waters (Milford, MA) liquid chromatograph equipped with a WISP automated injector, M6000 and M45 solvent delivery systems, M660 solvent programmer, M440 ultraviolet light detector monitored at 254 nm, a Waters Data Module, and a McPherson spectrofluorimeter. Class fractionation was performed by normal-phase liquid chromatography using an amino-cyano column (Perkin Elmer) with a solution of 3 percent *tert*-butyl methyl ether in heptane as the mobile phase, operated at 2 mL/minute. Aliphatic hydrocarbons eluted first, between 2 and 4 mL after injection; olefinic and aromatic compounds eluted with varying retention times over the next 20 mL. Fifteen minutes after the sample injection, the flow through the column was reversed automatically with an Autochrome (Milford, MA) solenoid valve programmed with a ChronTrol timer (Lindburg Enterprises, San Diego, CA), and the mobile phase changed to 100 percent *tert*-butyl methyl ether; this step backflushed the polar compounds from the column into the fraction collector. The four fractions, one aliphatic, two aromatic, and one polar, were concentrated to approximately 5 mL under nitrogen and stored in amber vials at −20°C.

Analysis for phenanthrene was performed isocratically by reverse-phase liquid chromatography on a C_{18} column (no end capping) (Vydac, 201TP54; SeParations Group, Hesperion, CA) with an aqueous solution of 62 percent acetonitrile. Phenanthrene was measured with the spectrofluorimeter, which was set to monitor excitation wavelength at 253 nm and emission wavelength at 372 nm.

The entire sampling train—filter, treated filter, and polyurethane foam plugs (Figure 2)—was tested by adding known amounts of the standard PAH solutions to a blank filter and then sampling in the environmental chamber while volunteer smokers were generating environmental tobacco smoke. A second set of samplers without the treated filter was tested at the same time to insure that the treated filters did not affect PAHs collected on the polyurethane foam plugs. Spiked filters and sampling trains were tested in the railroad repair shops, where diesel exhaust concentrations were expected to be highest, with an extra, fourth, polyurethane foam plug in series to check for possible breakthrough. For selected personal samples collected in the field, each of the three polyurethane foam plugs in series in the cassette extenders was analyzed separately to check for breakthrough.

Sampling Strategy

Our previous work in railroads showed that railroad workers' job titles, work locations, and the outdoor temperature during the exposure measurements were major determinants of their level of exposure and the composition of air contaminants (Woskie et al. 1988a). Clerks, who work in offices, had the highest level of environmental tobacco smoke exposure but no exposure to diesel exhaust. Mechanics and electricians, who work in large open repair shops, had the highest exposures to diesel exhaust. Outdoor temperature was an important factor in exposure, because doors and windows that provide ventilation indoors are closed when the outdoor temperature drops. As a result, air contaminants emitted indoors accumulate more and ex-

posures are higher when outdoor temperature is below approximately 50°F.

Our goal was to contrast the urinary mutagenicity of workers with no diesel exhaust exposure with that of workers who had a wide range of exposures. Therefore, our sampling strategy was to choose workers who worked away from operating locomotives, such as clerks and carmen, as our unexposed group, and mostly shop workers as our diesel exhaust-exposed group. Shop workers and engineers were oversampled in the cold weather trips to maximize potential diesel exhaust exposure in these categories (Table 1).

MUTAGENICITY

Chemicals

Benzo[*a*]pyrene (Gold Label), 2-aminofluorene (2-AF), 2-nitrofluorene (2-NF), 4-nitroquinoline-*N*-oxide (4-NQO), and dimethylsulfoxide (DMSO, Gold Label) were purchased from Aldrich Chemical Co. (Milwaukee, WI), and used without further purification. β-Glucuronidase was purchased from Sigma Chemical Co. (St. Louis, MO).

Microsuspension Assay Method

A microsuspension (micro preincubation) procedure, previously developed to detect mutagens in the urine of cigarette smokers and nonsmokers (Kado et al. 1983, 1986), was used throughout the study. The method is at least 10 times more sensitive than the standard plate incorporation test (Ames et al. 1975), as judged by absolute amounts of mutagen required for a specific mutagenic response. The method has been tested using a number of standard mutagens and complex mixtures.

For our modification, bacteria were grown overnight in Oxoid Nutrient Broth No. 2 (Oxoid Ltd., Hants, England) to approximately 1 to 2 × 10^9 cells/mL, and harvested by centrifugation (10,000 × *g*, 4°C, 10 minutes). Cells were resuspended in ice-cold phosphate-buffered saline (PBS, 0.15 M, pH 7.4) to a concentration of approximately 1 × 10^{10} cells/mL, as determined by the number of surviving bacteria and the optical density at 550 nm.

The metabolic enzyme S9 and the S9 mix were prepared according to the procedure of Ames and colleagues (1975). The S9 from male Sprague-Dawley rats pretreated with Aroclor 1254 was used throughout. This fraction contained 52.4 mg of protein/mL, as determined using the modified Biuret method of Ohnishi and Barr (1978).

For the microsuspension assay, the following ingredients were added, in the order stated, to 12- × 75-mm sterile glass culture tubes on ice: 0.1 mL of S9 mix; 0.005 mL of urine extract in DMSO; and 0.1 mL of concentrated bacteria in PBS (1 × 10^{10}/mL of PBS). Mutagen standards and air filter extracts were also dissolved in DMSO and added, at 0.005 mL volume, in place of the urine extract. The mixture was incubated in the dark, at 37°C, with rapid shaking. After 90 minutes, the tubes were placed in an ice bath, then taken out one at a time, and 2 mL of molten top agar (Ames et al. 1975) containing 90 nmol of histidine and biotin was added immediately. The combined solutions were vortex-mixed and poured onto minimal glucose plates. Plates were incubated at 37°C in the dark for 48 hours. Strain markers were determined for each experiment. All procedures were carried out in a room fitted with yellow fluorescent lights (G.E.N. F40Go) to minimize potential photooxidation.

Diesel Extracts

A number of dichloromethane extracts of high-volume filters, used for collecting particulate matter at three different railroad facilities, were screened for mutagenic activity using bacterial tester strains TA98 and TA100, with and without S9 metabolic activation. Dose-response curves were established for a selected number of the extracts that demonstrated the highest revertant values in the preliminary screening assay. Bacterial tester strains TA98 and TA98 NR were used for the dose-response curves. The extracts were tested in tester strain TA98 NR, because this strain has been reported to be less sensitive to certain nitropolycyclic aromatic hydrocarbons (nitro-PAHs) (Rosenkranz and Mermelstein 1983).

Dose-response curves were also determined for an extract from heavy-duty diesel particulate matter. The extract was obtained from D. Schuetzle of the Ford Motor Co. (Dearborn, MI), and tested directly. The heavy-duty diesel particulate matter from which the extract was obtained was a composite of exhaust from a number of heavy-duty diesel engines. (The same particles are also available for purchase from the National Bureau of Standards, Standard Reference Sample No. 1650.)

A selected number of personal filters from clerks and shop workers were also extracted with dichloromethane (HPLC grade, Fisher Scientific, Springfield, NJ) and tested for mutagenic activity. We wanted to determine whether or not mutagenicity from these filters could be measured for these relatively short sampling times and thus, if possible, to provide an individual index of exposure to mutagens during the work shift.

Urine Collection and Extraction Methods

Urine was collected in washed, sterile, amber-colored glass or polyethylene plastic bottles. The bottles were immediately placed on ice at the collection site. For creatinine

determination, a 5-mL aliquot of urine from each sample was dispensed into a scintillation vial fitted with a Teflon-lined screw cap. Both the main collection bottle and the scintillation vials were frozen at −20°C. Frozen samples were then shipped in dry ice to the University of California, Davis, Department of Environmental Toxicology, for extraction and testing. Urine samples were stored at −20°C until processed and tested. Extracts were stored in amber glass vials, sealed with nitrogen, at −20°C. Extraction and testing of urine samples were delayed because of the need to validate the sensitivity of the methods used (see Appendix B). The approximate time from collection to extraction ranged from 1 to 10 months, with a median time of 8.5 months (n = 29 separate extraction sets); the approximate time from extraction to testing ranged from 1 to 16 weeks, with a median time of 6 weeks (n = 13).

The urine samples were extracted using the methods of Yamasaki and Ames (1977), incorporating the modification of Putzrath and workers (1981), except that we eliminated the use of sodium sulfate to dry the extract. The sorbent XAD-2 (Applied Science Laboratories, Inc., State College, PA) was repurified by Soxhlet extraction (24 hours acetone, 24 hours methanol) before use. Concentrated urine was obtained by redissolving the acetone extract (eluted from the XAD-2 column) in water (20 mL/100 mL of original urine), neutralizing the solution to pH 7.0 with 0.2 N sodium hydroxide, and extracting three times with dichloromethane (7 mL/mL of original urine each time). The extracts were combined, dried in vacuo at 45°C, capped with nitrogen, and stored at −20°C until testing. Deionized glass-distilled water that had been passed through a charcoal adsorption column was used throughout, and an extraction control in which PBS (0.15 M, pH 7.0) was substituted for urine was routinely included for every extraction. Extracts were reconstituted with DMSO to make a 1,000-fold concentrate just before testing. Urine was extracted in lots of 11 samples, and each lot had its own PBS extraction control.

The XAD-2 resin was a good initial candidate for use in extracting mutagens from urine samples, since much of the work on urine mutagenicity, especially from cigarette smokers, has been done using this adsorbent. Belisario and associates (1985) found that XAD-2 used in series with XAD-4 was most efficient in extracting mutagens from the urine of rats that were injected with diesel particles.

Mutagenicity Testing of Urine

All urine samples were tested at a minimum of three urine equivalent doses of 2.5, 5, and 10 mL, in duplicate, both with and without S9 mix. A single experiment could test three batches of urine (11 samples/batch), each with its own extraction control using PBS (0.15 M, pH 7.0). The mutagens 2-AF and BaP served as positive controls for +S9, and 2-NF and 4-NQO served as positive controls for −S9. All positive control mutagens were tested at three doses in triplicate and were included in every experiment. Four to five plates were routinely used for the DMSO standard that provided the zero concentration.

The order of the samples was as follows for the +S9 test: (1) batch samples at two or three concentrations; (2) PBS blank; (3) +S9 positive controls (BaP and 2-AF); and (4) DMSO blank (zero concentration for the batch). The same order (1 through 4) was repeated for each batch. After the +S9 samples, the −S9 tests were carried out in the same order, except that the positive controls for −S9 were 2-NF and 4-NQO. Because the DMSO blank was located last in each batch, it was not possible to test for a drift within the batch.

The number of revertants per milliliter equivalent of urine extracted was normalized for urinary concentration of mutagens by dividing by the concentration of creatinine. Creatinine was determined in each urine sample using a standard clinical chemistry kit (Sigma Chemical Co., St. Louis, MO). Normalization by creatinine concentration was considered since the urinary concentration of compounds could vary depending on each worker's fluid intake for the day. This approach has been reported to improve the accuracy of the analysis of toxic compounds in the urine (Barber and Wallis 1986).

(See the Clinical Chemistry section below for further discussion of urine creatinine measurement and its use for urine marker normalization.) Graphs of the mean number of revertants per micromole of creatinine of urine plotted against concentration frequently show downward curvature at the higher concentrations, presumably as a result of compounds in the urine extract that are toxic to bacteria or of competitively binding metabolic enzymes (data not shown). In *Salmonella* testing, particularly with the standard plate incorporation test, it is not common practice to test the toxicity of the sample preparations directly. Therefore, it became necessary to estimate indirectly the number of revertants per micromole of creatinine of urine.

The number of revertants per milliliter equivalent of urine was calculated as follows: least-squares regression fitted the number of revertants per plate to a constant, a linear term in plate concentration, and a squared term in plate concentration. If the p value for the squared term was less than or equal to 0.15 and the coefficient of the squared term was negative, indicating a downward curvature at high doses, samples with the highest dose (usually 10.0-mL equivalents) were dropped and the process was repeated until the squared term for nonlinearity was "nonsignifi-

cant," with $p > 0.15$. Finally, the number of revertants per milliliter of urine was estimated from a simple linear fit to the remaining dose points.

This procedure is common practice in the toxicology literature, but it can be criticized because of the subjective nature of the significance level chosen for detection of curvature (0.15 here) and because of the elimination of information from the higher doses. To evaluate the sensitivity of our results to the exact procedure chosen, two other methods of calculating the dose-response relation were attempted. In the first, the p value for detection of nonlinear trend was changed to the more stringent 0.10. This added a higher dose to fewer than a dozen samples for mutagenicity, both with and without S9, out of more than 600 tested. Second, a quadratic model was fitted to all doses for each sample, and the number of revertants per milliliter of urine was estimated as the slope of the tangent to the fitted curve at zero.

The tangent slopes from the quadratic fit were higher, sometimes much higher, than those calculated after deletion of observations to obtain a linear fit (see Appendix C and Table C.1). This was especially true for the + S9 results. The between-sample variability of the tangent slopes was greater, and the individual standard errors of the tangent slopes were higher, than those from the point-deletion calculation. (All results are significant by the paired Wilcoxon rank sum test at $p < 0.01$.) The Spearman rank correlation of the + S9 tangent and the point-deletion slopes was 0.73, and that of the − S9 slopes was 0.82. Because of the greater variability in the tangent slopes, the point-deletion slopes were used throughout.

To correct for possible batch differences, the slope for the PBS blank was subtracted from the slopes for individual samples in each batch. This calculation resulted in negative values if a sample result was lower than that for the blank. Negative results were not forced to zero, because this would have distorted the observed variability.

Descriptive mutagenicity statistics in this monograph are not adjusted for differential sensitivity of the bacterial strains over the course of the experiments. However, the multiple regression analyses for predicting mutagenic activity do adjust for differential batch response to the positive mutagenic control 2-AF. (See Statistical Methods, Model Form section for details, and Appendix D for the results.)

CLINICAL CHEMISTRY

Markers for tobacco smoke inhalation and absorption were measured. Thiocyanate, a compound derived from cyanide components in tobacco, has been suggested for use as a convenient measure of tobacco smoke exposure (Borgers and Junge 1979). Urinary thiocyanate concentrations were determined using the procedures outlined by Bowler (1944) and Butts and coworkers (1974), with slight modifications. Four milliliters of each urine sample were placed in 13 × 100-mm tubes, and the urine was centrifuged in a clinical table-top centrifuge (15 minutes, approximately 2,000 rpm). One milliliter of each sample (in triplicate) was placed in a 13 × 100-mm culture tube, 1 mL of water was added, and the absorbance was read at 460 nm. Standards of potassium thiocyanate (2 mL) were prepared in triplicate at 62.5, 125, 250, and 500 μmol. To all samples and standards, 1 mL of 0.5 M ferric nitrate solution was added and vortex-mixed, and absorbance was read at 460 nm. Absorbance recorded before the addition of ferric nitrate was subtracted from absorbance values after ferric nitrate was added. Although thiocyanate standards were stable at 4°C for at least two months, we do not know how specific the assay is for thiocyanate, and our measurement may reflect "thiocyanate equivalents." With the more sensitive and specific nicotine and cotinine measurements, the thiocyanate results were not used in the final analyses to control for cigarette smoking.

Urinary concentrations of nicotine and cotinine (a metabolite of nicotine) were determined by Peyton Jacobs, Lisa Yu, and Neal Benowitz (San Francisco, CA) by gas chromatographic analyses, as described by Jacobs and colleagues (1981), with modifications. One milliliter of urine was added to a 13 × 100-mm culture tube, to which was added 0.1 mL of internal standards (5-methyl nicotine and 5-methyl cotinine), followed by 0.5 mL of sodium hydroxide (2 N) in ammonium hydroxide (0.2 N). Three milliliters of toluene:butanol (7:3) were then added and the solution was vortex-mixed. The tubes were then placed in a dry-ice and acetone bath until the bottom layer was frozen; the top layer was poured into a new tube containing 0.5 mL of sulfuric acid (1.0 N). The solution was again frozen and the top layer discarded; 0.5 mL of 50 percent potassium carbonate (in 0.2 N ammonium hydroxide) and 0.4 mL of toluene:butanol (9:1) were added. The resulting solution was vortex-mixed, frozen again, and the top layer poured into a sampling vial for gas chromatographic analysis.

One microliter each of nicotine and cotinine extracts was analyzed by gas chromatography (model 580, with nitrogen-phosphorus detector, Hewlett Packard, Sunnyvale, CA) on a 25-m × 0.31-mm inner diameter capillary column (5 percent phenylmethyl silicon, Hewlett Packárd) at a temperature program of 90° to 275°C at 25°C per minute. Creatinine concentrations were determined using a diagnostic kit (Sigma Chemical Co., St. Louis, MO). Triplicate samples of urine were routinely tested.

All urine concentrations (smoking markers, mutagenicity) were corrected for creatinine concentration. This procedure has been shown to improve the accuracy of assays for toxic substances in the urine (Barber and Wallis 1986). One potential problem with this adjustment is that correction for excessively dilute urine (very low concentration of creatinine) may overcorrect for the marker of interest. We used the previously described cutoff of 0.3 g/L and found that all cigarette smoking markers in the dilute urine samples (< 0.3) were outliers (too high) from predicted levels (Barber and Wallis 1986). Therefore, all samples with urine creatinine concentration of less than 0.3 g/L (six nonsmoker, two smoker) were excluded from analyses that used urine markers.

DATA MANAGEMENT

Data input forms were designed for each source of information. The five-digit key identifier was assigned by the field staff at the time of enrollment. The identifier (ID) consisted of three digits unique to each participant in the study, a single digit designating how many repeat two-day field protocols the worker had, and an "a" or "b" designating the first or second day of the protocol. For quality control, the trip, date of sample, and code number of the two-day protocol were also included. Each sample vial and questionnaire was labeled by the field staff.

To guard against errors in the key identifier variables, a master file was created by hand, and all entries were checked against this list. All data forms were checked by eye before being sent to the University Keypunch Service, where they were entered independently by two operators onto a computer disk. The data were transferred by computer tape to the Occupational Health Program VAX 11/750. The Statistical Analysis System (SAS Institute 1985) and BMDP Statistical Software (1983) were used for all data management.

Printouts of all transferred laboratory data were checked against the original forms by the study research associate and laboratory technician. Any errors detected at this stage were corrected in the raw data file. Subsequently, plots and histograms were used to detect possibly unlikely values. Frequency counts and other checks were used primarily to find out-of-sequence samples. Those found were relocated to the standard order.

SAS data sets were produced for each data type and merged for final analyses. To guard against possible inadvertent modification, no merged analysis data set could be deleted once created. Tape backups were regularly made.

STATISTICAL METHODS

General Strategy

The statistical analysis generally proceeded from simple descriptive procedures and tables to more complex models. Since multiple samples were collected from each worker, problems of dependent observations arose at the outset. Our strategy was, generally, to ignore observation dependence for most of the descriptive tables. When it was clearly inappropriate to use the daily sample as the reporting unit, results by two-day protocol or by individual worker were displayed.

Preliminary screening for variable selection used the daily samples as the analytic unit, without allowance for dependence of repeat observations. After a reduced set of variables and models was chosen for intensive analysis, the data were transferred to BMDP files, where the appropriate generalized techniques could be employed.

For simple descriptive techniques, we relied upon means, standard deviations, medians, and quartiles (25th and 75th percentiles). A frequent technique was to group continuous variables into two, three, or four categories with approximately equal numbers in each. For studying distributions of two or more variables at a time, rank correlations and scatter plots were used.

Preliminary Smoking Analyses

Data on cigarette smoking characteristics were examined to choose factors predictive of mutagenicity in smokers who had not been exposed to diesel exhaust. "All-subsets" multiple regression techniques examined a large number of predictive models for factors such as numbers of cigarettes smoked, tar content of cigarettes, depth of smoke inhalation, urine cotinine, thiocyanate, and nicotine levels, and interactions. Regression residuals and Cook's D-statistic (Cook 1977) were regularly examined for evidence of outliers and unduly influential observations.

Indices of Diesel Exhaust Exposure

For assessing the response of urine mutagenicity to diesel exhaust exposure, two different methods were used: (1) construction of ordered groups, based on an index of the amount of diesel particulate matter in the air; and (2) a full multiple-regression dummy-variable model in which the effect of "exposure" was expressed as an interaction between the amount of respirable particles and nicotine in the air, and work at jobs with known potential for diesel exhaust exposure. In addition, some analyses attempted to detect a dose-response effect only within diesel exhaust-exposed job groups.

Table 2. Respirable Particle, Environmental Tobacco Smoke, and Adjusted Respirable Particle Exposures by Job Group

Exposure Assignment[c] and Job Group	Respirable Particle Concentration ($\mu g/m^3$)				Environmental Tobacco Smoke[a] ($\mu g/m^3$)				Adjusted Respirable Particle Concentration[b] ($\mu g/m^3$)			
	n	Mean ± SD	Median	25th and 75th Percentiles	*n*	Mean ± SD	Median	25th and 75th Percentiles	*n*	Mean ± SD	Median	25th and 75th Percentiles
Unexposed												
Clerks	57	119 ± 71	115	(70, 164)	44	71 ± 69	58	(17, 101)	44	60 ± 54	54	(25, 98)
Carmen welders	14	285 ± 170	251	(137, 445)	14	88 ± 108	52	(6, 131)	14	210 ± 178	196	(75, 319)
Carmen nonwelders[d]	21	194 ± 115	201	(102, 264)	20	108 ± 132	64	(2, 157)	20	104 ± 101	89	(44, 189)
Moderate exposure												
Engineers	14	183 ± 182	157	(61, 244)	14	114 ± 143	46	(0, 257)	12	73 ± 133	54	(10, 78)
Brakers	33	156 ± 168	91	(75, 190)	30	48 ± 83	5	(0, 83)	27	99 ± 85	73	(63, 88)
High exposure												
Shop workers	156	153 ± 99	132	(87, 200)	158	29 ± 69	2	(0, 14)	156	127 ± 94	113	(71, 163)

[a] Environmental tobacco smoke = nicotine concentration × 8.6.
[b] Adjusted respirable particle concentration = respirable particles − (8.6 × nicotine concentration) (see text).
[c] A priori diesel exhaust exposure assignment based on previous work (Woskie et al. 1988a,b) and observation of job locations and tasks.
[d] Car repair workers excluding those doing cutting, burning, or welding.

From our previous work (Woskie et al. 1988a), workers employed in the railroad shop were known to have the highest potential exposure to diesel particles. Brakers and engineers were exposed to lower concentrations of diesel exhaust, and clerical workers and carmen were classified as unexposed. Rather than use the average exposure of a job group as an estimate of a worker's exposure on a given day (Woskie et al. 1988a,b), the study design for this project involved actual measurement of an individual's exposure on the day that his urine was collected for mutagenicity testing. In addition, through improvements in the sampling method, we were able to develop more accurate estimates of exposure. However, estimates of the diesel exhaust exposures of the brakers and carmen were difficult to make because both groups were exposed to nondiesel particulate matter, such as dirt and sand, that would increase the particulate matter exposures but not reflect diesel exhaust (see the Discussion, Diesel Exhaust Exposure section). When the methods developed in this study were used on this study population, it was possible to obtain better estimates of the actual exposure to diesel exhaust (Table 2).

Index Construction

The construction of an index of diesel exposure was based on the primary equation:

Respirable Particles =
Tobacco Smoke + Diesel Particles + Remainder.

By assumption, there are no diesel particles in unexposed jobs. In jobs potentially exposed to diesel fumes, the equation can be rewritten:

Diesel Particles =
Respirable Particles − Tobacco Smoke − Remainder.

In this form, the quantity of diesel particles could not be estimated in an unbiased fashion for two reasons. First, in two subgroups of workers, the brakers and the carmen welders, the remainder term was apt to be systematically large (see the Methods, Mutagenicity section). For the brakers, dust in the air was occasionally substantial; for the carmen welders, welding fumes were present in high concentrations. Corrections for the presence of dust or welding fumes were not possible, as neither type of particle had been directly measured. Therefore, both brakers and carmen welders were excluded from the analysis.

However, even with these exclusions, another source of error still remained: Only one of the constituents of environmental tobacco smoke had been measured directly, vapor-phase nicotine (NICOTINE). Therefore, it became necessary to estimate environmental tobacco smoke.

Two different data sets were available for this estimate. First, chamber studies of environmental tobacco smoke (see the Results, Specific Aim 1 section) showed approximately that

Respirable Particles in the Chamber/m^3 =
Constant + 8.6 × [NICOTINE].

If it is assumed that in the chamber studies the background level of respirable particles is uniform, the same equation holds true for environmental tobacco smoke with a different constant. This relationship was validated on data in smokers and nonsmokers in this study who were unex-

posed to diesel exhaust. A regression of respirable particles on nicotine in these samples (excluding brakers and carmen welders) yielded the same nicotine coefficient, 8.6 (SE = 1.3), as the estimate from the chamber studies.

These considerations, and the concordance of the two methods for estimating the nicotine coefficient, led us to the following index of exposure to diesel particles:

Adjusted Respirable Particles =
Respirable Particles − 8.6 × [NICOTINE].

It is not necessary to subtract a constant term in this equation, because any regression model will include a constant term. The adjusted respirable particle index indicates the excess of respirable particles over the amount to be expected based on the nicotine concentration in the air of men not exposed to diesel particles. It accounts for diesel exposure only in men with potential diesel exhaust exposure.

This index of diesel particles is subject to error. After exclusion of the brakers and carmen welders, the error should be less systematically related to job category. One consequence of the (random) error for estimating diesel particles is that the observed association between mutagenicity and adjusted respirable particles will be of lower magnitude than any true association, as will the probability of detecting an association (Judge et al. 1980). This is known in statistics as the "errors in variables" problem.

To study both unexposed and exposed men in the same analysis, and to reduce the effect of error in measuring diesel exhaust exposure, we grouped air samples according to concentration of adjusted respirable particles. All samples taken from men in unexposed jobs constituted the first group. Samples from exposed men were grouped roughly into thirds, for a total of four groups. The samples from exposed workers with the highest exposure to adjusted respirable particles (group 4) should have higher true diesel exhaust exposure than the samples in the unexposed group (group 1) and those in group 2. However, the random error in adjusted respirable particles makes it likely that some overlap and misclassification in true diesel exhaust exposure remains. The differences among the four exposure groups are tested with an F or chi-squared test using three degrees of freedom in the regression models.

Interaction Model for Exposure Effects

As an alternative to the adjusted respirable particle index discussed above, we also used a multiple regression model to detect a differential effect of measured respirable particles and nicotine in exposed jobs.

Formally, we included as predictors in the multiple regression model a dummy variable for potential exposure to diesel exhaust (EXPOSED = 1 for exposed jobs, EXPOSED = 0 for unexposed), and two interaction variables: EXPOSED with respirable particles (Respirable Particles × EXPOSED) or with air nicotine (NICOTINE × EXPOSED).

Any differential effect of respirable particles and nicotine in exposed jobs in this model is expressed through the significant interaction terms. The test of the three interaction terms has three degrees of freedom. This approach bypasses entirely the need to construct an index.

Phenanthrene Models

Phenanthrene was measured in fewer than half the study air samples. Although it was used as an indicator for diesel exhaust in some of the multiple regression analyses, we do not report the results. However, for a subset of observations on nonsmokers with repeated observations on different days, mutagenicity is plotted against phenanthrene.

Other Predictive Factors for Regression Models

For preliminary analyses, we screened a number of predictive factors for inclusion with the grouped and interaction exposure models. These analyses showed very different effects in smokers and in nonsmokers, and so we report results separately by smoking category. Besides the exposure-definition variables discussed above, the following predictive factors are included in one or more of the reported models: (1) urinary cotinine (control for internal dose of tobacco smoke) for all models; (2) "protective food" consumption (cabbage, Brussels sprouts, or fish) (1 = yes, 0 = no) for nonsmoker models; (3) cigarettes smoked on study day for smoker models.

We also considered including dummy variables for differential effects of the six trips made to the railroad facility (a compounding of batch, handling, seasonal, storage, and processing effects). However, the samples from most visits were from workers who were all exposed or all unexposed (Table 1). Therefore, exposure status was inextricably confounded with visit effect, and controlling for visit differences would have made the finding of exposure effects impossible.

Urinary thiocyanate was also entered into preliminary models as an indicator of active and passive smoking. However, it was not as successful as urinary cotinine and the number of cigarettes actually smoked. Urinary nicotine was also considered, but was rejected as less predictive than the combination of cotinine alone and the number of cigarettes smoked on the study day.

Transformations and Model Diagnostics

Formal diagnostic tests for a power transformation (Box and Cox 1964) of the form

Response Variable = Mutagenicity$^{\lambda}$

were tried (Atkinson 1986), but the results proved unsatisfactory. Most of the observed responses were little higher than the background (blank) counts; indeed, many were negative. Familiar transformations, such as the logarithm (corresponding to $\lambda = 0$), square root ($\lambda = 0.5$), or inverse ($\lambda = -1$), sometimes suggested by the diagnostic gave strongest emphasis to these very low counts. This phenomenon was unappealing in the face of the strong background component. Suggested power transformations with power greater than 1, such as $\lambda = 4$, were also implausible. As a result of these considerations, only the original, untransformed mutagenicity rate, in revertants per milliliter of creatinine, was chosen as a response.

To detect unduly influential observations, Cook's D-statistic, which measures the effect of omitting an observation, was calculated (Cook 1977). We eliminated observations with $D > 0.5$, because these generally appeared clearly separated from observations with lower values. After each round of elimination, the model was refit and Cook's D-statistic was recalculated until no unduly influential observations remained. Although this criterion was somewhat subjective, it led to the elimination of no more than two observations from any analysis.

Model Form: Accounting for Differential Sensitivity of Bacterial Strains

The urine mutagenicity samples were processed on 13 different occasions as the study progressed. As described in the Mutagenicity section above, four different standard mutagens were evaluated on each occasion as a positive control for bacterial strain sensitivity: 2-AF and BaP for +S9, and 2-NF and 4-NQO for −S9.

Descriptive statistics for these control experiments are described in Appendix E, Tables E.1 and E.2. As can be seen, the between-experiment coefficients of variation for 2-AF and BaP were greater than 20 percent. Table E.3 shows the Spearman rank correlation of the standard means on each occasion with the study sample results assayed on that occasion. Somewhat surprisingly, the two +S9 controls, 2-AF and BaP, were more highly correlated with the −S9 results than were the −S9 controls, 2-NF and 4-NQO.

On the basis of these findings, we sought ways of correcting the regression analyses for what appeared to be varying sensitivity of the TA98 strain on different experimental occasions. Let S indicate the mean mutagenicity of a standard on an experimental date. The following three different types of regression models were fit to the data in preliminary analyses:

$$\text{Response} = \text{Mutagenicity Rate}/S = b_0 + (b_1 \times Z_1) + (b_2 \times Z_2) + \ldots + (b_k \times Z_k) + \text{Error Terms.} \quad (1)$$

Here $Z_1, Z_2, \ldots, Z_k$ are covariates. All four standards were tried in this model, but the fits were unsatisfactory. Not only were the suggested power transformations implausible (for example, $p = -3/4$), but up to 5 of 65 observations were identified as unduly influential for some analyses, a number that seemed too high. This approach, then, was abandoned, and the results are not reported.

Response = Mutagenicity Rate (standard means added as covariates). (2)

This model was attractive because mutagenic activity of more than one standard could be included at one time. Following this approach, the original values were used for response, and no more than two observations were identified as unduly influential in any analysis. From a biological point of view, however, this formulation was unsatisfactory because it extrapolates a positive response even when the standards give no response. In addition, the model fits were no better and the conclusions did not differ from the third type of mode, reported below. Therefore, the results of this approach are not reported.

Response = Mutagenicity Rate (multiply original covariates by one standard mean). (3)

This model amounted to using the following linear model for mutagenicity (or its transform):

$$\text{Response} = S \times [b_0 + (b_1 \times Z_1) + (b_2 \times Z_2) + \ldots + (b_k \times Z_k)] + \text{Error Terms.} \quad (4)$$

(In fact, the computer program we used, BMDP3V [see next section], required that a separate intercept, say, a_0, be fitted.)

Models (1) and (4) are very similar. If both sides of the equation for model (1) are multiplied by S, the form for the response mean is identical to the form for model type (4). However, in model type (1), the error terms are multiplied by S. This form of model appeared to give smaller residual standard deviations than did the models of form (2), above. Furthermore, use of 2-AF as the standard mutagen resulted in the smallest standard deviations of all the standards. As

with model type (2), only one or two unduly influential observations were detected and eliminated from any analysis. Results for model type (4) are reported.

To calculate predicted effects from model (4), the estimated coefficients from the model were multiplied by the overall study mean for mutagenicity of 2-AF, 3.46 revertants/ng (Table E.1). This was used throughout as the value of S in model (4).

Generalized Least Squares

To accommodate the multiple observations per person that were recorded in the study, an estimated generalized least-squares model was fit (Judge et al. 1980). In this model, the response was modeled as the sum of a fixed regression mean and two independent random terms. The fixed mean is the same as would be fit by ordinary multiple regression. The first random term is assumed to be common to all the samples taken from the same person on the same trip, varying between subjects with a variance called the "between-person" variance component. The second random term represents the differences between daily samples taken from the same person, and is called the "within-person" or "error" variance component.

Statistical p values and standard errors are more valid under the generalized model than under the ordinary multiple regression model, which assumes that all observations are independent (Judge et al. 1980). The point estimates for regression effects are weighted to have minimum variance, and so are also somewhat different from the effects under the independence model.

The computer program BMDP3V (General Mixed Model) Analysis of Variance was used for these calculations (BMDP Statistical Software 1983). The restricted maximum likelihood estimates are presented because (1) estimated coefficients are likely to be robust to the formal assumption of Gaussian distributions for the random terms (Jennrich and Sampson 1976; Harville 1977), and (2) estimated variance components are less biased. One drawback to this program is that it requires the fitting of a separate intercept.

For testing a subset of variables, one model was fit with the subset ("full model") and one without it ("reduced model"). Log-likelihood statistics (based on a nominal assumption of normality) were computed for full and reduced models by the computer program. Two times the difference in the log-likelihoods was referred to a chi-squared distribution with degrees of freedom equal to the number of parameters in the subset. For the analyses with strata formed on the basis of diesel exhaust exposure and adjusted respirable particles, the resulting test had three degrees of freedom. This was also true of the test for the three interaction factors in the exposure-interaction model. For regular maximum likelihood problems, the log-likelihood for a reduced model is always less than that for a full model. During the analysis, it was discovered that log-likelihoods from restricted maximum likelihood fits do not always have this ordering. As a result, all likelihood tests are based on maximum likelihood fits, not on restricted maximum likelihood fits.

RESULTS

SPECIFIC AIM 1

Phenanthrene as a Marker for Diesel Exhaust

The polyurethane foam plugs were determined to be efficient at collecting phenanthrene from the breathing zone of railroad workers as a possible marker for diesel exhaust exposure. One extraction of a polyurethane foam plug spiked with PAHs removed nearly all the PAHs added, and no PAHs were found in the third extraction. Laboratory experiments with the different sampling schemes (filter plus either XAD-2 resin or polyurethane foam) showed that all of the chrysene and B*a*P remained on the filter during sampling, but 35 percent of the pyrene, 62 percent of the phenanthrene, 66 percent of the fluorene, and 100 percent of the naphthalene were lost from the filter after eight hours of sampling at 1.7 Lpm. Neither the XAD-2 resin tubes nor the polyurethane foam plugs collected the naphthalene efficiently; both collected the pyrene that had been lost. While the polyurethane foam collected virtually all of the fluorene and phenanthrene that was lost from the filter, the XAD-2 resin collected only half as much phenanthrene and three quarters as much fluorene as the polyurethane foam plugs. All of the phenanthrene was found on the first two polyurethane foam sections, and none on the third. Experiments in the environmental chamber with environmental tobacco smoke demonstrated that no difference in PAH content of the polyurethane foam plugs was found between samplers with the treated filter (which collects nicotine) and those without. Thus, the treated filter did not interfere with the collection of phenanthrene.

A two-step analysis was developed to measure PAHs in the polyurethane foam samples. Liquid chromatographic methods first fractionated the filter and polyurethane foam extract solutions into the following chemical fractions: aliphatic compounds; two-ring or three-ring aromatics; larger aromatic compounds; and polar compounds. The small aromatic fraction was then analyzed by isocratic separation on a reverse-phase column. Although gradient chro-

Table 3. Respirable Particles and Adjusted Respirable Particle Exposures for Carmen and Shop Worker Welders and Nonwelders

	Respirable Particle Concentration ($\mu g/m^3$)				Adjusted Respirable Particle Concentration ($\mu g/m^3$)			
Job Group	*n*	Mean ± SD	Median	25th and 75th Percentiles	*n*	Mean ± SD	Median	25th and 75th Percentiles
Carmen welders[a]	14	285 ± 170	251	(137, 445)	14	210 ± 178	196	(75, 319)
Carmen nonwelders	21	194 ± 115	201	(102, 264)	20	104 ± 102	89	(44, 189)
Shop worker welders[a]	7	173 ± 71	163	(138, 251)	7	102 ± 100	147	(57, 161)
Shop worker nonwelders	149	152 ± 100	129	(86, 200)	149	129 ± 94	112	(71, 164)

[a] "Welding" includes cutting, burning, and welding of metal parts.

matography achieved better separation, it was too time-consuming to analyze the large number of samples involved. Finally, a spectrofluorimeter was used to increase specificity and sensitivity for phenanthrene relative to the standard ultraviolet spectrophotometric method. For standard solutions, the limit of detection was approximately 5 ng/mL. Field blank filters had about 5 ng of phenanthrene. Field blank polyurethane foam plugs had much more phenanthrene, and the values were quite variable (53 ± 30 ng).

All of the phenanthrene concentrations from field samples were less than 1 $\mu g/m^3$, and near the limit of detection (limit of detection = mean + 2 SD of the blank samples). Most of the samples from clerks and carmen were at or below the limit of detection, while most of the samples from shop workers were above the limit of detection. To use phenanthrene as a marker for diesel exhaust, the ratio of phenanthrene to respirable particle concentration had to be established. The area samples from the repair shops, which are virtually all diesel exhaust, showed a surprisingly constant ratio of phenanthrene to respirable particles of 0.006. This would be expected if the samples came from the same source and the source had an approximately constant output of phenanthrene and respirable particles. If this is true, and if repair workers have exposures to other sources of airborne particles, such as through grinding, sanding, and mechanical activities, then the ratios of phenanthrene to respirable particle concentration in the diesel exhaust-exposed workers' personal samples should be equal to or less than 0.006 or 0.0076 (mean + 2 SD). Calculation of the ratio of phenanthrene to respirable particle concentration for each personal sample on which both measurements were made (n = 62) showed this to be true. The mean ratio for these personal samples was 0.003, suggesting that, on average, approximately 50 percent of the adjusted respirable particles in nonsmokers' samples was not diesel exhaust. (A similar ratio does not result from the cumulative data, which give average values of respirable particle concentrations [Tables 2 through 4] and phenanthrene [see Figure 8] for job categories, and not the observed ratios for individual samples.) We also examined the ratio of adjusted respirable particles to phenanthrene (see Figure 9), because respirable particles may include environmental tobacco smoke particles that do not contribute significantly to phenanthrene levels. Because environmental tobacco smoke exposures of nonsmoking shop workers were low (Table 4), the ratio of phenanthrene to adjusted respirable particles (0.003) in personal versus area samples was similar to the ratio of phenanthrene to respirable particles. These data should be interpreted cautiously because there were few area samples analyzed. However, the data suggest that phenanthrene should be pursued as a marker for diesel exhaust.

Nicotine as a Marker for Environmental Tobacco Smoke

Optimal conditions for nicotine recovery from sample filters were developed in several stages. The addition of sodium hydroxide deprotonates and neutralizes the nicotine so that it can be concentrated by liquid/liquid extraction into heptane. The liquid/liquid extraction yielded good recoveries when 2 mL of 10 N sodium hydroxide was added to the solution, but less base led to inefficient recovery of nicotine. Filters were spiked with varying amounts of nicotine. Without the addition of ethanol during the initial extraction, the extraction efficiency was approximately 75 percent, but it was highly variable. With the addition of ethanol to help wet the filter, extraction of nicotine was complete, averaging 98 percent for 5 µg of nicotine.

Nicotine is stabilized during collection by reaction with

Table 4. Workplace Exposures by Job Location and Cigarette Smoking Status

	Respirable Particle Concentration ($\mu g/m^3$)			Environmental Tobacco Smoke Concentration ($\mu g/m^3$)			Adjusted Respirable Particle Concentration ($\mu g/m^3$)		
Job Group	*n*	Median	25th and 75th Percentiles	*n*	Median	25th and 75th Percentiles	*n*	Median	25th and 75th Percentiles
Inside workers[a]									
Smokers	17	133	(103, 169)	11	53	(13, 63)	11	66	(38, 111)
Nonsmokers	40	104	(45, 164)	33	59	(18, 106)	33	44	(23, 96)
Outside workers									
Brakers									
Smokers	14	190	(93, 312)	12	124	(35, 158)	11	73	(60, 292)
Nonsmokers	17	83	(65, 90)	18	0	(0, 2)	16	74	(64, 85)
Carmen									
Smokers	24	247	(124, 348)	24	96	(39, 160)	24	138	(53, 259)
Nonsmokers	9	143	(56, 203)	8	1	(0, 3)	8	116	(51, 204)
Shop workers[b]									
Smokers	42	233	(145, 305)	42	94	(3, 131)	42	136	(59, 213)
Nonsmokers	108	113	(72, 161)	110	1	(0, 5)	108	110	(71, 155)

[a] Clerks.
[b] Work in large, indoor repair facility.

the bisulfate ion. To check for possible breakthrough of nicotine from treated filters during sampling, 13 samplers placed in the cigarette exposure chamber contained backup treated filters. All of these filters contained less than 1 percent of the nicotine found in the first treated filter. Thus, the method of treated filters was found to collect nicotine vapor efficiently and desorb the nicotine quantitatively for gas chromatographic analysis. The limit of detection by gas chromatography with a nitrogen-selective detector was 0.06 μg of nicotine per treated filter. Recovery studies were performed with as little as 0.2 μg of nicotine added. The overall limit of detection of nicotine in air would depend on the flow rates and sampling times; in these studies, field samples collected at 1.7 Lpm for eight hours would have a limit of detection of nicotine of 0.2 $\mu g/m^3$; a one-hour sample would have a limit of detection of 2 $\mu g/m^3$.

Reduction of nicotine content in mainstream smoke is achieved by filtering the smoke, by increasing ventilation, and by other changes in the design of the cigarette (Schlotzhauer and Chortyk 1983). Sidestream smoke, which is emitted from the tip of the cigarette directly into the air, is the major component of environmental tobacco smoke. Little variability in the nicotine levels of sidestream smoke is found among different brands of cigarettes, regardless of their degree of mainstream smoke ventilation (Rickert et al. 1984). Several different brands of cigarettes, with varying tar and nicotine contents in mainstream smoke (Federal Trade Commission Report 1985), were tested under similar conditions in the environmental chamber. The ratio of nicotine to particulate matter in environmental tobacco smoke did not vary significantly among the brands. Thus, nicotine would appear to be an appropriate marker for environmental cigarette smoke.

Most of the environmental chamber experiments were conducted at relatively high particle concentrations, of around 1,000 $\mu g/m^3$. Because the environmental tobacco smoke exposures of the railroad workers were at much lower levels, only those chamber experiments in which particulate concentration was less than 1,200 $\mu g/m^3$ (11 such experiments) were used to calculate the relationship of nicotine and particles from environmental tobacco smoke. A principal components analysis yielded the following relation:

$$\text{Respirable Particles} = 8.6 \times [\text{NICOTINE}] + 50.5.$$

The value of this intercept reflects the statistical uncertainty in extrapolating the chamber data to an airborne nicotine level of zero. By jackknife resubstitution, the estimated intercept had a SE = 125 for a coefficient of variation of 250 percent. Thus, the precision of the intercept estimate is poor compared to that of the slope (SE = 4.4, coefficient of variation = 50 percent).

The environmental tobacco smoke contribution to particulate matter was calculated by multiplying the ambient

Table 5. Comparison of Microsuspension and Forward Mutation Assays of Mutagenic Activity of Diesel Exhaust from Railroad Filters

Sample No.	Diesel Railroad Study No.[a]	Total (T) or Respirable (R)[b]	Mutagenic Activity from Microsuspension Assay (revertants/100 µg of extract) TA98 + S9	TA98 − S9	TA100 + S9	TA100 − S9	Activity Previously Reported for *Salmonella* Forward Mutation Assay[c]
D0681	III	T	1,280	717	686	199	Positive
D0682	III	R	1,290	400	820	198	Positive
D0689A	III	R	448	54	348	103	Positive (+ S9) Negative (− S9)
D0690A	III	T	159	108	195	130	Negative
D1080D	IV	T	1,388	1,012	774	453	Positive
D1085A	IV	R	384	196	336	179	Negative
D0134C	II	T	110	78	279	141	Negative
D0691	III	Blank filter	32	19			
DMSO		Solvent Blank	26 ± 5	16 ± 5	130 ± 9	106 ± 13	

[a] Refers to previous study of railroad diesel exhaust (Woskie et al. 1988a).

[b] Total = total particulate matter collected in a filter with no size-selective device. Respirable = particles collected on a filter with a cyclone preselection device with 50 percent collection efficiency at 3.5 µm aerodynamic diameter.

[c] Positive = 3 × SD of the assay performed at Massachusetts Institute of Technology (Skopek et al. 1978).

nicotine concentration by 8.6. This value was then subtracted from the respirable particle concentration to yield an adjusted respirable particle concentration. The nicotine concentration can be used as a marker of exposure to environmental tobacco smoke and the adjusted respirable particles can be used as a marker of diesel exhaust exposure, although other particles (sand, gravel, welding fumes, air pollution) may also contribute to the adjusted respirable particles. To minimize bias contributed from samples with a systematic excess of nondiesel particles, brakers and carmen welders were excluded from some analyses (see Statistical Methods section).

SPECIFIC AIM 2

Microsuspension Assay Sensitivity for Diesel Extracts

As a validation of the sensitivity of the microsuspension method for diesel extracts, dichloromethane extracts of high-volume filter samples of particles collected at a variety of railroad facilities were tested for mutagenic activity, using the micro procedure. These same extracts were previously tested at the Massachusetts Institute of Technology by William Thilly's group, using the *Salmonella* forward mutation assay (Skopek et al. 1978) (Table 5). A significant (positive) response in the forward mutation assay was defined as an increased mutation frequency, exceeding the historical 99 percent upper confidence limit of the untreated control cultures. A positive response in the microsuspension method was defined as a doubling of the background number of revertants. The samples that tested positive in the forward mutation assay, both with and without S9, were also positive in the microsuspension assay in both tester strains TA98 and TA100, with and without S9. However, all of the samples that tested negative in the forward mutation assay were determined to be positive in strain TA98, with and without S9, using the microsuspension assay. Strain TA100 was less sensitive in detecting mutagenic activity, especially without the addition of S9 (Table 5).

Dose-Response Relationships of Diesel Extracts

Dose-response relationships were determined for some of the particle extracts from two railroad facilities (Figure 3). Tester strain TA98, with and without S9, was chosen for the dose-response determinations on the basis of the strain sensitivity found in the screening procedure, and on historical sensitivity to the mutagens in diesel particulate matter. Another tester strain, TA98 NR, was used without S9 because the TA98 NR strain is insensitive to certain nitro-PAHs (Rosenkranz and Mermelstein 1983). We wanted to examine if nitro-PAHs or related compounds were present in the diesel railroad extracts.

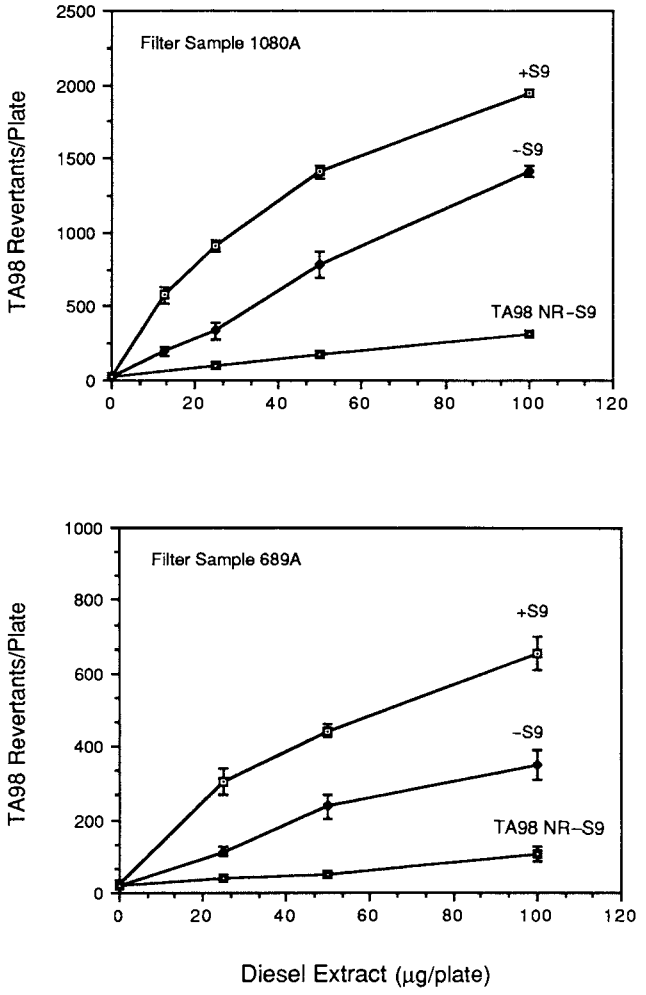


Figure 3. Mutagenic activity using the microsuspension procedure for total particulate matter collected at two railroad facilities. Each data point represents the mean (± SD) of triplicate plates.

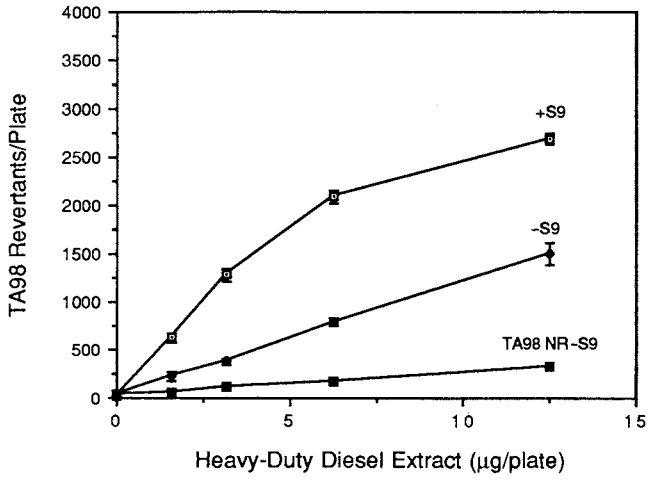


Figure 4. Mutagenic activity of heavy-duty diesel engine exhaust extracts (National Bureau of Standards Reference No. 1650). Each data point represents the mean (± SD) of triplicate plates.

Extract from a single railroad (Filter Sample 1080A in Figure 3) was derived from the respirable sized particles and was mutagenic with and without S9 liver homogenate. The activity greatly decreased with strain TA98 NR. The other railroad sample (Filter Sample 689A in Figure 3) was from the total suspended particles collected by a high-volume sampler, and was also mutagenic with and without S9. Again, as in the case of the first sample, the mutagenic activity in strain TA98 NR was significantly less than that in strain TA98. The insensitivity of tester strain TA98 NR to the railroad diesel extracts indicates that the compounds in the diesel mixtures are nitro-substituted compounds such as 1-nitropyrene, which is highly mutagenic and has been identified with several other nitro-substituted compounds in automobile and diesel exhaust (National Research Council 1981; Pitts et al. 1982; Schuetzle et al. 1982; Xu et al. 1982; Nakagawa et al. 1983). The chemical composition of the complex mixture of diesel extract tested with strains TA98 and TA98 NR can be inferred by the response of the strains to known compounds such as 2-NF and 1-nitropyrene. Generally, other nitro-substituted PAHs (such as the dinitropyrenes) respond differently in that the responses of the two strains do not decrease in potency (Rosenkranz et al. 1981).

Dose-response curves were determined for the extracts (from D. Schuetzle, Ford Motor Co., Dearborn, MI) from the heavy-duty diesel engines using bacterial tester strains TA98 and TA98 NR (Figure 4). The dose-response curves follow the pattern of response reported for other diesel extracts (Claxton 1981; National Research Council 1981) in which mutagenic activity, with and without S9, is similar and the activity with strain TA98 NR is substantially decreased. The dose-response curves for the extracts from railroad-diesel-particulate matter are similar to those for heavy-duty diesel engines. Direct comparisons of mutagenic activity between railroad diesel and heavy-duty diesel engine particulate matter (per milligram of particulate matter collected) are difficult, because the conditions of collection for the particles are quite different. The particles from heavy-duty diesel engines (the extracts from Ford Motor Co. were tested) were direct emissions collected from a dilution tunnel, whereas the particles collected in the work areas of diesel railroad employees were airborne samples, which are considerably more dilute.

Mutagenicity of Respirable Particles from Personal Samplers

Mutagenic activity was determined from extracts of respirable particles on a few personal filters collected in identical two-day sampling periods (Figure 5). In this pilot study of 12 filters (6 from clerks and 6 from shop workers), the range of activity (TA98 + S9) was approximately 250 to 1,500 revertants/m^3 of air sampled. The range of activity without the addition of S9 was approximately 750 to 2,000 revertants/m^3 of air. The mutagenic activity in these filter extracts was higher without metabolic activation (−S9) than with metabolic activation (+S9). This is not the normal pattern seen in pilot studies of sidestream and mainstream cigarette smoke, using the microsuspension assay (Ling et al. 1987). Our pilot study is an early effort toward examining mutagenic activity in collected personal airborne samples. The elevated mutagenic activity without S9 over that with S9 is not unprecedented. There are reports, for example, in which extracts of particulate matter collected from ambient air have greater activity without S9 than with S9 (Louis et al. 1987).

The volume of air sampled per filter was approximately 0.8 m^3 for all filters tested here. The approximate mutagenic activity per filter can be calculated by multiplying the revertants per cubic meter by 0.8 m^3. Since the data set was small, no relationship with airborne nicotine, urine nicotine and cotinine, or urine mutagenicity could be established. Mutagenic activities of two filters acquired from a single clerk were compared to the activities of six filters from three diesel shop workers (Figure 5). The filters were from the same two-day sampling period. The mutagenic activity of the filters from the clerk was higher than for the filters from shop workers, but no generalizations can be made because of the small number of samples.

Although an association between the airborne and the urine mutagenic activity could not be evaluated with the small number of personal filter samples (n = 8) measured here, this pilot study of the particulate matter collected on filters attached to low-volume sampling pumps indicates that measurements of mutagenic activity at the "personal sampling" level are possible. Further studies with larger numbers of samples will be necessary to characterize and evaluate this methodology in epidemiological studies.

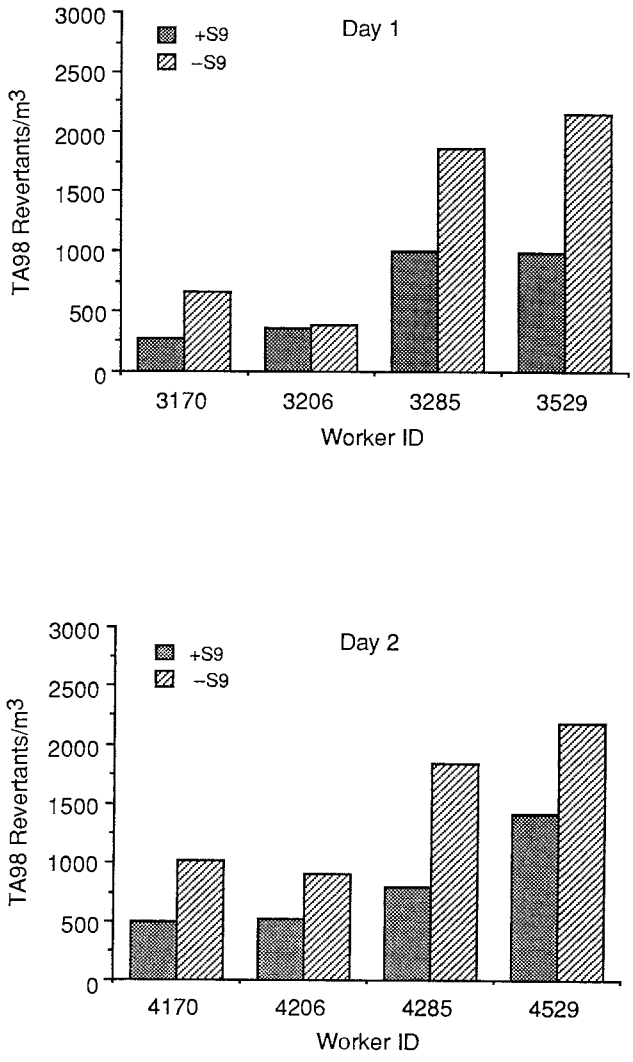


Figure 5. Mutagenic activity from extracts of respirable particles on personal filters from both shop workers and clerks. Personal samples were collected on identical sampling day 1 or day 2.

SPECIFIC AIM 3

Study Population

Eighty-seven male subjects were studied during the project. The mean age of subjects was 47.2 ± 9.7 years, and they had worked for 16.1 ± 10.8 years on the railroad. Because of the limited size of the population within this railroad, and the desire to oversample the highest diesel exhaust-exposed workers, it was decided early in the project to do repeat sampling on the same study subjects. The most resampling was done among shop workers, who had the highest diesel exhaust exposure.

A total of 151 two-day protocols were completed by the 87 subjects. Another four subjects did not finish the second day's collection, but provided usable data for a single day. The 306 samples [(2 × 151) + 4)] represent the total data base (Tables 1 and 6). The job categories, mean ages, smoking prevalence, and total number of samples for the study are given in Table 7.

Some samples were missing or excluded from each source in this large, multidisciplinary field investigation

Table 6. Number of Samples Analyzed by Smoking Group (Before Exclusions)

	Nonusers of Tobacco	Only Cigarette Smokers	Cigarette and Other Tobacco Users	Only Other Tobacco Users	Missing (%)	Total
Urine						
Mutagenicity + S9	178	92	9	9	6	288
Mutagenicity - S9	170	89	9	8	10	276
Thiocyanate	188	93	10	11	1	302
Nicotine	167	84	8	10	12	269
Cotinine	168	84	8	10	12	270
Personal Sampling Pump						
Respirable particles	181	92	10	12	4	295
Nicotine	175	85	10	10	8	280
Total Number Possible	189	95	10	12		306

(Table 6). The reasons for this were different for the specific variables. The number of missing samples ranged from 1 percent for urinary thiocyanate to 12 percent for urinary nicotine and cotinine. Missing samples were high for these latter measurements because they were done after all other urine assays, and there was often an insufficient quantity of urine remaining. Mutagenicity was not measured with S9 in 6 percent of samples and without S9 in 10 percent of samples; in most cases this was because the quantity of urine was inadequate. Ambient nicotine was not measured in 12 percent of the samples, primarily from the first field visit, when the method had not yet been developed. Because data were missing for different subjects on different variables, analyzing only the data set with complete information would have seriously reduced the study size and power. Therefore, we elected to use the data subsets providing the maximum number of samples for separate analyses.

Cigarette Smoking

Thirty-seven percent of the subjects (n = 32) were cigarette smokers. Three nonsmokers and two smokers used other forms of tobacco. This yielded 97 urine samples for cigarette smokers (32 percent), 10 samples for cigarette smokers who used other tobacco products (3 percent), 189 samples for nonusers of tobacco (62 percent), and 12 samples for nonsmokers who used other tobacco products (4 percent). Smoking status was unknown on the study day for four subjects. Results for cigarette smokers who used other tobacco products were similar to those for cigarette smokers, and they were analyzed together. Results for urine analyses of nonsmokers who used other tobacco products were excluded or considered separately.

Table 7. Subject and Sample Numbers by Job Category

Job Category	Subjects	Samples	Age (Mean ± SD)	Nonsmokers
Brakers	17	37	45.3 ± 9.7	11
Carmen	18	35	45.3 ± 9.9	5
Clerks	21	60	51.0 ± 7.6	15
Engineers	8	16	55.7 ± 5.2	4
Shop workers	23	158	43.2 ± 10.2	16
Total	87	306	47.1 ± 9.7	51

The highest rate of cigarette smoking was found among the carmen studied (68 percent of samples). Lower rates of smoking were present for the other job categories (27 to 40 percent of samples). Among smokers, the average number of cigarettes smoked during the study shift was 11.8 ± 6.8. The mean number of cigarettes smoked for the entire day prior to urine sample collection was 14.4 ± 8.1. For most analyses, the number of cigarettes smoked during the day prior to urine collection was used, because it reflected better the markers present in the urine at the end of the shift. The mean tar per cigarette was 13.1 ± 5.2 mg (n = 98) (Federal Trade Commission Report 1985), range 1.2 to 23.3. However, only two samples were from subjects who smoked cigarettes with "ultra-low" levels of tar per cigarette (less than 5 mg). No association was seen between the number of cigarettes smoked on the study shift and self-reported depth of smoke inhalation, but tar per cigarette was positively correlated with reported depth of smoke inhalation.

Respirable Particle Exposure

A total of 303 of the possible 306 personal exposure samples were collected. However, 8 were missing particulate

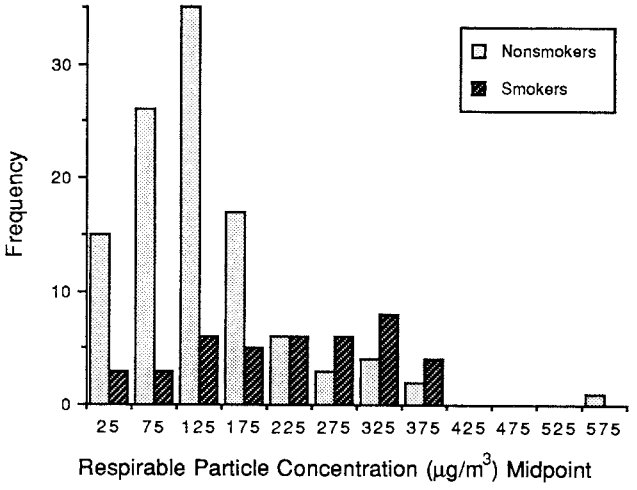


Figure 6. Frequency distribution of respirable particle concentrations among locomotive shop workers, by smoking status.

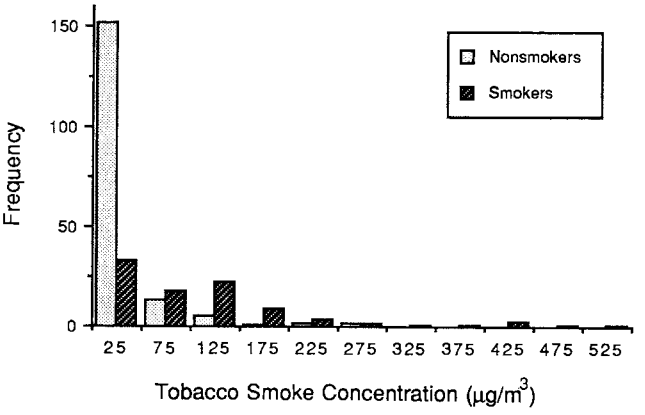


Figure 7. Frequency distribution of environmental tobacco smoke exposures for all subjects, by smoking status. Tobacco smoke concentration is calculated as nicotine × 8.6.

mass values as a result of filter damage, and 23 were missing nicotine values. The final data set was 295 measurements of personal exposure to respirable particles and 280 measurements of nicotine (Table 6). Summary statistics for the concentrations of respirable particle mass, environmental tobacco smoke, and adjusted respirable particle mass (respirable particle concentration minus environmental tobacco smoke) are shown in Table 2. The medians and quartiles reveal the skewness commonly observed in the distributions of airborne exposures. This skewness can be seen in the histograms of respirable particle concentrations of the locomotive shop workers (Figure 6), and in the ambient environmental tobacco smoke concentration for all workers (Figure 7). All of the job groups showed skewed distribution in ambient nicotine concentrations (environmental tobacco smoke), which was caused in part by the relatively large number of samples from nonsmokers that contained little or no nicotine. The respirable particle concentrations did not show a consistent or clear pattern of skewed distributions, which may be due to the relatively small number of samples in most job groups.

The carmen had the highest exposures to respirable particles, even though they have little or no diesel exhaust exposure. This resulted, in part, because some of them were exposed to particulate matter generated by welding or by using an acetylene torch for cutting and burning the chassis of train cars during repairs; this type of activity occupied one-half hour to four hours per day. A large difference was seen in the average exposures of the welders and nonwelders among the carmen. This difference was much smaller among locomotive shop workers, who generally weld for short periods of 1 to 15 minutes (Table 3). Also, cutting generates substantially more metal fume than does welding. If the welders (including those doing torch work) are removed from the carmen group, the respirable particle concentrations for the carmen are within the range of the other groups, although still high (Table 2).

Despite the wide range in diesel exhaust exposure between the shop workers and the clerks, the respirable particle concentrations of the job groups (Table 2) were similar, which is consistent with our previous findings from U.S. railroads (Woskie et al. 1988a). We attribute this similarity of respirable particle concentrations between job groups largely to the respirable particle mass contributed by environmental tobacco smoke (Table 4). The highest mean concentrations of environmental tobacco smoke were found among the carmen, followed by the engineers and the clerks. The carmen had the highest percentage of smokers.

If the contribution of environmental tobacco smoke to each respirable particle value is subtracted to give an adjusted respirable particle value, the relative diesel exhaust exposures are more apparent. The relative ranking of the jobs by concentration of adjusted respirable particles (Table 2), excluding welding carmen, was close to that expected by the relative contact these groups have with operating diesel locomotives, assuming that all workers are exposed to a background air concentration of about 30 $\mu g/m^3$. However, this cannot explain all of the difference seen between the job groups.

Another important factor in exposure is the degree of enclosure of the work area. We assigned job groups to three categories based on enclosure characteristics: work indoors in relatively small enclosed spaces; work indoors in large open areas; and work outdoors. Clerks and engineers both work in small indoor areas, carmen and brakers tend to work outdoors, and the shop workers work in large garages that house locomotives during repair.

The environmental tobacco smoke exposures of the indoor, small-space nonsmokers were higher than the exposures of either the outside or shop nonsmokers, demonstrating that environmental tobacco smoke can build up in small enclosed spaces and significantly contribute to a nonsmoker's particle exposure (Table 4). The concentrations for environmental tobacco smoke for nonsmokers working inside were equivalent to environmental tobacco smoke concentrations for smokers in these same small enclosed work areas. This may be due, in part, to the smoking characteristics of indoor workers; for example, burning cigarettes are left in ashtrays for long periods of time. Among workers in large spaces (outdoors or in repair shops), a distinct difference was present for environmental tobacco smoke exposures in smokers and nonsmokers. As expected, the respirable particle exposures of the smokers were higher than those of the nonsmokers. Adjustment of respirable particle values by correcting for environmental tobacco smoke reduced these differences considerably. For inside workers, the adjusted respirable particle values were similar for smokers and nonsmokers. Outside and shop smokers had approximately double the respirable particle exposures of their nonsmoking colleagues, but the adjusted respirable particle exposures were also similar for smokers and nonsmokers.

Phenanthrene Exposure

Three types of samples were collected to determine the phenanthrene exposure in the railroad jobs. First, several hundred personal samples were collected in the workers' breathing zone; second, the same sampling devices were used to collect area samples in the repair shop; finally, a few high-volume samples (430 L/minute) that used three large-diameter polyurethane foam plugs were collected in the repair shop. As in the laboratory tests, in which 68 percent of the phenanthrene spiked onto filters was found on the polyurethane foam plugs after sampling, the analyses of the first 20 or so field samples showed that virtually all (more than 90 percent) of the phenanthrene was on the polyurethane foam plugs rather than the filters, so for all remaining field samples only the polyurethane foam plugs were analyzed. Polyurethane foam plugs from 105 personal samples, one set of high-volume samples, and three sets of area samples from the repair shop were analyzed. The personal samples were chosen from three major job groups: clerks, carmen, and shop workers. Seventeen field blank polyurethane foam plug samples (16 personal samples and one high-volume sample) also were analyzed. The high-volume sampler collected substantially more total mass of phenanthrene than any of the personal samplers, and so provided a more precise estimate of concentration; the

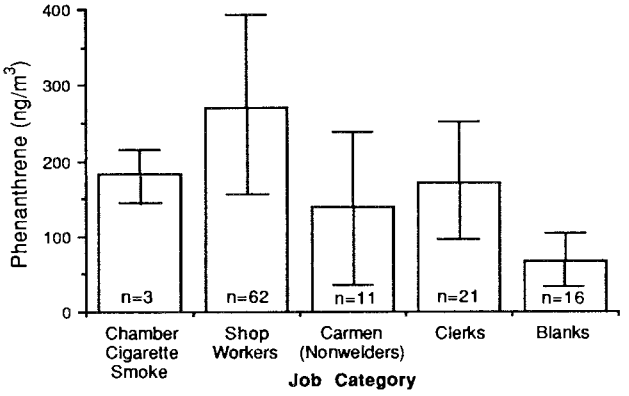


Figure 8. Distribution of phenanthrene concentrations by job group. Phenanthrene values are given in means ± 2 SD. For chamber cigarette smoke, respirable particle concentration = 1,200 μg/m^3 or approximately 10 times the respirable particle concentrations of job categories (see text and Table 4). Blanks are expressed as air concentrations in the same volume as the personal samples.

phenanthrene concentration was 420 ng/m^3, and the respirable particle concentration was 75 μg/m^3. Two area samples, collected with the personal sampling train on a day other than the high-volume collection day in the same repair shed, averaged 230 ng/m^3 phenanthrene and 40 μg/m^3 respirable particles, while a personal sampler on the third day of the same week collected 176 ng/m^3 phenanthrene and 34 μg/m^3 respirable particles. These limited samples had very similar ratios of phenanthrene to respirable particles (with mean = 0.006 and SD = 0.0008), and represent, predominantly, the phenanthrene content of aged diesel exhaust.

Of the 104 personal samples analyzed for phenanthrene, 94 were taken in the three major job groups: clerks, carmen (nonwelders), and repair shop workers. Figure 8 shows the range of phenanthrene concentration (mean ± SD) for each of these groups and for the blank polyurethane foam plugs (expressed as if they were samples of the same amount of air, 0.8 m^3, as the personal filter samples). The limit of detection was defined as the value for the blank mean + 2 SD: 66 + 2(38) = 142 ng/m^3. The exposures of nearly all the shop workers were well above the limit of detection (Figure 8); most of the carmen were exposed to less than detectable amounts of phenanthrene; and most of the clerks had exposures that were just above the limit of detection. Although exposure chamber cigarette smoke samples contained phenanthrene above the limit of detection, the respirable particle concentrations in the chamber were approximately 1,200 μg/m^3, which was about 10 times the level found in the offices; therefore, the phenanthrene level in 120 μg/m^3 environmental tobacco smoke particles would be only 11 ng/m^3, and would not be a significant source of phenanthrene. Only a weak relationship was found be-

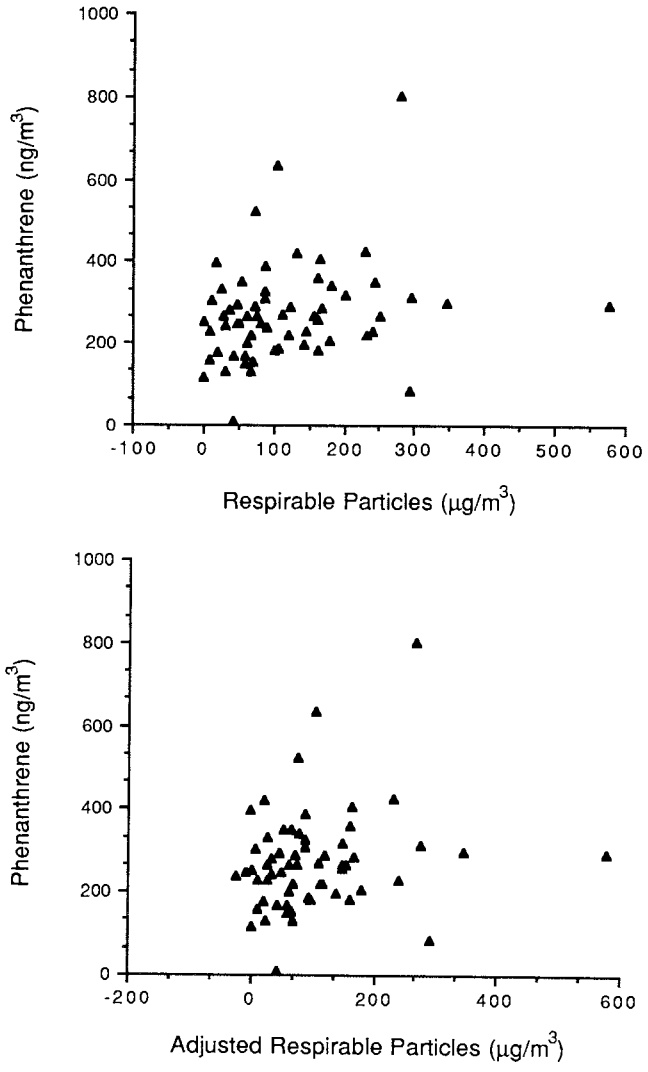


Figure 9. Phenanthrene versus respirable particle concentration and adjusted respirable particle concentration for shop workers only.

tween phenanthrene concentration and either respirable particle or adjusted respirable particle concentrations (Figure 9). The ratio of phenanthrene to adjusted respirable particles in the personal samples of nonsmoking shop workers averaged 0.003. Only three samples had ratios larger than the area sample mean + 2 SD, and all three had unreasonably low respirable particle values. The distributions of phenanthrene concentrations were approximately log-normal, and the data gave nearly linear plots on log-probability paper.

SPECIFIC AIM 4

Markers of Active Cigarette Smoking

Among cigarette smokers, the number of cigarettes smoked during the work shift studied showed a strong dose-response relationship with urine mutagenicity with S9 (Spearman $r = 0.38$, $p < 0.001$), and a weak but significant association with urine mutagenicity without S9 ($r = 0.15$, $p < 0.05$) (Table 8; Figure 10). A strong relationship was also seen between the number of cigarettes smoked on the study day and urinary nicotine and cotinine (Table 8; Figure 11). This association was stronger for urinary nicotine ($r = 0.58$, $p < 0.001$) than for urinary cotinine ($r = 0.38$, $p < 0.01$), reflecting the shorter biologic half-life of urinary nicotine. Urinary thiocyanate discriminated between smokers and nonsmokers; among smokers, however, there was no association between number of cigarettes smoked on the study day and urinary thiocyanate ($r = 0.08$, $p > 0.05$). Thiocyanate did show a high correlation with cotinine ($r = 0.46$, $p < 0.001$) but none with nicotine ($r = -0.06$, $p > 0.05$), perhaps reflecting that the half-lives of thiocyanate and cotinine are longer than the half-life of nicotine. Because of the lower correlation of thiocyanate and smoking dose or urinary mutagenicity, thiocyanate was not used in the sub-

Table 8. Spearman Rank Order Correlation Coefficients for Samples from Cigarette Smokers

	Mutagenicity		Cigarettes Smoked	Urinary Thiocyanate	Urinary Cotinine	Urinary Nicotine
	+ S9	- S9				
Urinary mutagenicity						
+ S9	–	0.52[a]	0.38[a]	0.20[b]	0.51[a]	0.54[a]
- S9	–	–	0.15[b]	0.15	0.07	0.18
Cigarettes smoked on day of sample	–	–	–	0.08	0.38[a]	0.58[a]
Urinary thiocyanate	–	–	–	–	0.46[a]	- 0.06
Urinary cotinine	–	–	–	–	–	0.47[a]

[a] $p \leq 0.001$.

[b] $0.01 < p \leq 0.05$.

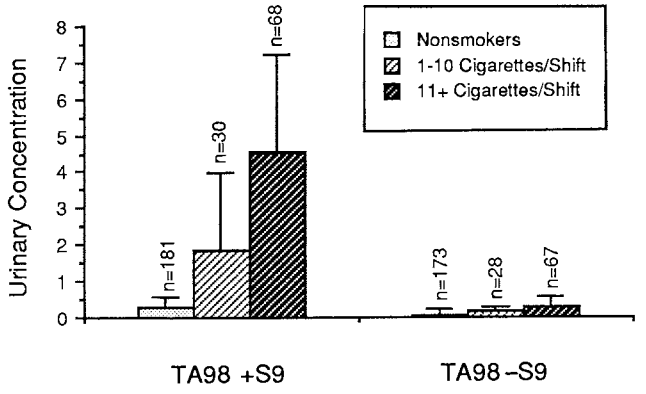


Figure 10. Urine mutagenicity by number of cigarettes smoked on study shift. Urine mutagenicity is expressed as median and quartile mutagenicity (revertants/μmol of creatinine). The data are based on number of cigarettes smoked on the study shift and exclude smokers who did not smoke during the study shift.

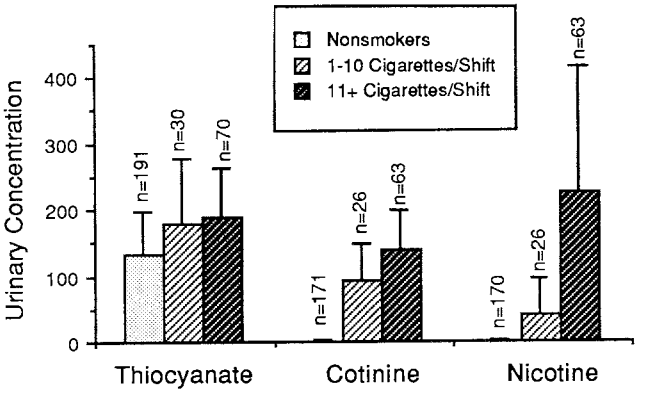


Figure 11. Urinary markers of cigarette smoking by number of cigarettes smoked on study shift. Urine mutagenicity is expressed as median and quartile concentration in urine calculated as follows: thiocyanate = (mg/μmol of creatinine) × 10; cotinine, nicotine = μg/μmol of creatinine. Data are based on number of cigarettes smoked on the study shift and exclude smokers who did not smoke on the study shift.

sequent multivariate models that controlled for the effect of cigarette smoking in testing for an association of diesel exposure with urinary mutagenicity.

All of the smoking variables (cigarettes smoked on the study day, tar per cigarette, nicotine per cigarette, and smoke inhalation) were next tested as independent determinants of urinary mutagenicity. When urinary markers were excluded from the prediction equations, the number of cigarettes smoked and smoke inhalation were significant independent predictors of mutagenicity with S9. A weak association was also present for these two variables and mutagenicity without S9. Tar and nicotine per cigarette (Federal Trade Commission Report 1985) were not significant independent predictors in these models.

When urinary cotinine (or nicotine) was added to the regression model, the other smoking markers no longer achieved the same level of statistical significance. The highest predictive value of the urine markers was generally achieved with cotinine, which was used, therefore, to adjust for smoking when testing for a diesel effect among smokers. However, the number of cigarettes smoked on the study day remained a significant independent predictor for urinary mutagenicity (+ S9) in addition to urinary cotinine. To control maximally for cigarette smoking in models of mutagenicity among smokers, both cotinine and the number of cigarettes smoked on the study day were entered in the models before testing for an effect of diesel exhaust.

Markers of Passive Cigarette Smoking

Nicotine concentration on the personal sampling filters was used as an indicator of passive smoking to evaluate the various urinary markers. Among nonsmokers, a weak dose-response relationship was seen for nicotine on the filter and for urinary nicotine ($r = 0.15$, $p = 0.06$) (Table 9; Figure 12). Urinary cotinine and thiocyanate showed increases for the highest environmental tobacco smoke exposure, but dose-response associations were not present. Among indoor workers, the group with the highest exposure to environmental tobacco smoke, a stronger association was seen between ambient and urinary nicotine, but the association did not achieve the same level of statistical significance because of the smaller sample size ($r = 0.26$, $p = 0.13$; data not shown). This association is consistent with the shorter biologic half-life of nicotine, and appears to correspond to the number of cigarettes smoked by coworkers during the work day up until the urine sample collection. Urine cotinine, with its longer half-life, may reflect nonoccupational exposures prior to the study work day and, therefore, not show the same strength of association with ambient nicotine measured on the work shift.

In the multivariate modeling of urinary mutagenicity markers among nonsmokers, passive smoking was controlled for by entering both ambient nicotine and urinary cotinine. Ambient nicotine may be considered a marker of environmental tobacco smoke exposure during the study work shift, and urinary cotinine among nonsmokers reflects a longer-term average of environmental tobacco smoke dose, including nonworkplace and previous-work-shift exposures. Ambient nicotine also adjusts for the contribution of environmental tobacco smoke to measured respirable particle concentration in the regression models. Both variables were entered in the full regression models even when they did not achieve statistical significance.

Table 9. Spearman Rank Order Correlation Coefficients for Samples from Nonusers of Tobacco

	Urinary Mutagenicity		Urinary Thiocyanate	Urinary Cotinine	Urinary Nicotine	Ambient Nicotine
	+ S9	- S9				
Urinary mutagenicity						
+ S9	-	0.45[a]	- 0.06	0.13	0.09	0.08
- S9	-	-	- 0.12	0.00	- 0.06	- 0.03
Urinary thiocyanate	-	-	-	0.19[b]	0.30[a]	0.05
Urinary cotinine	-	-	-	-	0.66[a]	0.07
Urinary nicotine						0.15

[a] $p \leq 0.001$.

[b] $0.001 < p \leq 0.01$.

Other Non-Diesel Exhaust Predictors of Urinary Mutagenicity

Several other variables were considered potential determinants of urinary mutagenicity a priori and were tested as independent predictors. They consisted of clinical conditions (for example, cancer, psoriasis), dietary intake during the previous two days (for example, grilled meat), and nonoccupational exposures (for example, pesticides, welding, hair dyes, hobbies with chemical exposures). Only self-reported ingestion of "protective foods" (that is, cabbage, Brussels sprouts, or fish) in the two days prior to the study was associated with lower urinary mutagenicity with S9 among smokers and nonsmokers. The mean level of mutagenicity (± SE) for samples from nonsmokers who consumed protective foods in the two days prior to sample collection (n = 25) was 0.20 ± 0.12, and for samples from nonsmokers who did not consume the same foods (n = 153), it was 0.51 ± 0.07. Similarly, for samples from smokers who consumed protective foods (n = 17), mean mutagenicity was 3.58 ± 0.55, and for those who did not, it was 5.23 ± 0.46. In the multivariate models, this protective food factor remained a statistically significant determinant of lower urinary mutagenicity with S9 only among nonsmokers (Appendix D, Tables D.1 and D.2). The protective food variable was not predictive of mutagenicity without S9 in smokers or nonsmokers.

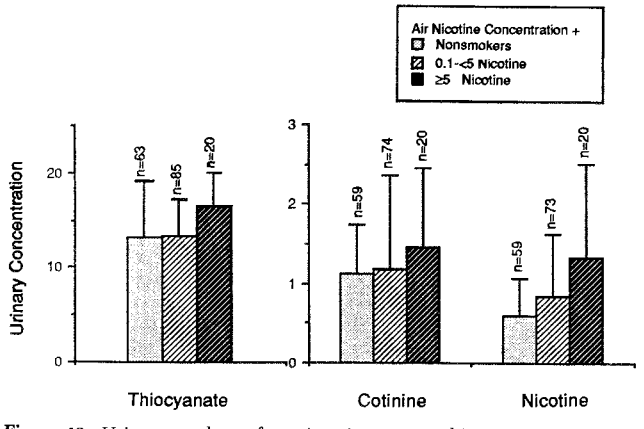


Figure 12. Urinary markers of passive cigarette smoking among nonsmokers, by strata of nicotine on personal sampling pump. Concentrations in urine are given as medians and upper quartiles. Thiocyanate values are mg/μmol of creatinine; cotinine and nicotine values are μg/μmol of creatinine.

Diesel Exhaust Exposure

Nonsmokers. After exclusions, 124 samples from nonusers of tobacco could be analyzed for mutagenicity with S9, and 121 samples could be analyzed for mutagenicity without S9. For these samples, diesel exhaust exposure was not a significant independent predictor of urinary mutagenicity either with or without S9 (Tables 10, D.2, and D.3). The regression models allowed for within- and between-person variances, and controlled for protective food, urinary cotinine (for environmental tobacco smoke), and response to standard mutagen control, 2-AF (to normalize for between-assay variability).

Stratification of the adjusted respirable particle variable showed no evidence of an effect on mutagenicity, with or without S9, among the nonusers of tobacco (Table 10). The median levels of adjusted respirable particles in the low, medium, and high strata of diesel exhaust exposure used in the models were 50.2, 110.0, and 181.4 $\mu g/m^3$, respectively (Table 11). The likelihood ratio chi-squared statistics (3 degrees of freedom) for differences among the exposure groups are 2.09 and 0.21 ($p > 0.05$) for mutagenicity with and without S9, respectively. The predicted means showed no evidence of dose-response association, and for every exposed group the estimated mean difference from the unexposed group was not more than half of its standard error.

For the exposure-interaction models, the conclusions are similar (Tables D.1 and D.2). In these models, the effect of

Table 10. Observed and Predicted Mutagenicity from Multivariate Regression Models by Strata of Adjusted Respirable Particle Concentrations[a]

	n	Observed Mean	Predicted Mean[b]	Difference from Unexposed (SE)
Nonsmokers[c]				
+ S9				
Diesel exhaust-unexposed	30	0.274	0.274	–
Diesel exhaust-exposed				
Low adjusted respirable particles	32	0.266	0.392	0.118 (0.121)
Medium adjusted respirable particles	40	0.369	0.395	0.121 (0.093)
High adjusted respirable particles	22	0.343	0.326	0.052 (0.125)
		Chi-squared for exposure (3 degrees of freedom) = 2.09.		
– S9				
Diesel exhaust-unexposed	29	0.109	0.109	–
Diesel exhaust-exposed				
Low adjusted respirable particles	30	0.057	0.119	0.010 (0.111)
Medium adjusted respirable particles	38	0.112	0.130	0.021 (0.090)
High adjusted respirable particles	22	0.105	0.092	– 0.017 (0.104)
		Chi-squared for exposure (3 degrees of freedom) = 0.21.		
Smokers[d]				
+ S9				
Diesel exhaust-unexposed	20	3.579	3.579	–
Diesel exhaust-exposed				
Low adjusted respirable particles	13	4.553	3.946	0.367 (0.917)
Medium adjusted respirable particles	11	3.908	3.755	0.176 (0.668)
High adjusted respirable particles	19	4.298	3.977	0.398 (0.644)
		Chi-squared for exposure (3 degrees of freedom) = 0.48.		
– S9				
Diesel exhaust-unexposed	16	0.248	0.248	–
Diesel exhaust-exposed				
Low adjusted respirable particles	13	0.282	0.251	0.003 (0.156)
Medium adjusted respirable particles	11	0.178	0.158	– 0.090 (0.118)
High adjusted respirable particles	19	0.300	0.238	– 0.010 (0.114)
		Chi-squared for exposure (3 degrees of freedom) = 1.10.		

[a] Mutagenicity is expressed in revertants/μmol of creatinine.

[b] Covariates for all models: 2-AF (standard mutagen revertants/ng), 2-AF × urinary cotinine, 2-AF × airborne nicotine, 2-AF × dummy variables comparing each exposed group to base line. Differences and standard errors were obtained by multiplying dummy-variable effects and standard errors by 3.46, the study mean for the mutagenicity of 2-AF.

[c] Nonsmoker models also included protective foods dummy variable.

[d] Models for smokers also included the number of cigarettes smoked on the study day.

exposure was measured by three variables: diesel exhaust exposure status (yes or no) and the interaction of diesel exhaust exposure status with respirable particle concentration and with respirable nicotine. For mutagenicity with and without S9, the chi-squared values for the diesel exhaust exposure term and the two interaction terms (3 degrees of freedom) are 1.87 and 0.82, respectively ($p > 0.05$).

The ratio of the between-person variance to the within-person (between-sample) variance is about 1:2.6 for mutagenicity, both with and without S9 (Tables D.1 and D.2). This indicates a definite lack of independence in observations taken from the same worker. The between-person variance components are both more than twice their standard errors (Tables D.1 and D.2). However, these standard errors are very sensitive to the assumption of normally distributed

Table 11. Strata of Adjusted Respirable Particles Used in Index Regression Models with Associated Concentrations of Respirable Particles and Environmental Tobacco Smoke[a]

Diesel Exhaust Exposure	*n*	Adjusted Respirable Particle Concentration ($\mu g/m^3$) Median	25th and 75th Percentiles	Respirable Particle Concentration ($\mu g/m^3$) Median	25th and 75th Percentiles	Environmental Tobacco Smoke ($\mu g/m^3$) Median	25th and 75th Percentiles
Unexposed							
Nonsmokers	39	51.4	(26.7, 99.3)	107.0	(66, 182)	48.2	(4.3, 80.0)
Smokers	23	78.8	(43.0, 141.6)	162.0	(111.0, 253.0)	54.2	(28.4, 117.8)
Low adjusted respirable particles							
Nonsmokers	34	50.2	(25.6, 65.9)	51.5	(26.8, 68.5)	0.9	(0.0, 3.4)
Smokers	15	10.0	(−26.1, 32.0)	147.0	(80.0, 267.0)	119.5	(78.3, 244.2)
Medium adjusted respirable particles							
Nonsmokers	52	110.0	(96.0, 129.0)	118.5	(103.3, 139.5)	3.4	(0.0, 6.7)
Smokers	13	115.8	(93.0, 136.0)	183.0	(139.0, 241.0)	81.7	(14.6, 110.5)
High adjusted respirable particles							
Nonsmokers	27	181.4	(166.0, 311.0)	200.0	(177.0, 311.0)	0.9	(0.0, 2.6)
Smokers	19	222.3	(190.9, 305.1)	305.0	(257.0, 339.0)	50.7	(2.6, 95.5)

[a] Environmental tobacco smoke = 8.6 × ambient nicotine (see text).

random terms (unlike the standard errors of the regression effects) and, therefore, can be taken only as a rough guide.

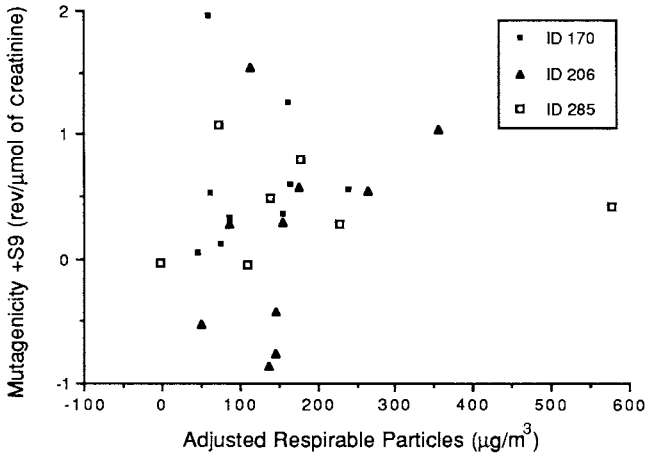
To control for individual differences in urinary mutagen excretion, a subset of workers who had been studied on different days and who had low environmental tobacco smoke and potentially high diesel exhaust exposure was selected for detailed analysis. Three repair shop workers were identified who did not smoke and who were not exposed to much environmental tobacco smoke, based on the ambient nicotine levels in their personal samplers. Each of these workers had been sampled on 8 to 10 days, and phenanthrene exposures of these workers were determined.

Simple plots for the repeated samples from these three workers of urinary mutagenicity with and without S9 (revertants/μmol of creatinine) versus the two markers of diesel exposure (adjusted respirable particles and phenanthrene values) show no suggestion of a positive association (Figures 13 and 14). This observation provides additional support for the multiple regression results.

Cigarette Smokers. After exclusions, 61 smokers' samples with S9 and 59 samples without S9 were part of the multivariate analysis. Two outliers were excluded from the +S9 models, and three were excluded from the −S9 models. Cigarette smoking was controlled for by urinary cotinine (corrected for creatinine) and by the number of cigarettes smoked on the study day up to the time of sample collection. Although these two variables are correlated ($r = 0.38$), they each contributed, at $p < 0.05$, to the regression equation for mutagenicity with S9 (Table D.3). They also differ in biological significance. Cotinine, with a long half-life, is weighted by cigarette smoking prior to the study day, while the number of cigarettes smoked on the study day reflects recent smoking exposure.

In the regression models using strata of adjusted respirable particles for exposure groups, no evidence was found of a dose-response relation between mutagenicity (with or without S9) and level of adjusted respirable particles (Table 10). No individual exposed group has a statistically significant increase in mutagenicity, either with or without S9, compared to the unexposed group (clerks, carmen). These are confirmed by the log-likelihood chi-squared statistics, which are 0.48 and 1.10 for +S9 and −S9 mutagenicity, respectively ($p > 0.05$).

Results for the exposure-interaction model for +S9 mutagenicity in smokers are shown in Table D.3. The overall test for the three exposure-interaction terms is not significant at the 0.05 level ($\chi^2 = 6.15$, $p < 0.25$). An interesting phenomenon was noted for the fit of the respirable particles and the main effects and interactions of ambient nicotine: The main-effect terms are almost *equal* in absolute magni-



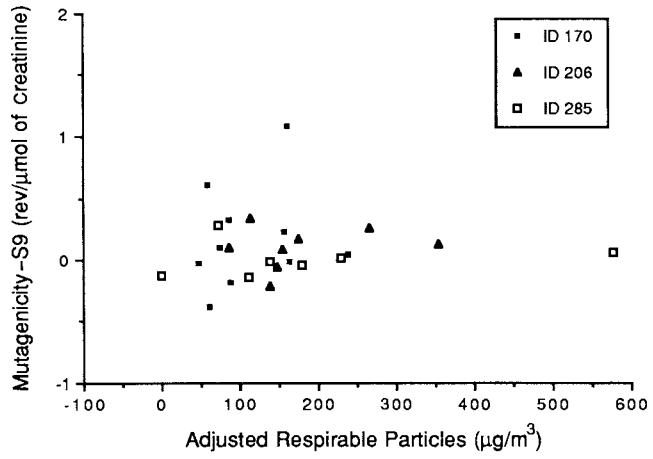
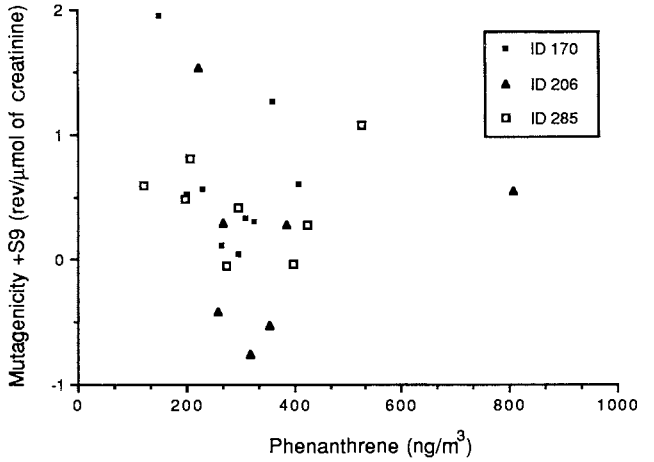


Figure 13. Urine mutagenicity + S9 (top) and - S9 (bottom) versus adjusted respirable particle concentration for three nonsmoking shop workers with repeated measurements.



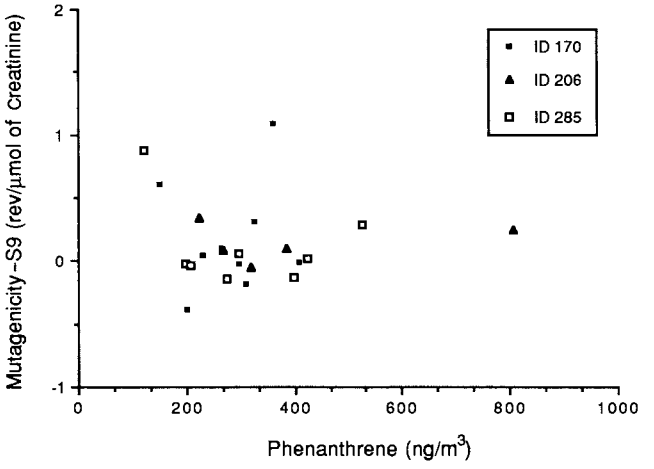


Figure 14. Urine mutagenicity + S9 (top) and - S9 (bottom) versus phenanthrene for three nonsmoking shop workers with repeated measurements.

tude to the corresponding exposure-interaction terms, but *opposite* in sign. Thus, the main-effect and interaction terms for respirable particles are - 2.656 and 2.971, respectively; the main-effect and interaction terms for ambient nicotine are 39.724 and - 38.788, respectively.

In unexposed men, the joint effect of respirable particles and ambient nicotine is (- 2.66 × Respirable Particles) + 39.72 Ambient Nicotine. That is, the predicted effect of increasing respirable particles is to decrease mutagenicity. This was borne out by analyses in unexposed men alone, which also showed a negative coefficient for respirable particles. This puzzling finding is probably due to the correlations among total respirable particles, ambient nicotine, cigarettes smoked, and urinary cotinine. With such correlated variables, the individual coefficients can behave in counterintuitive ways.

In men with potential diesel exhaust exposure, the effects of respirable particles and ambient nicotine are found by adding the main-effect and interaction terms. Thus, in exposed men, the corresponding joint effect is (- 2.66 + 2.97) × Respirable Particles + (39.72 - 38.79) × Ambient Nicotine = 0.31 × Respirable Particles + 0.93 × Ambient Nicotine. These coefficients are negligible, corresponding to no dose-response association in exposed samples. This is exactly what was observed in the three exposure-group models of Table 10. In fact, the predicted effect of increasing respirable particles in terms of revertants/µmol of creatinine of respirable particles among exposed samples is 0.76 more revertants/µmol of creatinine for each 1,000 extra µg/m^3 of respirable particles (assuming a 2-AF control response of 3.46 revertants/ng).

For mutagenicity with S9, Table D.3 shows that both uri-

nary cotinine and number of cigarettes smoked on the day of the study were individually predictive of mutagenicity. The between-person variance component was 0.235, (SE = 0.644), negligible or zero, compared to the within-person variance of 3.152 (SE = 0.794). A zero between-person component corresponds to complete independence of samples taken from the same person, once all covariates are known. In this case, generalized least-squares corresponds to ordinary least-squares multiple regression.

For mutagenicity without S9 also, no evidence was found of a dose-response association within exposure strata of respirable particles (Table 10). The log-likelihood ratio chi-squared test statistic was 1.10 ($p > 0.05$). In the full multiple-regression exposure-interaction model (Table D.4), the diesel exhaust exposure parameters also did not contribute significantly to the model ($\chi^2 = 5.39$, $p > 0.05$). Inspection of the interaction parameters shows, again, no separate effects of respirable particles in either diesel exhaust-exposed or unexposed men. In fact, the predicted coefficient in exposed men is negative ($-0.085 + 0.006 = -0.079$). For mutagenicity without S9 in smokers, the between-person variance component (0.070) is larger than the within-person component (0.011).

Other Analyses

In addition to the analyses displayed in the tables, a number of other analyses were carried out. We employed a two-stage technique for utilizing samples in which urine cotinine had not been measured. In all samples with full data, we regressed urine cotinine on cigarettes smoked, degree of smoke inhalation, and urinary thiocyanate. The predicted value of urinary cotinine from these regressions was used for those samples in which cotinine was not measured. Urine nicotine was also used in place of urine cotinine. Neither of these changes affected the regression results.

Subgroups of men working in exposed jobs were examined for an independent effect of respiratory particles; no effect of diesel exhaust exposure was observed. Models using the urine mutagenicity results of day 1 to predict urine mutagenicity on day 2 had too few samples for regression analysis.

Alternative model forms were considered, as summarized in the Statistical Methods section. Still other analyses included the effects of excluding and including possible outlier observations. The trip and protective food effects were removed. The tangent line to a quadratic fit for the calculation of the number of revertants per milliliter equivalent of urine was also tried. In none of these alternative analyses was an effect of diesel exhaust exposure detected.

DISCUSSION

DIESEL EXHAUST EXPOSURE

Respirable Particle and Environmental Tobacco Smoke Exposure

There are several sources of particles in the air breathed by railroad workers. They include diesel exhaust, environmental tobacco smoke, particles from other combustion sources, and mechanically generated dust from sand and gravel. Since diesel exhaust could not be measured directly, several strategies were used to develop markers for diesel exhaust that minimized the positive interference of other airborne contaminant sources. These included size-selective sampling to yield respirable particle concentration, subtracting the environmental tobacco smoke contribution to mass (adjusted respirable particles), and measuring exposure to phenanthrene, one of the most abundant PAHs in diesel exhaust.

Air concentrations of particles in a work area depend on the output rate of the contaminant source and on the layout, size, and ventilation characteristics of the work environment. In the case of cigarette smoking, they also depend on the worker density and availability of time for smoking.

Railroad workers' respirable particle exposures are a complex mixture of ambient particulate matter, dust, dirt or other particulate matter generated during work, environmental tobacco smoke, and diesel exhaust. We have demonstrated a method for measuring the environmental tobacco smoke exposure and how it can be used to remove one of the interfering contaminants in estimating a worker's diesel exhaust exposure. As judged by observation of the work patterns of the job groups we sampled, the clerks and carmen are unexposed to diesel exhaust from the trains, except in rare circumstances, but they are exposed to other particulates. The train crew, made up of the engineers, brakers, and conductors, either are inside or outside near the trains, and so are exposed to diluted diesel exhaust. The shop workers are in an enclosed or partially enclosed space with running locomotives, and would be expected to have the highest diesel exposures (Table 4).

The adjusted respirable particle exposures are not a direct measure of diesel exhaust because they still include other sources of particulate matter. All samples include background respirable particulate matter found in the ambient air. Air quality data from the area where we sampled showed an annual geometric mean of 25 $\mu g/m^3$ of total particulate matter, of which approximately 55 percent would be in the fine or respirable fraction of a dichotomous sampler, on the basis of limited data from the area. Also in-

cluded in the adjusted respirable particle measurement are dirt and dust generated by the work itself. The carmen spend much of their time outdoors along the dusty tracks where the freight cars are repaired. The brakers also work outside along the train tracks, where they are exposed to the dirt and dust produced by their own and the locomotives' activities, one of which is to apply sand to the tracks frequently in the winter to increase traction. In our previous study, we found that freight brakers' respirable particle samples were only 23 percent extractable in dichloromethane, while other job groups' samples were about 40 to 45 percent extractable, which implies that a substantial portion of the sample mass may be inorganic dirt and dust (Hammond et al. 1988). Nevertheless, even though the adjusted respirable particle exposure may overestimate the diesel exhaust exposure of these workers, it is a useful marker of the relative diesel exhaust exposure found among these railroad workers.

Phenanthrene Exposure

Our observation of a constant ratio of phenanthrene to respirable particles on area samples from the diesel exhaust-exposed repair shops suggests that phenanthrene should be pursued as a marker for diesel exhaust. Many other markers have been suggested for diesel exhaust, none of which is yet established as a "gold standard," and even less was known about these alternatives at the time this study was begun. A complete discussion of alternative markers is beyond the scope of this report, but a discussion of why phenanthrene was selected as a marker of diesel exhaust exposure is contained in Appendix F.

We selected phenanthrene in part because our earlier analyses of railroad diesel exhaust found it to be the PAH present in the highest concentration. This was an important consideration in designing a study that used personal sampling pumps that collect less than 1 percent of the volume of air collected by the high-volume samplers.

URINARY MUTAGENICITY

Diesel Exhaust

We did not observe an effect of diesel exhaust exposure on urinary mutagenicity. Several factors may have contributed to this negative result. Despite our attempt to maximize diesel exhaust exposure, the median level of respirable particle concentration from diesel exhaust among nonsmoking subjects in our highest exposed job group (shop workers) was only 113 $\mu g/m^3$, and 75 percent of samples were below 161 $\mu g/m^3$. These concentrations are lower than many historical measurements of diesel exhaust exposure among railroad workers (Woskie et al. 1988b). These low levels may reflect the decline in railroad activity in this country, with associated reductions in diesel engine use and exposure to diesel exhaust.

Willems and associates (1989) have reported no increase in fecal or urinary mutagenicity attributable to diesel exhaust exposure among car mechanics. The *Salmonella* plate incorporation assay (Ames et al. 1975) was used to measure mutagenicity.

The absence of an association of mutagenic activity in the urine with diesel exhaust exposure does not necessarily mean that diesel workers did not excrete diesel-associated mutagens. First, the level of mutagenic activity could be below the level of detection. For example, a worker who was exposed to a mutagen concentration of 1,000 revertant equivalents per cubic meter of air and who breathed 7 to 10 m^3 of air during the seven-hour exposure period received a total mutagen dose of approximately 7,000 to 10,000 revertant equivalents (1,000 revertants/m^3 × m^3/hour × 7 to 10 hours). The value of 1,000 revertant equivalents per cubic meter of air was estimated from a diesel shop worker's personal filters and was measured using the microsuspension procedure (Figure 5). If we assume that a small fraction of mutagens is excreted in the urine in a mutagenic form, then the measureable revertant equivalents can be approximated. This may not be an unreasonable assumption, since the mutagens adsorbed on diesel particulate matter will have to be desorbed in physiologic fluids. The bioavailability of mutagens on diesel particulate matter has been reported to be a relatively slow desorption process (King et al. 1981). The work by Belisario and colleagues (1984), who injected rats with whole diesel particles and measured urinary mutagenic activity, indicated that only a small fraction of measurable mutagens is excreted in a 24-hour period.

For illustrative purposes, if 1 percent of the total revertant equivalents is excreted in the afternoon urine sample collected, 70 to 100 revertant equivalents would be in 100 to 200 mL of urine (0.01 × 7,000 to 10,000 revertant equivalents). The concentration would therefore add, at the most, 0.7 to 1 revertant equivalent per milliliter of urine (70 to 100 revertant equivalents/100 mL of urine). This is the level of activity generally determined in the urine of nonsmokers (Kado et al. 1983).

Two important considerations in evaluating these results are the unknown kinetics of diesel exhaust absorption and excretion in humans, and the limited ability to evaluate them in the field setting. For example, it was feasible to collect only a single spot urine at the end of the work shift. Although the spot urine samples showed a good dose-response relation with smoking dose on the work day, if diesel mutagens were excreted more slowly than mutagens

from cigarette smoke, the assay might not have been sensitive enough to detect the diesel exhaust mutagens. It is also possible that the diesel mutagens are, in part, excreted by another route, such as the feces, or are excreted in a form that is not mutagenic in the urine. The diesel exhaust-associated mutagens may be conjugated to polar molecules, such as glucuronides or sulfates. In this conjugated form, the mutagens may not be mutagenic, or they may not be efficiently extracted by XAD, since they are polar. Deconjugation steps may not result in releasing the parent (unmetabolized) form of the mutagen.

The use of personal measurements for respirable particle concentrations adjusted for environmental tobacco smoke and of phenanthrene (on a subset of workers) improved the accuracy of our diesel exhaust exposure estimates, but some error in exposure assessment certainly remained. For adjusted respirable particles, this error may be due to imprecision in the correction for tobacco markers, as noted above. Phenanthrene seemed to be an improved marker of diesel exhaust, but it may represent mutagenic compounds in the vapor phase and may not be the best marker for all mutagenic components of diesel exhaust. It is likely that both of these errors would reduce the ability to observe an association of diesel exhaust exposure with urinary mutagenicity, if one were present. Mutagenic activity of respirable particles from personal samplers offers another potential marker for measuring exposure to the biologically active component of diesel exhaust. Unfortunately, we did not do enough measurements of particle mutagenicity to evaluate this possibility, but further studies should address this question.

Another approach to monitoring dose is to measure actual DNA or protein adducts of the reactive compounds in diesel exhaust. A number of methods have been used for biological monitoring of humans such as the ^{32}P-postlabeling methods of Randerath and associates (1984). The application and significance of this and other methods for human monitoring have been reviewed (Perea 1988; Santella 1988).

Although the mutagenic activity with metabolic activation (+S9) of environmental tobacco smoke extracts (using strain TA98) is usually higher than without metabolic activation (-S9), this was not the case for the personal filter samples from the clerk and shop workers. First, the purpose of these data was to show that measurement of mutagenicity on personal filter samples could be done. This is a significant point, since only a very limited sample is available on the personal filters in approximately eight hours of sampling time. Second, only very few personal filters were made available to us in the time frame of analyses. As a pilot study to examine the feasibility of testing such filters, we felt that this approach was promising.

Finally, the power of the study may not have been great enough to observe the effect of diesel exhaust on urinary mutagenicity, if one were present. The increased risk of lung cancer among diesel exhaust-exposed railroad workers observed in some studies (Howe et al. 1983; Garshick et al. 1987, 1988) is only a fraction of the risk from cigarette smoking, and likely occurred with higher levels of diesel exhaust exposure.

Dietary Factors

A number of reports on the effects of food consumption on urinary mutagenicity have been published. For example, Baker and colleagues (1982) reported that mutagenicity was elevated in the urine of volunteers who consumed meals including fried pork or bacon. Dolara and associates (1984) confirmed these results, but reported much lower mutagenic activity and concluded that "urinary mutagenic activity related to exposure to meat is marginal, and much lower than that reported after exposure to cigarette smoke or mutagenic chemicals in the work environment." Sousa and coworkers (1985) reported that mutagenic activity was detected in the urine after a fried beef meal, and that peak activity appeared approximately two to six hours after consumption. The level of activity reached two to five times the background level of revertants. In the studies by Baker and coworkers (1982) and Sousa and coworkers (1984), meat prepared by microwave cooking was not mutagenic, as was reported earlier by Nader and colleagues (1981).

The mutagenic values reported in the studies in which individuals consumed fried meats were based on the plate incorporation test. Since the microsuspension procedure has not yet been used in a study involving fried meats, the mutagenic levels anticipated to be in railroad workers' urine from eating fried meats are difficult to estimate.

Antimutagenic Activity of Naturally Occurring Compounds

Compounds found naturally in foods have been tested for their antimutagenic activity in the *Salmonella* plate incorporation test. For example, Lai and associates (1980) reported that chlorophyll inhibited 3-methylcholanthrene mutagenicity. Kada and colleagues (1978) reported that a protein-bound compound inhibited the mutagenic activity of highly mutagenic pyrolysis products, and the mutagenicity of B*a*P and cigarette smoke condensate was also decreased by either ellagic acid, riboflavin, or chlorophyllin (Terwel and van der Hoeven 1985). Ong and coworkers (1986) found that chlorophyllin inhibited the mutagenicity of a number of complex mixtures, including extracts from diesel emission particles. Hayatsu and colleagues (1988)

have reviewed dietary inhibitors of mutagenesis and carcinogenesis.

Since some naturally occurring compounds modulate the activity of mutagens, a study examining the effect of these "modulators" on excretion of mutagenic compounds in the urine would be informative. Our finding that mutagenic activity was decreased in the urine of nonsmoking diesel workers who consumed fish, cabbage, or Brussels sprouts is consistent with numerous reports on the protective effect of these foods (National Academy of Sciences 1982). For example, Graham and colleagues (1978) reported that there was a decreased risk of colon cancer in people who frequently ingest raw vegetables, especially cabbage, Brussels sprouts, and broccoli. Baker and associates (1986), however, reported that there were no effects of ingested fruits and vegetables on the urinary clearance of mutagens in fried bacon meals. Although our measure of dietary intake was based on self-report and was not validated, the consistency of the findings with the results of other studies suggests a causal association. Further controlled studies are necessary to evaluate this association.

Study Power

Study power was evaluated for +S9 analyses of both smokers and nonsmokers. The multiple regression models in Table 10, with the four adjusted respirable particle strata of exposure, were used. Power was calculated for the partial *F* test (with three degrees of freedom) of differences in group means, after adjustment for other factors. For simplicity, between-person variability was ignored and all observations were treated as independent. The method of O'Brien (1982) was modified for post hoc calculations with the study data. This method estimates power conditional on the values of the covariates observed in the study. The residual standard deviations are also fixed at their estimated values. Synthetic data based on each alternative hypothesis about exposure are generated to calculate the power.

For nonsmokers, +S9 urinary mutagenicity in the study ranged roughly from −0.9 to 2.4 revertants/μmol of creatinine, with the middle 75 percent of observations falling between about 0.12 and 0.50 revertants/μmol of creatinine. The predicted +S9 means in Table 10 had a maximum difference of about 0.1 revertants/μmol of creatinine. Suppose that the true differences from base line for the three strata of exposed adjusted respirable particles (low, medium, and high) were +0.1, +0.3, and +0.4, respectively. These differences would have been detected with 93 percent probability in this study.

For smokers, observed +S9 urinary mutagenic activity ranged from 0 to 15 revertants/μmol of creatinine, with 75 percent of the observations occurring between 1.9 and 5.4 revertants/μmol of creatinine. In Table 10, the maximum difference between predicted means among the four exposure groups was about 0.4. Suppose the true differences from the base-line mean were +0.2, +1.5, and +2.0, for the low, medium, and high adjusted respirable particle groups. In this case, statistical significance at the 0.05 level would have been declared 84 percent of the time.

Given the ranges in which the observed data fell, such effects must be considered possible. Thus, it is unlikely that such large exposure-related effects would have gone undetected, although smaller differences might well have been missed.

IMPLICATIONS OF THE FINDINGS

The aims of this study were to develop methods for estimating diesel exhaust exposure in epidemiological studies and for using urinary mutagenicity to evaluate exposure to diesel exhaust, and to use these methods in a field study of railroad workers. We have addressed some of the complex issues in measuring exposure to diesel exhaust in field studies and have demonstrated two improved methods for estimating diesel exhaust exposure (adjusted respirable particles and phenanthrene). We have also used a sensitive assay for urinary mutagens in the field study and have validated the dose-response characteristics of the assay for cigarette smoking by comparison with other smoking markers.

In the full-regression models, we observed an association of cigarette smoking and of certain foods with urinary mutagens. However, we did not detect an independent association of environmental tobacco smoke or of diesel exhaust exposure with urinary mutagenicity. Our research suggests that at the levels of exposure to diesel exhaust and environmental tobacco smoke found in our study, the mutagens associated with these levels of exposure were undetectable. Several possible explanations for this absence of an association exist, as we have noted. Unfortunately, there is currently no specific biological marker for diesel exhaust, as cotinine is a marker for cigarette smoke. The use of urinary mutagenicity for epidemiologic studies, therefore, would require higher exposure levels or greater sensitivity of detection. This assumes that mutagens are excreted in detectable form in the urine. The measurement of mutagenicity in personal filter samples showed promise.

This study provides no basis on which to draw conclusions regarding cancer risks associated with diesel exhaust exposure. Other epidemiological studies of railroad workers (Garshick et al. 1987, 1988) have directly addressed this issue, and are more appropriate to evaluate such an associa-

tion. This study also has no bearing on quantitative risk assessments of diesel exhaust exposure.

ACKNOWLEDGMENTS

The authors would like to thank Rebecca Morrison for secretarial and editorial assistance; Faith Davis and Marilyn Arnold for epidemiologic and field-work assistance; Nancy Bettinger and Anne Roche for industrial hygiene assistance; Dennis Hsieh for his guidance and support; Neal Benowitz, Peyton Jacob, and Lisa Yu for the analyses of urine nicotine and cotinine; Mike Hughes, Bob Wiebe, Marianne Rasor, and Skip Kennedy for data handling and programming; Richard Childs, JoAnne Shatkin, and Laura Smith for technical assistance; and Alan Buckpitt, Leon Rosenblatt, Jerold Last, Suzanne Mitchell, Linda Beltran, and John Batchelder for editorial comments.

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APPENDIX A. Health Survey

CARD #

ID NUMBER

TODAY'S DATE
(mo/day/yr)

Please complete the following set of questions. If you are not sure of how to respond to a question, please ask the staff for clarification. Your responses are CONFIDENTIAL and will NOT be released to anyone except in summary form. Thank you in advance for your cooperation.

EXPOSURE HISTORY

1. What is your BIRTHDATE? ___(month)/___(day)/___(year)
2. What is your current JOB TITLE? _____
3. What are your current JOB DUTIES? _____
4. How long have you had this job? _____years
5. In the PAST TWO DAYS, have you worked in the presence of diesel exhaust fumes? ___YES NO___

   If YES, for each day please indicate the approximate length of time exposed and the level of intensity. Intensity is classified as:
   LIGHT--no smell or eye irritation
   MODERATE--occasional odor and/or eye irritation
   HEAVY--strong odor and intense eye irritation

	Time	Intensity
Yesterday	(a)___no exposure (b)___less than 1 hour (c)___1 up to 3 hours (d)___3 up to 5 hours (e)___5 or more hours	(a)___light (b)___moderate (c)___heavy
Today	(a)___no exposure (b)___less than 1 hour (c)___1 up to 3 hours (d)___3 up to 5 hours (e)___5 or more hours	(a)___light (b)___moderate (c)___heavy

6. Are any of the following HEATING SYSTEMS regularly (i.e. at least once per week) used in your home and/or working areas? (please check YES or NO for each type)

   wood stove ___YES NO___
   coal stove ___YES NO___
   fireplace ___YES NO___

7. What type of COOKING STOVE is regularly used in your home? (please check only one)

   (a)___electric stove
   (b)___gas stove
   (c)___wood stove
   (d)___other, please specify: _____

8. In the PAST WEEK, have you regularly worked with any of the following materials at WORK or at HOME? (please answer each part (a-j) by checking NO or YES for each)

	NO	YES Work	YES Home
(a) Raw Diesel Fuel . .			
(b) Gasoline.			
(c) Motor Oil			
(d) Paint Stripper. . .			
(e) Paint Thinner . . .			
(f) Welding Fumes . . .			
(g) Adhesives or Glue .			
(h) Solvents.			
(i) Cutting Oils. . . .			
(j) Pesticides.			

SMOKING HISTORY

9. In the PAST WEEK, have you regularly smoked cigarettes (at least one cigarette per day)? ___YES NO___

   If NO, please skip to question 10 on the next page

   If YES, please answer the following questions (a-h)

   (a) How MANY cigarettes did you smoke each day in the past TWO DAYS? (recall that there are 20 cigarettes in each package) _____Yesterday (all day)
   _____Today (all day until now)

   (b) What BRAND of cigarettes do you now smoke? (please be as specific as possible, e.g. Marlboro Light 100's) _____

   (c) What SIZE are your cigarettes?
   (1)___regular
   (2)___kingsize
   (3)___100's
   (4)___120's
   (5)___other, please specify: _____

CARD #

ID NUMBER

   (d) Are your cigarettes FILTERED TIP? ___YES NO___

   (e) Are your cigarettes MENTHOL? ___YES NO___

   (f) Are your cigarettes LIGHTS? ___YES NO___

   (g) Are your cigarettes ULTRALIGHTS? ___YES NO___

   (h) Do you INHALE? ___YES NO___

   If YES, how deeply? (1)___into your mouth only
   (2)___back of your throat
   (3)___top of your lungs
   (4)___deep into your lungs

10. In the PAST TWO DAYS, have you smoked a PIPE? ___YES NO___

    If YES, how many pipefills each day? _____Yesterday
    _____Today

    If YES, do you inhale? ___YES NO___

11. In the PAST TWO DAYS, have you smoked a CIGAR? ___YES NO___

    If YES, how many cigars each day? _____Yesterday
    _____Today

    If YES, do you inhale ___YES NO___

12. In the PAST TWO DAYS, have you used CHEWING TOBACCO or SNUFF? ___YES NO___

    If YES, how many ounces each day? _____Yesterday
    _____Today

13. LAST NIGHT, were people smoking in your presence? ___YES NO___

    If YES, how many people? _____people

14. In your OFFICE of WORK AREA, does anyone other than yourself REGULARLY smoke cigarettes? ___YES NO___

    If YES, how many people were smoking in your presence TODAY? _____people

MEDICAL HISTORY

15. In the PAST TWO DAYS, have you used any of the following? (please check YES or NO for each item)

    Aspirin. . . . . ___YES NO___
    Nicotine gum . . . . . ___YES NO___
    Vitamins . . . . . ___YES NO___
    Any other medication . ___YES NO___
    If YES, please specify (include both prescription and over-the-counter medications): _____

16. Has a doctor EVER told you that you have any of the following conditions? (please check YES or NO for each item)

    Asthma. . . . . ___YES NO___
    Diabetes. . . . . ___YES NO___
    Emphysema . . . . . ___YES NO___
    Psoriasis . . . . . ___YES NO___
    High Blood Pressure . . ___YES NO___
    Kidney Disease. . . . . ___YES NO___
    Bladder Disease . . . . ___YES NO___
    Cancer. . . . . ___YES NO___
    If YES, what type of cancer? _____

PERSONAL HISTORY

17. Are you currently a vegetarian? ___YES NO___

18. In the PAST TWO DAYS, have you eaten at least one serving of any of the following FOODS? (please check YES or NO for each food)

Grilled, barbequed or broiled BEEF.	___YES	NO___
Grilled, barbequed or broiled PORK.	___YES	NO___
Grilled, barbequed or broiled CHICKEN	___YES	NO___
Grilled, barbequed or broiled FISH.	___YES	NO___
Grilled, barbequed or broiled SHELLFISH . . .	___YES	NO___
PROCESSED MEATS (e.g. bologna, bacon, salami)	___YES	NO___
BRUSSEL SPROUTS	___YES	NO___
CABBAGE	___YES	NO___

19. Do you currently have a hobby that involves regular use of chemicals or sprays? ___YES NO___

If YES, please describe: _____

_____

20. What brand of SHAMPOO do you regularly use? _____

_____

Is this a TAR TYPE shampoo? ___YES NO___ NOT SURE___

21. Do you currenly use PERMANENT HAIR DYE (do not count dyes that rinse out with washing)? ___YES NO___

Thank you for your cooperation in completing the preceeding set of questions. Again, your responses are CONFIDENTIAL.

APPENDIX B. Effects of Storage on Urinary Mutagenicity and Thiocyanate Concentration

To examine the effect of storage on mutagenicity and thiocyanate concentration in the urine from smokers and nonsmokers, urine samples from seven smokers and seven nonsmokers were collected and split into two equal portions. One portion was immediately extracted with XAD-2 and tested for mutagenicity and for the concentration of thiocyanate. The other portion was immediately frozen; approximately six months after the initial collection day, it was extracted and tested for mutagenicity and thiocyanate concentration. To test for the effect of storage on the actual extract, any material remaining after the initial XAD-2 extraction of the first portion was stored at −20°C in an amber screw-cap vial sealed with nitrogen, and was retested for mutagenic activity six months after the initial collection day.

The results, summarized in Table B.1, indicate that the mutagenic activity in the urine of smokers and nonsmokers after storage for approximately six months did not have any consistent pattern of increasing or decreasing. There were no order-of-magnitude changes. The initial extracts that were retested also did not appear to change significantly. However, the concentrations of thiocyanate for both smokers and nonsmokers appeared to decrease over the period of six months (Table B.1).

The reextraction and retest mutagenicity data in Table B.1 were analyzed by components-of-variance models. Individual plate values (not shown) were corrected by subtraction of the mean of the phosphate-buffered saline blank plates. This led to negative values, which were removed by addition of 26 to each plate's corrected rate, x.

Following the suggestion of Snee and Irr (referenced in Moore and Felton 1983), the fifth root was taken to stabilize the variance. The final transformation was, therefore, $y = (x + 26)^{0.20}$.

The basic statistical model was:

$$y = \underset{\text{(Fixed)}}{\text{Overall Mean}} + \underset{\text{(Random)}}{\text{Retest Effect}} + \underset{\text{(Random)}}{\text{Reextraction Effect}} + \underset{}{\text{Person Effect}} + \underset{\text{(Random)}}{\text{Plate Effect}}$$

For smokers, we have shown that mutagenicity rates vary systematically with the number of cigarettes smoked. Therefore, the data were analyzed separately for smokers and nonsmokers. For the nonsmokers, the person effect was treated as randomly varying among persons. For the smokers, each person effect was treated as fixed, reflecting the systematic variation.

The variances of the test, extraction, person, and plate effects are known as components of variance (Snedecor and Cochran 1980). The sizes of the test and extraction components relative to the others are indications of the analytic repeatability. (Because a retest was performed on the original extract only, it was not possible to estimate a test-extraction interaction.) The data were analyzed by means of the type-I and maximum-likelihood options in the SAS variance-components procedure. The maximum-likelihood option has a nominal assumption of normality.

The estimated variance components for smokers and nonsmokers, and for both methods of estimation, are shown in Table B.2.

The results are similar for the two methods of estimation for smokers and nonsmokers. Variability between retests of the same extraction is comparable to that among plates for one sample. After accounting for other sources, the variation between different extracts of the same sample is negligible or zero. For nonsmokers, the between-person variance is about one-third that of the components for retest and plate effects.

These results have implications for study design. First, the plate-to-plate variation is substantial enough to increase the number beyond three, if possible. Sample batches should be analyzed together. The statistical treatment should block on each test batch to control for the large retest component.

The fifth-root transformation was successful here, as judged by its ability to equalize, roughly, the variance components for smokers and nonsmokers. To check, the analyses were repeated for the square-roots and logarithms of the rates +26. The results (not shown) led to the same conclu-

Table B.1. Effects of Storage on Mutagenic Activity and Thiocyanate Concentrations of Smokers' and Nonsmokers' Urine Extracts[a]

Sample Identifier	Smoker/Nonsmoker	Immediately Tested Urine Mutagenicity[b] (TA98 revertants/mL)	Immediately Tested Thiocyanate (nmol/mL)	Urine Mutagenicity After Six-Month Storage[c] (TA98 revertants/mL)	Thiocyanate After Six-Month Storage (nmol/mL)	Retest of Immediately Tested Urine Mutagenicity[d] (TA98 revertants/mL)
S-1	Smoker	21.2	248	14.2	73	32.0
S-2	Smoker	ND[e]	596	57.2	290	ND
S-3	Smoker	0	106	0	62	2.6
S-4	Smoker	22	106	5.8	87	16.4
S-5	Smoker	69	302	74.6	306	55.2
S-6	Smoker	21.6	128	34.2	96	38.6
S-7	Smoker	92.2	334	ND	198	99.4
NS100	Nonsmoker	0.6	208	4.6	148	4.8
NS101	Nonsmoker	4.4	216	7.6	154	5.8
NS102	Nonsmoker	6.8	120	4	ND	7.6
NS103	Nonsmoker	4.8	90	5.2	48	11.2
NS104	Nonsmoker	ND	184	7.6	158	17.2
NS105	Nonsmoker	ND	200	2	128	13
NS106	Nonsmoker	ND	160	7.2	ND	20.2
OT1	Nontobacco smoker	2.8	ND	10.4	133	4.4
PBS	Buffer extract blank	2	ND	3.8		5.2

[a] Each table entry is the mean of duplicate or triplicate determinations.

[b] Urine samples split: half immediately extracted and tested; half frozen at −20°C.

[c] Frozen urine sample thawed, extracted, and tested.

[d] Remainder of extract from immediately tested sample stored at −20°C for six months and retested.

[e] ND = not determined.

Table B.2. Components of Variance in Studies on Effects of Storage

	Nonsmokers		Smokers	
	Type I	Maximum Likelihood	Type I	Maximum Likelihood
Retest	0.0543	0.0324	0.0407	0.0191
Reextraction	0.0058	0.0000	-0.0025[a]	0.0000
Between persons	0.0161	0.0179	—[b]	—[b]
Between plates	0.0476	0.0476	0.0344	0.0288

[a] A true variance cannot be negative; the negative estimate is indicative of a small or zero component.

[b] Smokers were treated as fixed; no random variance term applies.

sions about the relative contributions of the variance components. The estimated components were not (with the exception of the negligible reextraction variance) similar for smokers and nonsmokers.

APPENDIX C. Comparison of Point-Deletion and Tangent-Slope Methods for Computing the Number of Revertants per Milliliter Equivalent of Urine

In this appendix, we compare the results of calculating in two ways the number of revertants per milliliter equivalent of urine. As described in the Methods section, each method began by fitting a quadratic equation relating the number of revertants per plate to the concentration (dose) of urine:

$$\text{Revertants} = a + b\,(\text{Dose}) + c\,(\text{Dose-Squared}) \qquad \text{(A1)}$$

Toxicological theory suggests a linear relationship between revertants and dose. Any nonlinear appearance to the curve may be due to chance, to toxicity (resulting in fewer cells), or to other factors.

Two methods of calculating the linear portion of the dose-response relation, in the absence of a direct test for toxicity, are compared here.

The first calculation, suggested by Paul Meier in a personal communication, estimated the number of revertants per milliliter equivalent of urine as the slope of the line tangent to curve (A1) when dose is 0.0. Simple calculus shows this to be the parameter b if curve (A1) is fitted.

The second method evaluated the p value of the dose-squared coefficient c after fitting curve (A1) by least squares. If the p value was less than 0.15, the highest dose point was dropped. A quadratic model was fit to the remaining dose points. If the p value for the squared term was again less than 0.15, the next highest dose point was also dropped. At the earliest stage at which the p value for nonlinearity was greater than 0.15, a linear model was fit to the remaining observations:

$$\text{Revertants} = a + b\,(\text{Dose}) \qquad \text{(A2)}$$

The number of revertants per milliliter of urine was then estimated by the fitted slope b to this linear fit. This method resulted in the use of two, three, or four dose points for the fit, whereas the tangent-slope method used all observations.

For the samples with S9, the Spearman rank correlation between the two methods was 0.73, while for the samples without S9, the rank correlation was 0.81.

Descriptive statistics for the two methods are shown in Table C.1. It can be seen that the tangent-slope method pro-

Table C.1. Comparison of Revertants per Milliliter Equivalent of Urine Calculated by Tangent-Slope and Point-Deletion Methods

	With S9[a]					Without S9[b]				
	Minimum	First Quartile	Median	Third Quartile	Maximum	Minimum	First Quartile	Median	Third Quartile	Maximum
Estimated Slopes										
Tangent line	-6.7	3.2	7.6	33.3	258.5	-8.5	1.9	4.4	8.9	39.9
Point deletion	-12.4	3.5	5.6	16.9	133.8	-8.6	0.7	2.7	6.2	25.5
Difference	-16.3	-1.1	2.0	16.7	160.0	-8.1	0.1	2.0	3.5	14.3
Standard Errors of Estimated Slopes										
Tangent line	0.8	2.2	3.3	5.2	25.5	0.6	1.7	2.3	3.5	14.9
Point deletion	0.3	0.7	1.0	2.3	17.5	0.2	0.6	0.9	1.5	4.6
Difference	-10.3	1.2	1.9	3.1	16.6	-0.8	0.9	1.3	2.3	10.8

[a] $n = 289$.

[b] $n = 277$.

duced systematically higher values than did the point-deletion method. Inspection of individual plots in which the discrepancy was large shows two reasons for this. First, where the plot showed strong evidence of toxicity only at the highest dose point (an "obvious" downward bending of the curve), the point-deletion method dropped the point. Quadratic equation (A1) did not fit the data well and so it overestimated the linear slope at the lower dose points. Second, when the observations showed only slight but consistent curvature, the point-deletion method sometimes did not drop one or both of the highest two doses. Yet, the curves appeared to be bending. In this case, the tangent-slope method appeared to be estimating a true line at zero.

APPENDIX D. Urinary Mutagenicity Correlation with Multivariate Regression Variables

Table D.1. Multivariate Regression Model of TA98 + S9 Mutagenicity Among Nonusers of Tobacco[a]

Parameter	Estimate	SE	Estimate/SE
Constant	- 0.407	0.162	- 2.51[b]
Standard mutagen 2-aminofluorene (revertants/ng)	0.282	0.063	4.45[c]
2-Aminofluorene ×			
Cotinine (μg/μmol of creatinine)/1,000	- 0.150	1.168	- 0.13
Protective food (1 = yes)	- 0.104	0.034	- 3.09[d]
Respirable particles (μg/m^3)/1,000	- 0.537	0.359	- 1.50
Ambient nicotine (μg/m^3)/1,000	- 1.175	3.824	- 0.31
Diesel exhaust exposure (1 = yes)[e]	- 0.031	0.046	- 0.68
Diesel exhaust exposure × respirable particles/1,000	0.471	0.379	1.24
Diesel exhaust exposure × ambient nicotine/1,000	- 1.838	7.853	- 0.23
Within-person variance	0.098	0.016	
Between-person variance	0.040	0.018	

[a] Mutagenicity is expressed as revertants/μmol of creatinine; total number of samples is 124.

[b] $0.01 < p \leq 0.05$.

[c] $p \leq 0.001$.

[d] $0.001 < p \leq 0.01$.

[e] Chi-squared (3 degrees of freedom) = 1.87 and $p > 0.05$ for diesel exhaust exposure parameters.

Table D.2. Multivariate Regression Model of TA98 - S9 Mutagenicity Among Nonusers of Tobacco[a]

Parameter	Estimate	SE	Estimate/SE
Constant	- 0.235	0.139	- 1.69
Standard mutagen 2-aminofluorene (revertants/ng)	0.126	0.054	2.33[b]
2-Aminofluorene ×			
Cotinine (μg/μmol of creatine)/1,000	0.020	1.021	0.02
Protective food (1 = yes)	- 0.014	0.029	- 0.48
Respirable particles (μg/m^3)/1,000	- 0.298	0.306	- 0.97
Ambient nicotine (μg/m^3)/1,000	1.035	3.325	0.31
Diesel exhaust exposure (1 = yes)[c]	- 0.017	0.039	- 0.45
Diesel exhaust exposure × respirable particles/1,000	0.264	0.323	0.82
Diesel exhaust exposure × ambient nicotine/1,000	- 1.457	6.675	- 0.22
Within-person variance	0.072	0.013	
Between-person variance	0.026	0.013	

[a] Mutagenicity is expressed as revertants/μmol of creatinine; total number of samples is 119.

[b] $0.01 < p \leq 0.05$.

[c] Chi-squared (3 degrees of freedom) = 0.82 and $p > 0.05$ for diesel exhaust exposure parameters.

Table D.3. Multivariate Regression Model of TA98 + S9 Mutagenicity Among Cigarette Smokers[a]

Parameter	Estimate	SE	Estimate/SE
Constant	0.199	1.169	0.17
Standard mutagen 2-aminofluorene (revertants/ng)	- 0.045	0.391	- 0.11
2-Aminofluorene ×			
Cotinine (μg/μmol of creatinine)/1,000	3.502	1.091	3.21
Cigarettes smoked on day/100	4.879	1.072	4.53[b]
Respirable particles ($\mu g/m^3$)/1,000	- 2.656	1.593	- 1.67
Ambient nicotine ($\mu g/m^3$)/1,000	39.724	14.868	2.67[c]
Diesel exhaust exposure (1 = yes)[d]	- 0.122	0.317	- 0.39
Diesel exhaust exposure × respirable particles/1,000	2.971	1.749	1.70
Diesel exhaust exposure × ambient nicotine/1,000	- 38.788	17.056	- 2.27[b]
Within-person variance	3.152	0.794	
Between-person variance	0.235	0.644	

[a] Mutagenicity is expressed as revertants/μmol of creatinine; total number of samples is 63.

[b] $0.01 < p \leq 0.05$.

[c] $0.001 < p \leq 0.01$.

[d] Chi-squared (3 degrees of freedom) = 6.15 and $p > 0.05$ for diesel exhaust exposure parameters.

Table D.4. Multivariate Regression Model of TA98 - S9 Mutagenicity Among Cigarette Smokers[a]

Parameter	Estimate	SE	Estimate/SE
Constant	- 0.048	0.191	- 0.25
Standard mutagen 2-aminofluorene (revertants/ng)	0.061	0.072	0.85
2-Aminofluorene ×			
Cotinine (μg/μmol of creatinine)/1,000	- 0.380	0.213	- 1.78
Cigarettes smoked on day/100	0.277	0.187	1.48
Respirable particles ($\mu g/m^3$)/1,000	- 0.085	0.297	- 0.29
Ambient nicotine ($\mu g/m^3$)/1,000	7.351	4.050	1.90
Diesel exhaust exposure (1 = yes)[b]	0.039	0.055	0.72
Diesel exhaust exposure × respirable particles/1,000	0.006	0.319	0.02
Diesel exhaust exposure × ambient nicotine/1,000	- 6.314	4.050	- 1.56
Within-person variance	0.011	0.019	
Between-person variance	0.070	0.019	

[a] Mutagenicity is expressed as revertants/μmol of creatinine; total number of samples is 59.

[b] Chi-squared (3 degrees of freedom) = 5.39 and $p > 0.05$ for diesel exhaust exposure parameters.

APPENDIX E. Statistical Descriptions of Mutagenic Activity Controls

Table E.1. Mutagenic Activity in Positive Standards

			Revertants/ng from Zero + Two Dose Points				
				Between-Experiment		Within-Experiment	
S9	Standard	*n*	Mean	SD	Coefficient of Variation (%)	SD	Coefficient of Variation (%)
–	2-Nitrofluorene	13	17.24	1.79	10.4	0.87	5.1
–	4-Nitroquinoline-*N*-oxide	13	7.30	0.35	8.9	0.35	4.8
+	2-Aminofluorene	13	3.46	0.76	22.0	0.25	7.1
+	Benzo[*a*]pyrene	13	2.86	0.84	28.7	0.19	6.6

Table E.2. Mutagenic Activity in Negative Standards

			Revertants/Plate				
				Between-Experiment		Within-Experiment	
S9	Standard	*n*	Mean	SD	Coefficient of Variation (%)	SD	Coefficient of Variation (%)
–	DMSO	13	37.68	21.52	57.1	7.70	20.4
+	DMSO	13	37.12	27.97	75.3	7.06	19.0

Table E.3. Spearman Rank Correlation of Mutagenicity (revertants/ng) of Standard Mutagens by Smoking Status[a]

		Standard Mutagen[b]			
		+ S9 Standards		– S9 Standards	
	n	Benzo[*a*]pyrene	2-Aminofluorene	2-Nitrofluorene	4-Nitroquinoline-*N*-oxide
Nonsmokers					
+ S9	160	0.276 (< 0.01)	0.309 (< 0.01)	0.008 (0.92)	0.092 (0.25)
– S9	152	0.200 (0.01)	0.204 (0.01)	– 0.049 (0.55)	0.052 (0.52)
Smokers					
+ S9	90	0.231 (0.03)	0.331 (< 0.01)	– 0.050 (0.64)	0.045 (0.68)
– S9	87	0.343 (<0.01)	0.229 (0.03)	– 0.120 (0.27)	0.272 (0.01)

[a] Each table entry is the correlation coefficient; the *p* value is given in parentheses.

[b] Assayed on 13 processing dates, with mutagenicity (revertants/μmol of creatinine) of study samples evaluated on the same dates.

APPENDIX F. Phenanthrene as a Marker for Diesel Exhaust

Prior to this study, the authors conducted an investigation of diesel exhaust exposures and lung cancer rates. The results of that study guided the selection of markers for diesel exhaust that were used in the study reported here. In particular, we examined the usefulness of respirable particles as a marker for diesel exhaust and the need to correct this marker for interference by environmental tobacco smoke (Hammond et al. 1988). In addition, we collected 23 high-volume samples of respirable particulate matter from the running repair shops, the areas of highest diesel exhaust exposure, for intensive chemical analysis. The respirable-mass concentrations ranged from 25 to 486 $\mu g/m^3$. Approximately 40 percent of the particle mass was extracted with dichloromethane. These extracts were then concentrated and separated into fractions (aliphatic hydrocarbons, aromatic hydrocarbons, and polar compounds) by liquid chromatography. For several of the samples with the greatest mass, aliquots of both the raw extract and the fractions were analyzed using gas chromatography and mass spectrometry (Hammond et al. 1985). The gas chromatography and mass spectrometry analyses of these samples identified phenanthrene and alkylated phenanthrenes (one sample had methylfluorene). Specifically, other PAHs suggested as potential markers for diesel exhaust (pyrene, nitropyrene, B*a*P, or fluoranthene) were not identified. This probably reflects the limit of detection of the method, which was approximately 20 µg/mL. Because the gas chromatography and mass spectrometry analyses did find phenanthrene and its alkylated derivatives, the researchers deemed it reasonable that these PAHs were the predominant ones present. This conclusion was reinforced by the fact that filters do not collect phenanthrenes well, but they do collect the other PAHs mentioned much better (see the laboratory experiment results in the Results, Specific Aim 1 section, in which 100 percent of the B*a*P remained on the filter, but less than half of the phenanthrene was efficiently collected by the filter). The literature studies of diesel exhaust from automobiles and trucks also indicated that phenanthrene and its derivatives are the major PAHs, followed by fluoranthene. All other PAHs tend to be present at lower levels, generally lower by one order of magnitude or more.

A second major finding from this earlier study of diesel exhaust exposure among railroad workers was that the particle concentrations were quite low. Almost all (of more than 500) personal samples collected less than 0.5 mg of mass, and most collected less than 0.1 mg of respirable particles; that is, less than one-thousandth the mass of the high-volume samples. Thus, at best, each personal sample would contain only nanogram levels of all PAHs, and much less than a nanogram of most PAHs.

For the reasons given above, phenanthrene became the target of the analyses in this study. The original plan had been to analyze the polyurethane foam plugs and filters for a range of PAHs, but this was beyond the scope of the study.

Analyses for B*a*P were performed on both the filter and the polyurethane foam plug extracts of two samples from the repair shop. All four of these samples had less than detectable levels of B*a*P (limit of detection was 0.5 ng B*a*P), while these samples had approximately 230 ng of phenanthrene on the polyurethane foam plugs and 4 ng on the filters. The low levels of B*a*P are in agreement with published data (National Research Council 1981), in which between 0.5 and 5 ng of B*a*P were found per milligram of particles from automobile diesel exhaust. One could predict from this that, from railroad workers exposed to high levels, between 0.05 and 0.5 ng of B*a*P would be collected on the personal samples; these values are below or just at the limit of detection. Detectable levels of B*a*P were found in samples from the chamber study of environmental tobacco smoke (Hammond et al. 1987).

The limits of detection for PAHs were improved from the earlier study (Hammond et al. 1985) by changing from gas chromatography and mass spectrometry methods, with a limit of detection of about 4 mg per sample, to a method using liquid chromatography with fluorescence detection, with a limit of detection of about 1 ng per sample. Specific antibodies to B*a*P are not likely to be significantly more sensitive, and cross reactions with other PAHs in the mixture are possible. One would have to validate the specificity of antibody reactions in the diesel exhaust extract matrix.

Since completion of this study, several other candidate markers have been suggested for diesel exhaust, but many of the suggested diesel exhaust markers had not been developed or validated at the time this study was undertaken. Elemental carbon is not specific for diesel exhaust particles, but shows good separation between diesel and cigarette smoke particles. It may prove to be a good marker for diesel exhaust, but it has not yet been validated. The ratio of pyrene to 1-nitropyrene has been suggested as a means of separating the contributions of diesel exhaust and environmental tobacco smoke to respirable particles mass, because the ratio may be higher in environmental tobacco smoke than in diesel exhaust. However, in 1 mg of automobile diesel exhaust, there are only 5 to 100 ng of pyrene and 6 to 10 ng of 1-nitropyrene (National Research Council 1981). Thus, in a personal diesel exhaust-exposed sample of 0.1 mg, we would expect only 0.5 to 10 ng of pyrene and less than 1 ng of 1-nitropyrene—levels that would be very diffi-

cult to measure in the highly exposed workers. This would result in large uncertainties in the calculated ratios.

Another category of proposed markers includes biological markers such as DNA or hemoglobin adducts. However, the use of a biological marker of diesel exhaust would alter the basic study design, namely, measurement of diesel exhaust exposure as a determinant of urine mutagenicity, a biological marker. Measurement of another biological marker of diesel exhaust, such as hemoglobin adducts, would itself have to be validated against some measure of diesel exhaust exposure. Furthermore, collection of blood samples in field epidemiologic studies is not as feasible as collection of urine samples, because blood collection is much more invasive. Attempts to collect blood samples would have resulted in lower subject-response rates. The situation is very different from using paid volunteers in a laboratory. Similarly, collecting 24-hour urine samples is also not feasible in this setting, even though it would be preferable to using spot samples.

ABOUT THE AUTHORS

Marc B. Schenker, M.D., is Associate Professor of Medicine at the University of California at Davis. He is Chief of the Division of Occupational and Environmental Medicine within the Department of Internal Medicine, and Director of the Davis Component of the Center for Occupational and Environmental Health. He received his M.D. degree from the University of California at San Francisco, and completed clinical training in internal medicine, pulmonary disease, and occupational medicine. He trained in epidemiology at the Channing Laboratory, Harvard Medical School, and received an M.P.H. from the Harvard School of Public Health. His research interests focus on the epidemiology of occupational and environmental hazards to the lung, including biological markers of hazardous exposure.

Steven J. Samuels Ph.D. is Associate Adjunct Professor in the Departments of Internal Medicine and of Obstetrics and Gynecology at the University of California, Davis. He received his Ph.D. from the Biomathematics program at the University of Washington (1978). His special interests are design and power considerations in reproductive health studies.

Norman Y. Kado, Ph.D., is a Staff Toxicologist for the California Air Resources Board and an Adjunct Assistant Professor in the Department of Environmental Toxicology, University of California, Davis. He received a Ph.D. in Environmental Health Sciences and Toxicology from the Department of Biomedical and Environmental Health Sciences, University of California, Berkeley, and received his postdoctoral training in the Department of Cancer Biology at the Harvard School of Public Health. Dr. Kado's research interests include monitoring of humans for mutagen and carcinogen exposures, and toxicological mechanisms of airborne contaminants.

S. Katharine Hammond, Ph.D. is an Associate Professor in the Departments of Family and Community Medicine and Pharmacology at the University of Massachusetts Medical School, Worcester, MA. She received a Ph.D. in chemistry from Brandeis University in 1976 and an M.S. in Environmental Health Sciences from the Harvard School of Public Health in 1981. Dr. Hammond's research interests focus on exposure assessment for epidemiological studies and include studies of exposures to diesel exhaust, environmental tobacco smoke, styrene, machining fluids, and gasoline. A related theme in her research has been studying the relationships of various biological markers of exposure to air concentrations of contaminants.

Thomas J. Smith, Ph.D., is Professor of Family and Community Medicine, Environmental Health Sciences Program, University of Massachusetts Medical School. Dr. Smith received both his M.S. and Ph.D. degrees in Environmental Health from the University of Minnesota. His special emphasis is the use of pharmacological modeling in epidemiological studies.

Susan R. Woskie, C.I.H., is a Research Industrial Hygienist in the Department of Family and Community Medicine at the University of Massachusetts Medical School. She received her M.S. in Industrial Hygiene from the Harvard University School of Public Health. Dr. Woskie is Board-certified in the comprehensive practice of industrial hygiene by the American Board of Industrial Hygiene. Her special emphasis is exposure characterization for health effects studies.

ABBREVIATIONS

2-AF	2-aminofluorene
B*a*P	benzo[*a*]pyrene
DMSO	dimethylsulfoxide
HPLC	high-pressure liquid chromatography
2-NF	2-nitrofluorene
4-NQO	4-nitroquinoline-*N*-oxide
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate-buffered saline
SD	standard deviation
SE	standard error
TA98 + S9	*Salmonella* tester strain with metabolic activation by S9 enzyme
TA98 - S9	*Salmonella* tester strain without metabolic activation

INTRODUCTION

In the summer of 1982, the Health Effects Institute (HEI) issued a Request for Applications (RFA 82-3) soliciting proposals for "Models of Susceptible Populations." In response to this RFA, Marc B. Schenker, then at the Brigham and Women's Hospital, Harvard University, Boston, MA, submitted a proposal entitled "Biologic Monitoring of Human Sensitivity to Diesel Exhaust Exposure." The HEI approved the original proposal; the project was then modified in the summer of 1983 because of staff changes that were necessary when Dr. Schenker moved to the University of California at Davis. The three-year project began in October 1983. Total expenditures were $388,638. The Investigators' Report was received at the HEI in May 1987 and was accepted by the Health Review Committee in January 1988, pending modifications that were subsequently made by Dr. Schenker and his colleagues.

During the review of the Investigators' Report, 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 Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective, as an aid to the sponsors of the HEI and to the public.

REGULATORY BACKGROUND

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

Research on the health effects of exposure to diesel exhaust also relates to the informed promulgation of occupational safety and health standards. Section 6(b)(5) of the Occupational Safety and Health Act of 1970 directs the Secretary of Labor to create occupational safety and health standards governing exposure to toxic materials and other harmful physical agents, with the goal of ensuring that no employee will suffer material impairment of health or functional capacity even if the employee has regular exposure to the harmful agent for the entire period of his or her working life. This section of the act directs the Secretary to promulgate such standards "on the basis of the best available evidence," including "research, demonstrations, experiments, and . . . the latest available scientific data in the field."

Pursuant to this directive, the Secretary, through the Occupational Safety and Health Administration, has promulgated numerous standards regulating occupational exposure to potentially harmful agents. In addition, the Administration has promulgated standards for the identification, classification, and regulation of potential occupational carcinogens (see 29 C.F.R. part 1990). These latter standards provide for the placement of potential carcinogens into risk categories "depending on the nature and extent of the available scientific evidence" (29 C.F.R. section 1990.111[f]). Recent research by the National Institute for Occupational Safety and Health culminated in the recommendation that whole diesel exhaust be listed as a "potential occupational carcinogen" under this section (National Institute for Occupational Safety and Health 1988).

Research relating to the health effects of exposure to diesel engine exhaust within a discrete occupational population, such as railroad workers, will contribute to informed decision making by the Administration in connection with the creation and modification of these and other occupational health standards.

SCIENTIFIC BACKGROUND

The health effects of exposure to diesel engine exhaust have been a focus of intense research in the United States and abroad. Increased use of diesel engines in motor vehicles, especially during the early 1970s, stimulated this effort. Today, although diesel-powered cars have not captured a large segment of the United States market, they represent a significant fraction of the European automotive fleet. Heavy-duty diesel engines have found wide use in trucks, buses, trains, farm equipment, machinery, and ships. Diesel engines are more fuel-efficient and emit lower levels of carbon monoxide and gaseous hydrocarbons than do

gasoline-fueled engines; however, they produce 30 to 100 times more particles or soot.

Diesel engine exhaust consists of a complex mixture of gaseous compounds and particulate matter. The composition of the exhaust varies considerably, depending on engine design, fuel, lubrication, emission control technology, and operating conditions (Johnson 1988). The available data base, which refers mainly to light-duty engine exhaust, was characterized over 10 years ago, and may not be representative of current emissions or of emissions from heavy-duty engines. Hundreds of chemicals, including polycyclic aromatic hydrocarbons, have been identified in the gaseous phase of diesel engine exhaust (International Agency for Research on Cancer 1989). The particle phase contains aggregates of small carbon particles with adsorbed organic and inorganic compounds. The fact that diesel engine exhaust particles are readily respirable (0.1 to 0.5 μm) and have genotoxic compounds adsorbed to their surfaces raises concerns regarding their effects on human health.

Numerous investigators, using molecular and cellular experimental systems, have demonstrated the capacity of constituents of diesel exhaust to act as mutagens, to produce chromosomal alterations, and to transform mammalian cells (Lewtas 1982; International Agency for Research on Cancer 1989). These test systems include the *Salmonella typhimurium* mutagenicity assays (Huisingh et al. 1978), the mouse lymphoma assay (Mitchell et al. 1980), and the determination of the induction of sister chromatid exchanges in Chinese hamster ovary cells and primary cultures from the lungs of exposed Syrian hamsters (Pereira 1982; Li et al. 1983). It has been shown that extracts from diesel particles and individual constituents of diesel emissions are tumorigenic in the mouse-skin bioassay, suggesting the potential carcinogenicity of diesel exhaust (Kotin et al. 1955; Nesnow et al. 1984). An increase in lung tumor incidence has been shown in rats chronically exposed to high levels of diesel engine exhaust (Brightwell et al. 1986; Ishinishi et al. 1986; Iwai et al. 1986; Mauderly et al. 1986; Stöber 1986). After reviewing the epidemiological, genotoxicity, and carcinogenicity data relating to diesel exhaust and its constituents, the International Agency for Research on Cancer evaluated diesel engine exhaust to be "probably carcinogenic to humans" (International Agency for Research on Cancer 1989). In the United States, the National Institute for Occupational Safety and Health has recommended that whole diesel exhaust be regarded as a "potential occupational carcinogen" (National Institute for Occupational Safety and Health 1988).

There are many difficulties in using epidemiological approaches to establish an association between diesel exhaust exposure and carcinogenicity in humans. The first difficulty has been to identify sufficiently large groups of individuals who have been exposed to diesel engine exhaust exclusive of other confounding exposures. Specific populations identified as being at risk include occupational groups such as transportation workers, operators of heavy construction equipment, railroad workers, and miners. A second difficulty has been to document the actual concentrations of diesel exhaust to which the individuals have been, or are, exposed.

In epidemiologic studies, exposure can be assigned either quite broadly, based on job classification and work site, or more precisely, by sampling ambient air over time. In early epidemiology studies of the health risks of diesel engine exhaust, past exposures were typically estimated by questionnaire, job description, or, occasionally, by air sampling at current job sites. Exposures that are known to confound estimates of diesel exhaust exposure include cigarette smoke, coal dust, inorganic dusts, and asbestos (Smith et al. 1984; Wynder and Higgins 1986; Woskie et al. 1988); cigarette smoke is, by far, the largest confounder.

Several recent reviews of epidemiologic investigations were designed to examine the possible association between cancer and occupational exposure to diesel engine exhaust (Wynder and Higgins 1986; McClellan 1987; International Agency for Research on Cancer 1989; McClellan et al. 1990). The exposed populations included employees in the railroad industry (Kaplan 1959; Howe et al. 1983; Garshick et al. 1987, 1988), transportation workers (Raffle 1957; Silverman et al. 1986) and miners (Waxweiler et al. 1973). These investigations focused on the risk of lung and bladder cancer. The earlier studies generally yielded negative or equivocal results, but because of their many limitations, particularly problems with exposure assessment and the failure to exclude cigarette smoking as a confounding factor, it is difficult to draw definitive conclusions.

Some of these limitations were addressed in more recent studies in which investigators examined the relationship between lung cancer and diesel engine exhaust exposure in a large population of American railroad workers. In the first study (Garshick et al. 1987), a case-control study, diesel exhaust exposure in workers who had at least ten years of railroad service was estimated from an evaluation of job descriptions and work areas, as provided by the U.S. Railroad Retirement Board. In a later report, the same group of investigators (Woskie et al. 1988) provided an analysis of the current levels of exposure to respirable particulate matter as determined from an analysis of personal and area samples collected from four railroad facilities in the United States. Cause of death was determined by death certificates, and next of kin were surveyed to obtain information on smoking. The relative hazard of lung cancer attributable to exposure

to diesel engine exhaust was calculated using a multiple conditional logistic regression to correct for smoking and asbestos exposure. A relative odds ratio of 1.41 (95 percent confidence intervals of 1.06 and 1.88) for lung cancer was found among workers who were 64 years of age or younger at the time of death and who had 20 years or more of exposure to diesel exhaust. No effect of diesel exhaust exposure was seen in the group of older workers (age 65 to 82 years at the time of death); this group was evaluated separately because they were considered by the authors to have had less chance of working in a job setting where they were exposed to diesel exhaust.

In the second study, a retrospective cohort study, Garshick and coworkers (1988) assessed the risk of lung cancer in a cohort of 55,407 railroad workers who were 40 to 64 years of age in 1959, and had been employed in railroad service at that time for at least ten years. Smoking histories were not obtained. The cohort was followed until 1980, at which time there had been 19,396 deaths. Examination of the death certificates (88 percent were obtained) revealed 1,694 cases of lung cancer. A proportional hazards model and directly standardized rate ratios were used to calculate the relative risk of lung cancer, which was significantly elevated in those workers with the longest period of exposure to diesel engine exhaust (relative risk of 1.45; 95 percent confidence intervals of 1.11 and 1.89).

Epidemiological studies, such as those discussed above, have frequently used job category descriptions to estimate exposure. The serious drawbacks that are apparent with reliance on the use of questionnaires and job descriptions include recall bias, misclassification, nonresponse, and imprecise interpretation of terminology. Collecting ambient air samples at each job site and identifying constituents of diesel engine exhaust and environmental tobacco smoke are steps that have been taken to improve our understanding of exposure. These measures, however, may not always relate to the actual dose to the worker because of individual variations in respiration rate, depth of inspiration, and activity patterns. Monitoring personal exposure in combination with monitoring ambient air has the potential to provide more accurate information on the actual exposure of different populations to airborne pollutants.

In the environmental health field, there has been increasing interest in developing markers to monitor personal exposure, and to relate exposure to internal dose. Biological markers have been defined as indicators of events in biologic systems or samples (National Research Council 1989). They can be classified as markers of exposure, markers of effect, and markers of susceptibility. A number of different approaches and different markers have been evaluated to obtain a quantitative index of exposure to xenobiotics.

Traditional analytical chemistry techniques have been used to quantify organic and inorganic xenobiotics in physiological tissues and fluids. Examples include measuring heavy metals in fingernails and hair and carboxyhemoglobin levels in blood, and analyzing nicotine and cotinine in body fluids. The latter compounds are unique markers for exposure to tobacco, tobacco smoke, or environmental tobacco smoke (National Research Council 1986). Cotinine is excreted in the urine and has been shown to correlate well with exposure to tobacco smoke (Wallace and O'Neil 1987; Jarvis et al. 1988). Such traditional analytical approaches have been used successfully with xenobiotic markers that are unique, or nearly unique, and that are present in amounts that are within the detection limits of available assays.

Other approaches take advantage of newly developed and highly sensitive biochemical techniques to monitor exposure to certain compounds, especially carcinogens. Examples of such biochemical markers are macromolecular adducts that are formed when metabolically activated agents react with critical macromolecules, such as DNA, RNA, or proteins. These adducts can be detected in target tissues, blood, or urine. Both diesel exhaust exposure (Jackson et al. 1985; Wong et al. 1986) and cigarette smoking (Everson et al. 1986; Randerath et al. 1986) have been shown to lead to the formation of DNA adducts. The challenge to researchers is to assign the relative fraction of adducts to the appropriate exposure source.

Analyzing urine for mutagenicity is another approach to monitoring exposure to genotoxic agents. Because urine is easily obtained, and represents a major route of excretion for xenobiotics, measuring urine mutagenicity is potentially a powerful tool for estimating exposure in field studies. While analytical assays are usually tailored to detect specific compounds, or classes of compounds, mutagenicity assays respond to a broad spectrum of chemicals and also detect as yet unidentified compounds.

The *Salmonella* mutagenicity assay (McCann et al. 1975) is the most widely used in vitro test for gene mutation, because it is rapid, inexpensive, and sensitive to a variety of chemical agents, including carcinogens. It is to be noted, however, that nonmutagenic carcinogens have been recognized as a separate class. In the *Salmonella* assay, a strain of *Salmonella typhimurium* that requires histidine to grow, and therefore cannot grow in the absence of histidine unless a back mutation (reversion) occurs, is incubated in a medium containing only a trace of histidine and the test substance. Only revertant cells that do not require histidine for growth divide and form colonies. An S9 homogenate (a 9,000 × g postmitochondrial fraction, generally from rat liver) is used as the metabolic activation system. The entire test consists of incubating *S. tymphimurium* in the pres-

ence and absence of S9, with appropriate controls. The standard *S. typhimurium* tester strains include the TA98 and TA100 strains, which detect frameshift and basepair substitution mutations, respectively. Resins are frequently used to concentrate body fluids and to separate mutagens from histidine and other potentially interfering compounds. Although determination of urinary mutagens is a promising technique to measure recent exposure to specific carcinogens, confounding factors such as diet, smoking, and other environmental exposures need to be carefully evaluated when interpreting the results.

Human exposure to mutagens can occur by a number of routes: air, water, food, and dermal contact. The contribution of diet to the total mutagenic burden is complex. Food may contain accidentally-occurring mutagenic contaminants, naturally occurring mutagens, as well as mutagens generated during food processing (Sugimura et al. 1981). The mutagenicity of individual food constituents depends on the cooking method and can be modulated by other components in the diet. The mutagenicity of human urine has been shown to be related to the composition of the diet consumed by the subjects (Doolittle et al. 1989). Recently Lioy and coworkers (1988) evaluated the relative contribution of the air, water, and food routes of exposure for benzo[*a*]pyrene and found that, in half of the individuals, diet was the predominant source of benzo[*a*]pyrene exposure.

Several investigators have examined the correlation between exposure to diesel engine exhaust or coal dust and urinary mutagens in experimental animal models. Belisario and colleagues (1984) measured mutagenic activity in the urine of rats 24 hours after administration of diesel particles and noted a dose-dependent increase in mutagenicity, as tested with a *Salmonella*/microsome assay; other groups, however, have not found any increase in urinary mutagens after exposure of rodents to diesel exhaust emissions (Pereira et al. 1981; Ong et al. 1985). Mutagenic activity has been detected in the urine of cigarette smokers using the *Salmonella* assay (Yamasaki and Ames 1977), but not all studies have produced positive results, possibly because of the lack of dietary controls (International Agency for Research on Cancer 1986).

The development of a method to evaluate exposure to diesel engine exhaust using urinary mutagens, supplemented by measurements of personal exposure to diesel and cigarette particulates, represents an experimental approach that warrants further investigation. The use of biomarkers as indicators of exposure to diesel exhaust would contribute to more accurate estimates of exposure in future epidemiological studies, both in the workplace and in the general environment.

JUSTIFICATION FOR THE STUDY

Under RFA 82–3, "Models of Susceptible Populations," the HEI solicited proposals for studies that would describe the existence and importance of susceptible human or animal subpopulations that may be particularly vulnerable to mobile-source emissions. In response to this RFA, Schenker and colleagues proposed to examine markers of exposure to diesel engine exhaust in a population of railroad workers. The investigators planned to explore the usefulness of assays of urine mutagenic activity, as well as the more conventional analysis of personal air samples, to monitor the exposure of individual workers to diesel engine exhaust. They also proposed to develop new approaches to control for the confounding effects of tobacco smoke and other particulate matter.

The study population was to be selected from railroad workers at a single railroad where the investigators had previously measured exposures (Woskie et al. 1988). This occupational group was considered a suitable population for studies of the health effects of exposure to diesel engine exhaust because typical occupation-related exposures are higher for this group than those encountered by the average individual. In previous surveys, the investigators identified a range of exposures to diesel engine exhaust in different work areas within individual railroad facilities (Woskie et al. 1988).

GOALS AND OBJECTIVES

The major objectives of this study were to refine and validate methods for estimating exposure to diesel engine exhaust in epidemiological studies, and to conduct pilot tests of these approaches in a population of railroad workers.

The investigators pursued their objectives through the following specific aims:

1. To use personal air samples to compare respirable particle concentration, a standard measure of exposure to diesel engine exhaust and other particles, to the concentration of phenanthrene, which may be a more specific marker for diesel emissions.
2. To refine methods to measure exposure to another major source of respirable particles, tobacco smoke.
3. To develop and validate methods to use assays of mutagenic activity in urine samples to assess exposure to diesel engine exhaust.
4. To conduct a pilot field survey of both diesel exhaust–exposed and unexposed railroad workers using the meth-

ods for measuring personal exposure to diesel engine exhaust and tobacco smoke that had been developed in specific aims 1 through 3. Nondiesel sources of respiratory particles and urinary mutagens also were to be evaluated.

5. To correlate the concentration of urinary mutagens with diesel exhaust exposure, cigarette smoking, and nonoccupational exposures.

STUDY DESIGN

Area and personal samples were used to determine the exposure of 87 male railroad workers to diesel engine exhaust. Subjects were selected on the basis of job category and work location. All subjects completed a health survey questionnaire that included a brief medical history, exposure history, smoking history, and questions about the consumption of specific dietary items over the preceding two days. Personal samplers were designed to measure respirable particles, nicotine, and phenanthrene. Airborne nicotine concentrations were measured to adjust the respirable particle concentration for active and passive cigarette smoking. Phenanthrene, a polycyclic aromatic hydrocarbon found in high concentrations in exhaust from railroad diesel engines, was evaluated as a potential specific marker for exposure to diesel emissions. A unique sampling system was designed that drew air through three collectors in series: a high efficiency Teflon filter that collected respirable particles, a bisulfate-treated filter that trapped nicotine, and three polyurethane foam plugs that collected volatile organic compounds including phenanthrene.

Additional chamber studies were carried out to determine the nicotine collection efficiency of the filters and the relation between nicotine and respirable particles. Personal air samples were taken over two consecutive work shifts, and urine samples were collected at the end of the two work shifts. Nicotine measurements on the personal air samples, urine concentrations of nicotine and cotinine, and the number of cigarettes smoked during the work shift were used to estimate the contribution of active and passive tobacco smoke exposure to both respirable particle concentration and urine mutagenicity. The relationship between respirable particle concentration, adjusted for cigarette smoke, and diesel exhaust exposure was investigated. Phenanthrene concentration was measured on a subset of personal monitors and correlated with the job grouping index. Mutagenic activity in both air sample filters and urine samples was analyzed using the bacterial tester strains *Salmonella* TA98 and TA100, with and without S9 metabolic activation.

TECHNICAL EVALUATION

This study was undertaken as a follow-up to a large epidemiological study on the health effects of diesel exhaust exposure in railroad workers (Garshick et al. 1987, 1988; Woskie et al. 1988). Previous reports indicated that subgroups in this population were exposed to a wide range of respirable particle concentrations from diesel exhaust: the lowest mean was for clerks (17 $\mu g/m^3$), and the highest means were for mechanics (114 $\mu g/m^3$) and electricians (134 $\mu g/m^3$) working in repair shops (Woskie et al. 1988). While the exposures of the railroad workers were high, they were not as high as those found in some mines where diesel-powered equipment is used (International Agency for Research on Cancer 1989). Locomotive diesel exhaust, in contrast to automotive diesel exhaust, has not been well characterized. However, the analysis of the mutagenic activity of automotive diesel exhaust and locomotive diesel exhaust, as presented in the current study (Figures 3 and 4), suggests that the pattern of the dose-response curves of mutagens in the two exhausts may be similar. Unfortunately, direct comparison of mutagenic activity was not possible because the diesel exhaust samples from the two sources were collected differently.

ENVIRONMENTAL MONITORING

The investigators developed a new personal air sampling device to obtain simultaneous measurements of total respirable particle concentration, concentration of phenanthrene, and levels of ambient nicotine in railroad workers in five different job categories: brakers, carmen (with and without welders), welders, clerks, engineers, and shop workers. The value for respirable particles, corrected for tobacco smoke, is referred to as the "adjusted respirable particle concentration" by the authors.

Total Respirable Particle Concentration

The data presented in the Investigators' Report do not appear to show clear-cut differences in exposures to respirable particle concentration among workers in the five job categories (see Tables 4 through 6). When the respirable particle levels were corrected for the contribution of tobacco smoke, and the welding carmen were excluded because of exposure to high levels of welding fumes, some correlation with diesel exhaust exposures estimated by job grouping was seen. From these data, it appears that all subjects worked in environments that had similar levels of total respirable particles. The contribution of cigarette smoke to the particle measurements partially obscured diesel ex-

haust exposure differences among the job groups. The number of smokers and the "degree of enclosure" of the work site were found to be important variables that determined respirable particle concentration on the filters.

Phenanthrene

Phenanthrene is one of the polycyclic aromatic hydrocarbons present in high concentrations in diesel exhaust particles. This compound is also present in cigarette smoke at concentrations of 0.08 to 0.62 μg/cigarette in mainstream smoke (International Agency for Research on Cancer 1983). The investigators developed a reliable and convenient method to measure very low concentrations of phenanthrene (0.1 μg or less) in samples of ambient particles collected on the polyurethane filters in the personal air samplers. They used these measurements to make independent estimates of the concentrations of diesel exhaust particles in the work environment. They controlled for phenanthrene originating from cigarette smoke by determining the concentration of phenanthrene and respirable particles in tobacco smoke and by doing subset analysis of personal filters only from nonsmoking shop workers. Phenanthrene concentration in ambient air showed a gradient of exposure with respect to proximity to sources of diesel emission. Thus, the repair shop workers were exposed to more phenanthrene than were clerks and carmen; however, as discussed below, the clerks were exposed to more phenanthrene than expected.

A constant ratio of phenanthrene to respirable particles (0.006 ng/μg) was found in particles collected in the repair shop, where diesel exhaust concentrations were expected to be highest. The investigators assumed that diesel particles generated by similar sources and under similar conditions would show a constant ratio of phenanthrene to particulate mass. Using the phenanthrene:particulate mass ratio from repair shop samples, they calculated that half of the respirable particles in the personal samples from nonsmoking shop workers were not derived from diesel exhaust, but were from other airborne particulate matter such as sand and dust particles. The personal samples from workers in different job categories (clerks, carmen, and repair shop workers) showed only a weak relationship between phenanthrene concentration and total or adjusted respirable particles (see Figure 9).

The data also show that the clerks, who did not work in the vicinity of any sources of diesel emissions, were exposed to approximately 160 ng/m^3 of phenanthrene, which is more than twice the concentration found on the field blanks (66 ng/m^3), which was also quite high. The investigators were aware that the value for field blanks was anomalously high, but had neither the time nor the resources to investigate this. In this connection, it should be noted that Cantreels and Van Cauwenberghe (1978) reported a gas-phase phenanthrene concentration of 45 ng/m^3 in the ambient atmosphere. In the current study, a comparison of the ratio of phenanthrene to particulate mass suggests that it is unlikely that the phenanthrene to which the clerks were exposed was derived from cigarette smoke; that is, for clerks the ratio of phenanthrene (160 ng/m^3) to respirable particles (120 μg/m^3) was 0.003, whereas in cigarette smoke the ratio of phenanthrene (180 ng/m^3) to respirable particles (1,200 μg/m^3) was 0.00015. The source of the phenanthrene to which the clerks were exposed is not known, and is one of the major unresolved questions of this study.

In summary, the investigators developed a technique for the detection and measurement of phenanthrene in environmental samples; however, the use of phenanthrene as a marker for exposure to diesel exhaust was not fully validated. Validation of this technique, and more extensive studies that use phenanthrene or other chemicals present in diesel exhaust, will be necessary to develop a reliable specific marker for diesel exhaust.

Cigarette Smoke

The levels of environmental tobacco smoke were estimated on the basis of the levels of nicotine collected on filters downstream of trapped particulate matter. The measurement of gas-phase nicotine was facilitated by the development of a filter-trapping technique. This technique appears to be a good method for determining the concentration of environmental tobacco smoke constituents relative to the concentration of respirable particles. The ambient concentration of environmental tobacco smoke was calculated on the basis of the following relationship, as determined in chamber studies: respirable particle concentration = 8.6 × [NICOTINE] + 50.5. The authors interpret the value of the intercept (50.5) to reflect the statistical uncertainty in extrapolating the chamber data to an airborne nicotine concentration of zero (the intercept is highly variable, with a standard error of 125).

Mutagenicity of Respirable Particles from Personal Samples

During the course of this study the investigators also developed a method for measuring the mutagenicity of particle extracts from personal filter samples. They successfully demonstrated that, despite the limited amount of particulate matter collected on the filters in an eight-hour sampling time, the microsuspension modification of the Ames assay is sufficiently sensitive to detect mutagenic activity.

The mutagenicity of the small number of personal parti-

cle samples tested in this study, however, is difficult to interpret. Using the *Salmonella* strain TA98, the mutagenicity of particulate matter obtained from the areas where the clerks worked was 750 to 2,000 revertants/m^3 without S9, and 250 to 1,500 revertants/m^3 with S9. Because the clerks do not work in the vicinity of any source of diesel emissions, the mutagenicity of these samples was tentatively attributed to environmental tobacco smoke or other ambient sources. However, in contrast to this observation, others have reported the mutagenic activity of cigarette smoke to be greater with S9 activation than without S9 activation, which is consistent with what is known about the composition of cigarette smoke (Löfroth and Lazaridis 1986; Ling et al. 1987). The study design did not include a systematic investigation of the relationship between mutagenicity and airborne nicotine, urinary nicotine, and urinary cotinine. These relationships, and the source of the high mutagenic activity in the clerks' samples, therefore, remain unknown.

URINE MONITORING

One of the objectives of this study was to explore whether or not urinary mutagens could be used as markers of exposure to diesel engine exhaust. Urine samples were collected at the end of two consecutive work shifts and monitored for markers of smoking (nicotine, cotinine, and thiocyanate) and for mutagenicity. Mutagenicity was tested by a microsuspension modification (Kado et al. 1986) of the Ames assay (using *S. typhimurium* strains TA98 and TA100, with and without S9 metabolic activation).

Cigarette Smoke Exposure

The investigators made a strong effort to determine the contribution of exposure to tobacco smoke to the observed mutagenicity of urine. Levels of nicotine, cotinine, and thiocyanate in urine samples, as well as ambient nicotine from personal air monitors, were measured to estimate active and passive exposure to cigarette smoke. For smokers, the number of cigarettes smoked was also taken into account. Regression analyses suggested that the highest correlate of mutagenic activity in the urine in smokers was cotinine, and that ambient nicotine and urinary cotinine levels provided the best indicators of environmental tobacco smoke exposure for nonsmokers. A report by Thuan and coworkers (1989) indicates that another nicotine metabolite, 3-hydroxycotinine, may actually account for 50 percent of all the metabolites of nicotine. Since 3-hydroxycotinine has been shown to comigrate with caffeine and cotinine in some high-performance liquid chromatography systems, appropriate controls are needed before identifying the gas-chromatographic peak as cotinine. In the present study, other metabolites of nicotine were not investigated and details of the chromatography procedures were not provided.

The number of cigarettes smoked during the work shift showed a strong dose-response relation with urine mutagenicity when tested with S9, and a weak dose-response relation when tested without S9. These findings are consistent with other reports in the literature that state that cigarette smoke contains very little direct-acting mutagenicity (Kier et al. 1974; Löfroth and Lazaridis 1986; Bryant et al. 1988; Löfroth et al. 1988; Löfroth 1989; Malaveille et al. 1989). No association was detected between markers of exposure to environmental tobacco smoke and mutagenicity of urine samples in nonsmokers, although the levels of cotinine metabolites were elevated in the urine of nonsmokers. It is possible that any increase in urine mutagenicity in nonsmokers was small and the assay was not sufficiently sensitive to detect it.

Diesel Exhaust Exposure

The investigators explored the relationship between urinary mutagens and several indicators of diesel exhaust exposure, including job category, respirable particle concentration, and phenanthrene level. Appropriate corrections were made for the contribution of exposure to cigarette smoke. In both smokers and nonsmokers, exposure to diesel exhaust was not a significant independent predictor of mutagenic activity in urine samples, either with or without S9.

In this report, the investigators present their data in summary form; the report would have been strengthened by the inclusion of the critical primary mutagenicity data on the urine samples. Others seeking to evaluate the genotoxicity of diesel emissions will, undoubtedly, want to examine both the primary data on the mutagenicity of urine samples, as well as the actual responses of the positive and blank controls, with and without S9 metabolic activation.

The investigators conclude that, although they found an association between the levels of mutagenicity in urine samples and active cigarette smoking, they were unable to discern a correlation between mutagenicity of urine and exposure to the emissions of diesel engines. As they correctly point out, this lack of correlation does not, in fact, rule out the possibility that the emissions of diesel engines are mutagenic, or that they are metabolically converted to mutagenic compounds. Several comments regarding these observations follow.

First, the investigators assumed that the bioavailability of mutagens from diesel exhaust follows a similar pattern to that of cigarette smoke. However, this assumption may not

be valid because in addition to differences in the size of the constituent particles, diesel engine exhaust is chemically different from cigarette smoke. Cigarette smoke contains very little elemental carbon (less than 10 percent), whereas diesel exhaust particulates contain more than 60 percent (Zaebst et al. 1988). It is possible that smaller amounts of mutagens may be present in the urine of diesel-exposed subjects than the investigators calculated, reducing the power of the study to detect mutagens originating from exposure to diesel engine exhaust. This possibility is reinforced by the difference in the nature of mutagens in cigarette smoke and diesel emissions; that is, polycyclic aromatic hydrocarbons are found in cigarette smoke, whereas nitropolycyclic aromatic hydrocarbons are found in diesel exhaust.

Second, it should be noted that the investigators stored urine samples (median time 8.5 months) while they were developing some of the methods used in this study. The storage time for each sample was documented in the report, as well as the results of a pilot study to examine the effect of storage on mutagenic activity and thiocyanate concentrations (Appendix B). Although storage for six months did not appear to affect the mutagenicity of the urine of smokers or of nonsmokers in a consistent direction, the small number of samples makes generalization difficult.

Third, because there is little information on the nature of the diesel-derived urinary mutagens, or on their pharmacokinetics, it would have been preferable if the experimental procedures for recovery and screening of the mutagens had been optimized by prior studies with animals exposed to diesel exhaust. Such experiments, however, were beyond the scope of the present work.

Finally, it has long been known that most polycyclic aromatic hydrocarbons are excreted in the urine after conjugation with carbohydrates or sulfates (Miller and Miller 1977; Conney 1982; Nebert 1982). In this study, some of the mutagens could have been converted to highly reactive electrophilic species, such as *N*-hydroxylaminoarenes, which are expected to react with target molecules prior to elimination. Alternatively, some might have been converted to species that are no longer mutagenic, or to species that exhibit decreased mutagenicity, such as the aminoarenes, which are much less mutagenic than their nitro analogs. The possibility that the negative results reported by the investigators are related to bioconversion of the mutagens cannot be ruled out.

Several other explanations, were offered by the authors for the lack of a correlation between diesel exhaust exposure and mutagenicity in the urine samples. They include lower than expected exposures to diesel exhaust, misclassification of exposures among job categories, and lack of study power due to both the lack of sensitivity of the mutagenicity test and the small numbers of urine and personal filter samples that were tested.

OTHER FACTORS

In addition to exposure to cigarette smoke, dietary factors had some effect on urine mutagenicity. Self-reported ingestion of those foods that have been shown to inhibit mutagenicity (such as cabbage or Brussels sprouts) was associated with decreased mutagenicity of the urine samples of nonsmokers in the S9-activated *S. typhimurium* TA98 assay. No significant effect of protective foods was found for nonsmokers in the absence of S9, or for smokers in either assay. Also, no enhancement of mutagenicity was seen in the urine of subjects who indicated that they had consumed foods that are reported to be mutagenic (broiled or grilled meats) (Doolittle et al. 1989). Although these findings are not always in agreement with other reports regarding the impact of individual dietary constituents on urinary mutagens (Doolittle et al. 1989), they suggest a need to consider dietary variables in the design of epidemiology studies.

IMPLICATIONS FOR FUTURE RESEARCH

From the results presented in this report, as well as from the work by several other groups (National Research Council 1986; U.S. Department of Health and Human Services 1989), it appears that the reliability of estimates of cigarette smoke exposure have been improved. Further research is needed, however, to develop reliable biomarkers for ambient levels of diesel engine exhaust. In addition to phenanthrene, other compounds present in diesel engine exhaust that may serve as markers include 2-nitrofluorene, 2-nitronaphthalene, 4-nitrobiphenyl, and 4-aminobiphenyl (hemoglobin adducts of 4-aminobiphenyl also serve as sensitive markers of cigarette smoke) (Jensen and Hites 1983; Schuetzle 1983; Tong and Karasek 1984; Daisy et al. 1986; Bryant et al. 1988). The ratio of pyrene to 1-nitropyrene in respirable particles may also be a useful marker of diesel exhaust because the concentration of pyrene is 20 times higher in cigarette smoke than in diesel exhaust. Finally, the use of elemental carbon as a marker for diesel particulates should be explored because diesel particulates contain a much larger percentage of elemental carbon than does cigarette smoke.

Further research is also needed to develop and validate biomarkers of the biologically effective dose of diesel engine emissions under specific exposure conditions. Because of the difficulties encountered in this study, it may be desirable to do a pilot study, perhaps in animals, to learn

the optimal conditions for monitoring assays. This could be followed by an investigation, using suitable controls, of exposure-dose relationships in a well characterized human population that has been exposed to relatively high levels of diesel exhaust, such as railroad workers or miners. In addition to measurements of mutagenic activity in urine, other surrogates for diesel exhaust exposure might include protein (for example, hemoglobin), adducts, or antibodies against components of diesel exhaust; some or all of these may be deserving of further investigation. In order to employ such markers as predictors of health risk, a large research effort would be needed to establish their specificity and validity.

As discussed by the investigators, as well as by the Review Committee, many problems were encountered during the course of this study; these difficulties illustrate the complexities in determining exposure to, and the biological dose of, environmental agents. The investigators were able to solve a number of the problems they faced; further research is needed to develop and refine the methods to a stage where they can be used to measure personal dose and to estimate risk from exposure to the emissions of diesel engines.

CONCLUSIONS

Schenker and his coworkers undertook the difficult task of developing methods to estimate ambient exposure levels, as well as the biological dose, of diesel engine exhaust in a well characterized population of railroad workers. The investigators developed and conducted pilot investigations on a sampling device to measure markers of personal exposure to locomotive diesel engine exhaust and tobacco smoke. New techniques were developed for measuring low concentrations of phenanthrene and nicotine that, although they require further refinement, have potential value for future field studies. Phenanthrene concentrations in personal samples from workers in different job categories reflected a gradient of exposure with respect to proximity to sources of diesel emissions; however, the anomalously high levels of phenanthrene found on the field blanks, and the higher-than-expected levels found in samples from clerks who had little or no exposure to diesel emissions, preclude the use of this compound as a specific marker for exposure to diesel engine exhaust without further validation and testing. The technique of collecting gas-phase nicotine downstream from trapped particulates appears to be a good method for determining the contribution of cigarette smoke to total respirable particles.

In a pilot study of 87 railroad workers, no clear-cut differences were noted in the respirable particle concentrations on the personal filter samples of workers in five different job categories, even though there was a wide range in the exposure to diesel emissions. Schenker and his colleagues attribute this finding to the contribution of tobacco smoke to the respirable particle burden.

The investigators demonstrated that a modification of the Kado microsuspension procedure, when used in conjunction with the *Salmonella* mutagenicity assay, is a sensitive test to detect mutagenicity of respirable particles from personal filters.

The investigators evaluated mutagenic activity in the urine of smoking and nonsmoking railroad workers in different diesel exposure job categories. In agreement with other literature reports (National Research Council 1986; U.S. Department of Health and Human Services 1989), they observed a positive correlation between urinary mutagenic activity and the number of cigarettes smoked during the work shift. In the population studied, they observed no association between urinary mutagenic activity and exposure to locomotive diesel engine exhaust. It would be premature, however, to conclude from these data that no such correlation exists. There are many technical factors, discussed in the preceding Investigators' Report and in the Commentary, that could have contributed to the negative findings.

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Special Reports

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

Research Reports

Report No.	Title	Principal Investigator	Publication Date
1	Estimation of Risk of Glucose 6-Phosphate Dehydrogenase-Deficient Red Cells to Ozone and Nitrogen Dioxide	M. Amoruso	August 1985
2	Disposition and Metabolism of Free and Particle-Associated Nitropyrenes After Inhalation	J. Bond	February 1986
3	Transport of Macromolecules and Particles at Target Sites for Deposition of Air Pollutants	T. Crocker	February 1986
4	The Metabolic Activation and DNA Adducts of Dinitropyrenes	F.A. Beland	August 1986
5	An Investigation into the Effect of a Ceramic Particle Trap on the Chemical Mutagens in Diesel Exhaust	S.T. Bagley	January 1987
6	Effect of Nitrogen Dioxide, Ozone, and Peroxyacetyl Nitrate on Metabolic and Pulmonary Function	D.M. Drechsler-Parks	April 1987
7	DNA Adducts of Nitropyrene Detected by Specific Antibodies	J.D. Groopman	April 1987
8	Effects of Inhaled Nitrogen Dioxide and Diesel Exhaust on Developing Lung	J.L. Mauderly	May 1987
9	Biochemical and Metabolic Response to Nitrogen Dioxide-Induced Endothelial Injury	J.M. Patel	June 1987
10	Predictive Models for Deposition of Inhaled Diesel Exhaust Particles in Humans and Laboratory Species	C.P. Yu	July 1987
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18	Respiratory Infections in Coal Miners Exposed to Nitrogen Oxides	M. Jacobsen	July 1988
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20	Modulation of Pulmonary Defense Mechanisms Against Viral and Bacterial Infections by Acute Exposures to Nitrogen Dioxide	G.J. Jakab	October 1988
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25	Acute Effects of Carbon Monoxide Exposure on Individuals with Coronary Artery Disease	HEI Multicenter CO Study Team	November 1989
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Research Report Number 33 October 1990