Diesel Emissions Symposium Proceedings

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16. ABSTRACT

The high fuel efficiency of diesel engines is expected to result in a significant increase in the production of diesel-powered passenger cars. Major research programs were initiated in the late 1970s by governments, industry, and the academic community in order to understand the physical and chemical characteristics of emissions from the diesel engine, and the biological effects of these emissions. In October of 1981, the U.S. Environmental Protection Agency sponsored a Diesel Emissions Symposium to report and review the major scientific and technical information developed from these research programs.

This proceedings volume contains 21 review papers and 79 short papers covering all the oral and poster presentations of the 1981 Diesel Emissions Symposium. The meeting spanned the following subject areas: diesel emissions characterization and control technology; chemical and bioassay characterization; pulmonary function, toxicology, and biochemistry; mutagenesis; carcinogenesis; exposure and risk assessment.

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FOREWORD

The Health Effects Research Laboratory conducts a coordinated environmental health research program in inhalation toxicology, genetic toxicology, neurotoxicity, developmental and experimental biology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis, and the toxicology of pesticides and other chemical pollutants.

The high fuel efficiency of diesel engines is expected to result in a significant increase in the production of diesel-powered passenger cars. Major research programs were initiated in the late 1970s by governments, industry, and the academic community in order to understand the physical and chemical characteristics of emissions from the diesel engine, and the potential biological effects of these emissions.

In December 1979, the U.S. Environmental Protection Agency Health Effects Laboratory at Cincinnati, Ohio, sponsored the first symposium on the Health Effects of Diesel Engine Emissions. The 1981 Diesel Emissions Symposium, sponsored by the U.S. Environmental Protection Agency Office of Research and Development during October 1981 in Raleigh, North Carolina, fostered the exchange of more recent scientific and technical information derived from the various research programs.

This proceedings volume contains 21 review papers and 79 short papers covering all the oral and poster presentations of the 1981 Diesel Emissions Symposium. The meeting spanned the following subject areas: diesel emissions characterization and control technology; chemical and bioassay characterization; pulmonary function, toxicology, and biochemistry; mutagenesis; carcinogenesis; exposure and risk assessment.

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ABSTRACT

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SECTION 1

DIESEL EMISSIONS CHARACTERIZATION AND CONTROL TECHNOLOGY

DIESEL EMISSIONS, A WORLDWIDE CONCERN

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Recent visits to Japan and Europe plus scores of visitors from other coun-
tries have convinced me that there is a worldwide concern over the possible
health effects of diesel exhaust. Not all of these visitors come to San
Antonio to visit the Alamo or stroll by the river.

Laboratory tests with bacteria, animal cells and tissues have shown some
components of diesel exhaust to be toxic, mutagenic or carcinogenic. In addi-
tion to gas phase compounds that have both direct and secondary effects in the
atmosphere, diesel exhaust contains particulate matter of both solid (soot) and
liquid (aerosol) type. The soot particles are less than one millionth of a
meter in size and provide a surface for the aerosols to condense or absorb.
For example, benzo(a)pyrene, a well known carcinogen, is but one of the mate-
rials that are in diesel particulate. Nitropyrenes are currently a popular
group of compounds for study.

Studies in 1977 by Southwest Research Institute's Emissions Research Depart-
ment proved that diesel passenger cars produce particulate on the order of 50
times their gasoline-fueled counterparts.[1] A 1981 report[2] gave 0.31 g/km (0.5
g/mile) as an emission rate from cars. On the average, about 15 percent of
the particulate weight is soluble organics (i.e., extractable with dichloro-
methane solvent).

- It is the soluble fraction of the particulate that has sounded the
  alarm since this contains the materials that have been found to be
  direct acting mutagens by the Ames bioassay test.
- It is this fraction, first collected by SwRI[3] and then evaluated in
  the Ames test in 1977, that resulted in the precautionary notice
  published by EPA that same year.
- It is this fraction which has caused this symposium to be held and
  the previous CRC Dearborn meeting in March 1981, the EPA Cincinnati
  Symposium in December 1979, and the EPA Ann Arbor Symposium in
  May 1978.
- It is this fraction which has caused the legislators, Federal
  officials in the DOE, DOT, EPA, Bureau of Mines and others so much
  frustration and confusion.

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- It is this fraction which has given executives of diesel engine manufacturers and car makers chronic nightmares.
- It is this fraction which has given the voices for environmental protection another item for argument.
- It is also this fraction which has given many of us a scientific challenge.

I loosely gather the governmental policymakers, the corporate executives, and all those for whom you work as "THEY Who Must Be Obeyed." THEY have supported us with money, facilities and the opportunity to investigate diesel particulate. Until recently, THEY have been, on the surface at least, patient, realizing that such research is tedious and long term in nature. Underneath that thin veneer of patience I sense the growing imperative to decide, to rule, and to move on. It is that basic impatience of the U.S. or us.

Can we decide at this conference if there is or is not a problem with that organic fraction? If there is, do we have enough data to prove it? If we don't, what is needed in time and money? Can we convince ourselves and others that the studies should continue? In the meantime, what about dieselization of the passenger cars and trucks in the U.S.? Is it business as usual? Do we suggest diesels be limited in urban and congested areas? Or do we give diesels the green light?

THEY, who must be obeyed, are faced with these and related questions. What to do? Do not think that because your research is incomplete or that you need another five years to complete a health survey of a specific population that THEY will necessarily wait. Diesels in cars and trucks are a quick way to reduce fuel consumption, energy costs and foreign dependence. It is clear from recent changes in environmental thinking that a new policy is emerging.

I am reminded of the analogy with the city traffic engineer who, as traffic at an intersection increases, decides to install a stop sign on the side street to assure orderly traffic flow and prevent accidents. Contrast that with the same traffic department, for whatever reasons, who must wait for three accidents within a year at that same intersection before making a traffic survey. Has environmental policy toward the diesel changed? To read the July 30, 1981 Wall Street Journal article.[4] it may be inferred that it has. The headline on the article by Andy Pasztor reads

"Studies That Find Diesel Fumes Benign Encourage the Easing of Engine Controls."

Incidentally, the medical definition of benign is "of a mild character." News of this article reached me by way of some visitors who began by saying, "Well,

I guess research on diesel exhaust particulate is over since the National Research Council says diesel exhaust is OK." Not having any response other than, "I don't think anyone as yet really knows that answer," I obtained a copy of the article thinking that if it were true, there would be little need for this speech, much less this symposium.

To illustrate my point, I want to read the first three paragraphs.

"WASHINGTON--Increasing numbers of prominent scientists appear ready to grant the diesel engine a clean bill of health. And that's bound to add fuel to the Reagan Administration's effort to roll back diesel pollution rules.

After years of controversy, many public health experts say they are becoming convinced the particles in diesel exhaust don't cause cancer or chronic respiratory difficulties as they once feared. Several scientists, in fact, are urging the administration to loosen a variety of pollution rules for diesel-powered cars and trucks, which are considerably more fuel-efficient than conventional models.

The latest sign of this trend is a report on the effect and future of diesel technology, just completed for the government by a blue-ribbon study group formed by the National Research Council. In the report, expected to be released in the next few weeks, more than a dozen prominent engineers, medical scholars and other experts conclude that air pollution from anticipated wide-spread use of diesel engines won't pose a major health hazard or environmental problem."

As of September 30, 1981, the National Research Council report had not yet been released.

From these recent pronouncements, it appears we have to prove to THEY, who must be obeyed, that there is a clear and present danger from diesel particulate. I wonder if we are able to do this in the next few years, much less in the next few days. In any event, this is the major challenge facing those of us in the health effects business. If we can not prove it, then, like the traffic department, we may have to wait for sufficient statistics before prescribing a cure. The case may be argued in many ways and without facts, it is hard to say who is right.

Regardless of the arguments, THEY, who must be obeyed, have an approach which goes essentially like this. A broad program has been in progress for, say, three to four years with no proof that diesel exhaust is hazardous beyond the

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positive Ames test (which we knew of in 1977). What are the chances of proving a hazard exists, given another three to four years?

It is the same question we ask of ourselves on many topics every day. I submit that the delegates to this conference have a job of convincing THEY, who must be obeyed, of the need to continue. Otherwise, THEY, who must be obeyed, quite likely will become impatient and make those decisions for us using whatever facts are available at the time. You may not agree, but the decision point is very near.

Let us now consider the importance of the diesel and its particulate contribution. Diesels are the workhorse of modern society. Practically all goods are transported by diesel locomotives, and diesel trucks. Practically all construction, farming and tunneling is by diesel tractors of one type or other. Practically all ships of the world are diesel-powered. Practically all emergency or standby power units are diesel. Yet their particulate emissions are a tiny fraction, less than 5 percent, of the particulate emissions from all sources.[5] The on-highway diesel constitutes about one-forth of this 5 percent. Of that, trucks and buses account for most of the particulates. To illustrate, Table 1 shows the annual particulate emission rate per vehicle for four different type vehicles. In one year, a diesel bus or truck generates about 17 times the particulate of a diesel car.

TABLE 1

ANNUAL PARTICULATE EMISSIONS FOR TYPICAL VEHICLES
Based on Three Year Old Vehicles[6]

Vehicle Type	Emission Rate g/mile	Miles/ Year	Pounds/ Year
Gasoline Car (Unleaded Fuel)	0.014	14,000	0.43
Diesel Car	0.50	14,000	15.4
Diesel Bus (2-Stroke Cycle)	1.77	69,000	273
Diesel Truck	1.61	69,000	248

Figure 1, from data in a recent APCA paper[7] by Ingalls and Bradow, projects several rates of dieselization of passenger cars. The 25 percent best estimate agrees with GM's prediction that by the late 1980's, nearly 25 percent of its new car fleet will be powered by diesels.

Figure 2 shows the expected sales of medium- and heavy-duty trucks. Note that 50 percent of medium-duty vehicles of 8500 to 10,000 lbs are expected to be diesel and by 1996, essentially all new trucks and buses over 10,000 lbs

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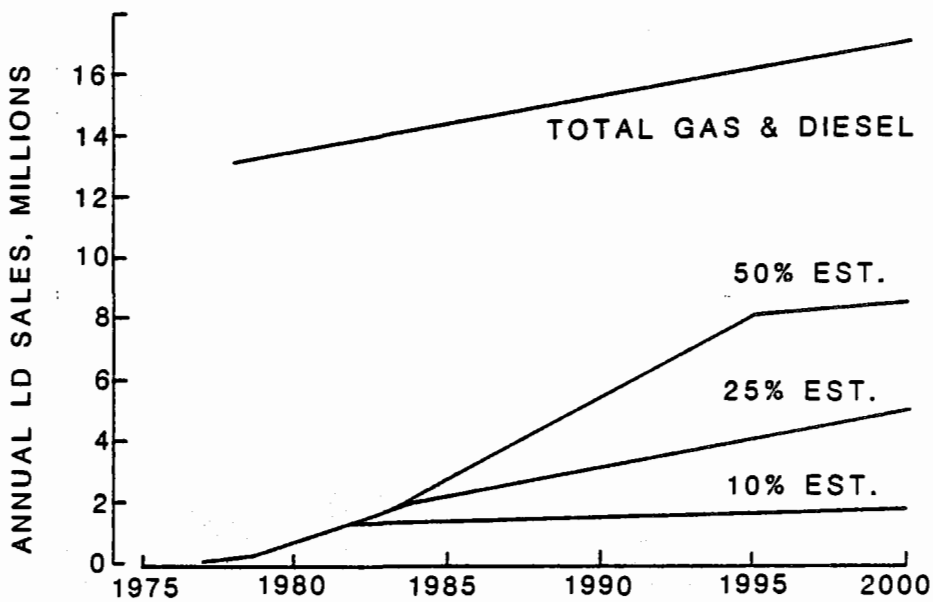
FIGURE 1. PROJECTED SALES PENETRATION
OF DIESEL POWERED LD VEHICLES
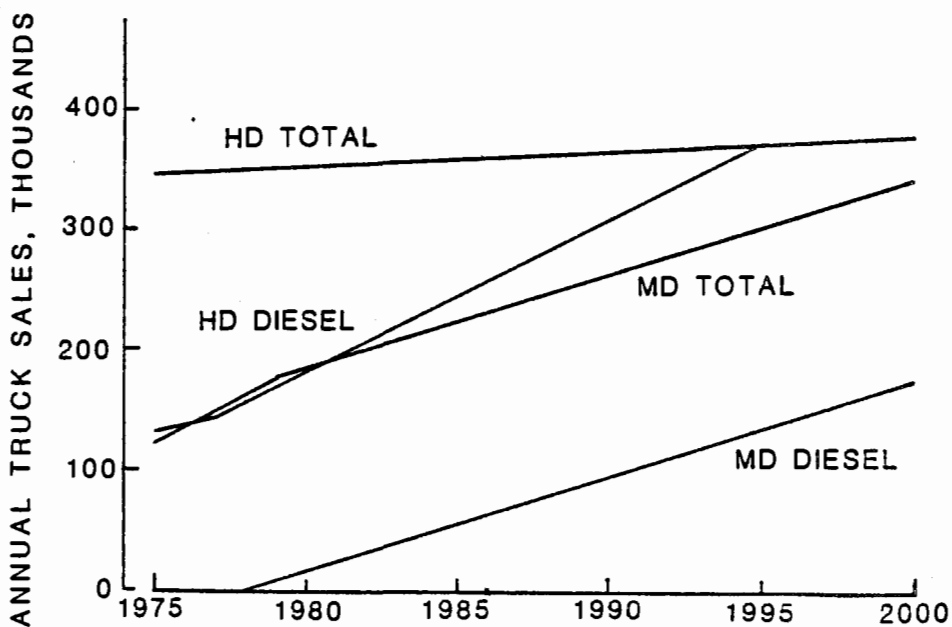
FIGURE 2. PROJECTED SALES OF MEDIUM AND HEAVY DUTY
DIESEL TRUCKS AND BUSES

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will be diesel-powered.

Figure 3 places the current interest in diesel passenger cars in perspective. Given a 25 percent sales penetration (by 1985 and thereafter) of cars powered by diesel engines, it will be 1998 before diesel cars equal diesel truck and bus particulates. With moderate particulate control of both classes of vehicles, particulate parity is not reached until after the year 2000. Diesel car particulates equal gasoline car particulates in 1986. With regulation, diesel cars reach the gasoline car total particulate level in 1989. Control assumes the 0.6 g/mile and 0.2 g/mile light-duty particulate standards take effect as proposed in 1982 and 1986. Control also assumes that new HD diesels must meet about a 30 percent reduction in particulate starting in 1985.

Figures 4 and 5, like Figure 3, project what may happen given a 10 percent and 50 percent penetration of diesels without and with some control of particulates. In the case of ten percent sales of diesels, which is achieved in 1982, the diesel car is only about one-third that of the truck and bus by the year 2000. With some regulation, the light-duty diesel car contribution is less than the gasoline car and is only one-sixth that of heavy-duty by the year 2000.

Assuming a very rapid rate of dieselization of 50 percent, Figure 5 shows that by 1991, light-duty diesel equals heavy-duty diesel and with regulation, 1996 is when diesel cars emit the same tons per year as heavy-duty vehicles. This rate of dieselization is greater than the most optimistic projections, but indicates what may occur if diesel cars become 50 percent of new car sales by 1995.

What do these graphs portray? First, diesel trucks and buses are the major producer of diesel particulate and will continue for many years, even with the best estimate of 25 percent car penetration by diesel engines. Second, comparisons to gasoline particulate are interesting from the total tonnage standpoint but tell us nothing of the relative hazards of each. Third, we still have a very viable alternative to diesel cars albeit a bit less energy efficient whereas we are hooked on the diesel in heavy-duty applications. There simply is no alternative. Fourth, more and more diesels are going to replace more and more gasoline engines in all types of highway vehicles, a trend that is gaining more momentum. Fifth, ought we not consider other circumstances than the total tonnage figures or the big picture?

In a recently published report[8] to EPA, the emphasis was on how to model small or microscale situations in which self-contamination might occur. Self-contamination can occur in parking garages, street canyons, tunnels, express-
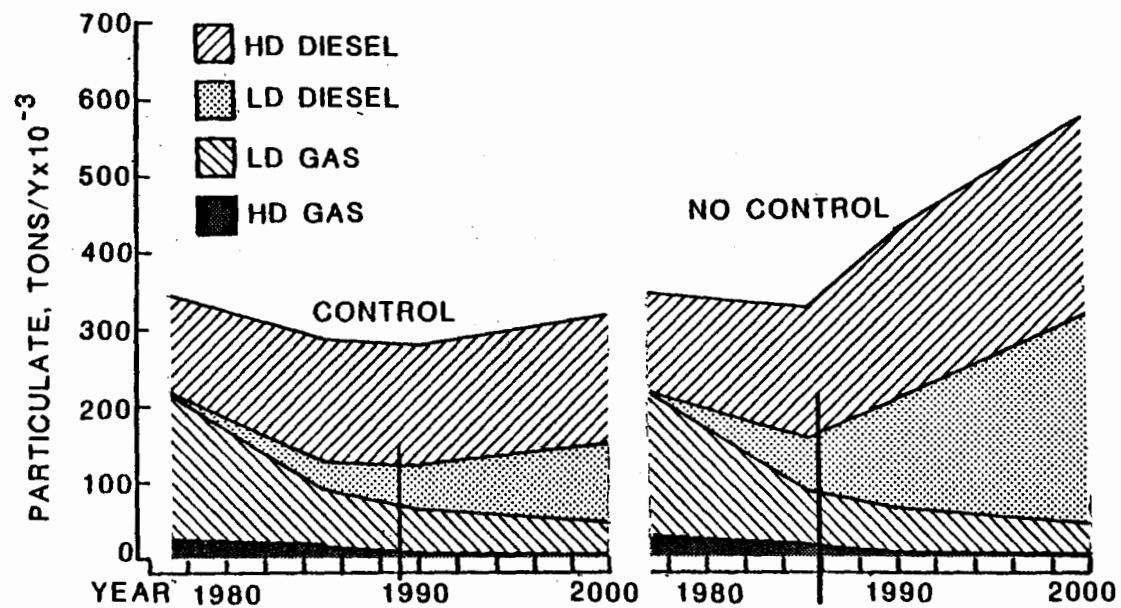
FIGURE 3. PROJECTED PARTICULATE POLLUTION
(25% LDD PENETRATION)
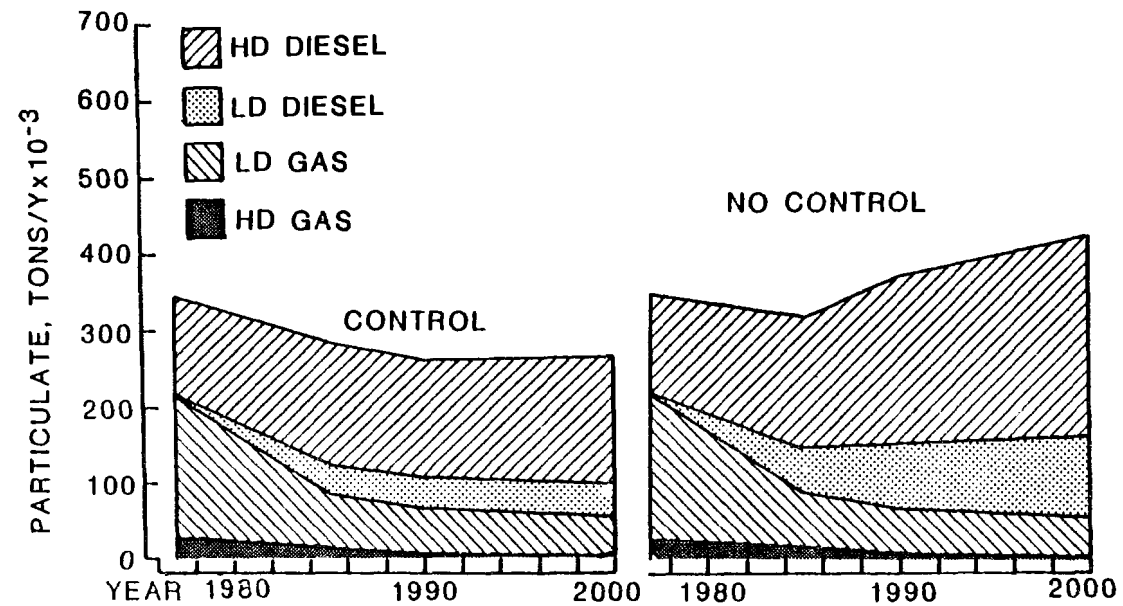
FIGURE 4. PROJECTED PARTICULATE POLLUTION
(10% LDD PENETRATION)
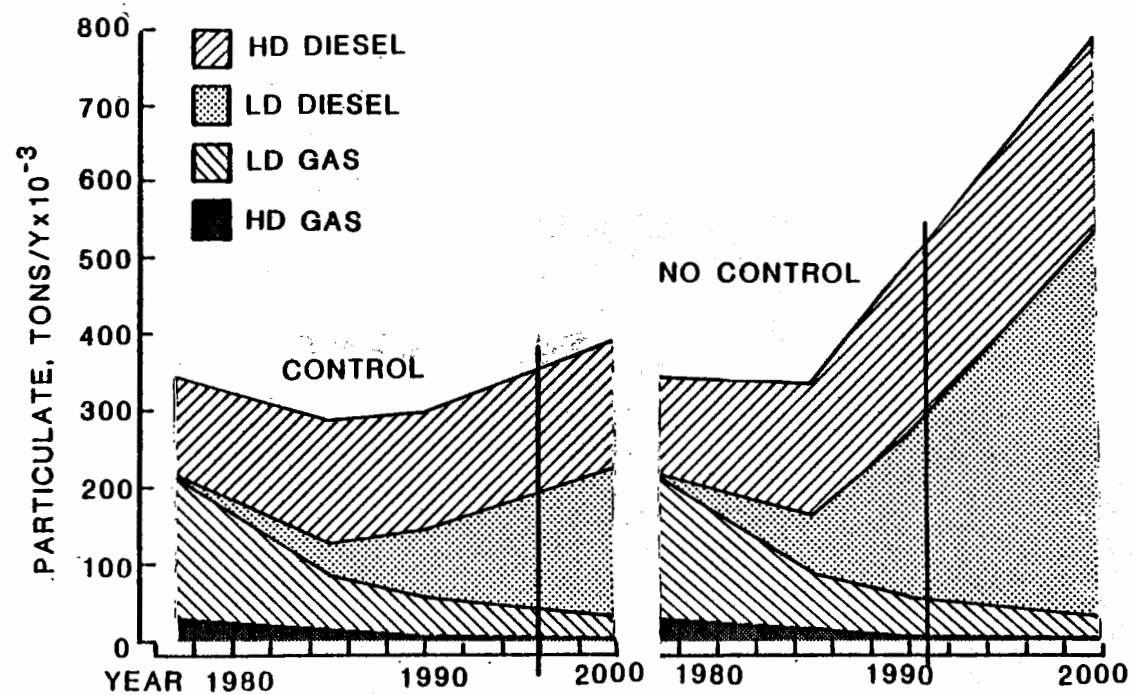
FIGURE 5. PROJECTED PARTICULATE POLLUTION
(50% LDD PENETRATION)

ways or almost anywhere crowded conditions occur with limited dilution. The current maximum 24 hour limit for total suspended particulate (TSP) is 260 $\mu g/m^3$. For example, in a typical above ground multilevel parking garage, the TSP can be exceeded by having as few as 12 percent of the cars diesel-powered. These localized concentrations may be important, if not from health, from public welfare, such as odor and eye irritation and reduced visibility, increased soiling, etc.

Most tunnels, underground parking garages and all underground mines have forced ventilation to dilute and remove exhaust fumes. This is an installation and operating cost of some significance and depends on the type and amount of exhaust generated. I know of a tunnel in Switzerland which must close occasionally due to reduced visibility from diesel traffic. I would assume that during peak conditions, one would be advised to take a deep breath on entering.

Earlier, I challenged this conference to decide whether a health problem exists with diesel exhaust or not. Now, I want to direct a few, more pointed remarks to the audience and especially THEY, who must be obeyed. I know you are here or will learn of this from the publication of this book. Your task is not to merely endure, although the veterans of the last such symposium in Cincinnati surely deserve a commendation for valor and courage above and beyond the call of duty. Your challenge is to assimilate or in plain English, to take up, absorb, incorporate, digest and compare. Then, you are to ruminate. In other words, turn it over in your mind, reflect on and think about it. Remember to assimilate and ruminate. When you are being shown a rat lung all black and sooty for the 92nd time, or the one-hundred and forty-sixth macrophage slide, don't forget to assimilate and ruminate.

My next challenge is to the speakers. Please note that the official language of the session is English. I have asked the symposium organizers to raise a white flag (to indicate surrender) when a speaker loses control with the very complex medical terms. Plain English is needed to summarize the importance and if we can not do this ourselves, our years of hard work may be of reduced value to "THEY, who must be obeyed."

My next message is to the organizers. You have done a superb job of bringing us a program that has 56 prepared papers in a three-day period. It is quite ambitious and by all standards, the topics to be covered are certainly adequate. By way of introduction, the topics are:

    Diesel Emission Characterization and Control Technology
    Chemical and Bioassay Characterization
    Pulmonary Function
    Pulmonary Toxicology and Biochemistry
    Mutagenesis and Carcinogenesis

Exposure and Risk Assessment

If this were not enough, we have poster sessions this evening and tomorrow evening. I asked someone what a poster session is, and was told that "It is an adult version of a high school science fair." As an ex judge of several senior division science fairs, I only hope our displays are as good.

Buried within this mountain of macrophages and mutagens are two most interesting developments. They are both relegated to Poster Session No. 2 on Tuesday evening. Two posters describe the CCMC (Committee of Common Market Automobile Constructors) Long-term Inhalation Health Effects program being conducted in Geneva, Switzerland. The other development is the planned Diesel Health Effects Study by the JARI (Japan Automotive Research Institute) for the Japanese automakers and government.

These are two major long-term programs that may well carry on much of the research that has been performed in the U.S. As the diesel health effects program in the U.S. is de-emphasized, and priorities revised, as appears to be happening, we may have to look to Europe and Japan for answers to the questions I posed earlier. Let us hope that these programs have sufficient pesos, patience and perseverance to finish the task.

There is little doubt that the worldwide worry over diesels has given health effects researchers a golden opportunity to study the lung in ways heretofore never tried. The pulmonary system has never been better understood and that in itself is a positive result of this work, although few of us emphasize its significance.

My final challenge is quite simply to

                        Assimilate

                        Ruminate

            and then

                        Communicate.

We must absorb, digest, compare and then think about it and get the meaning into simple terms so that THEY, who must be obeyed, will understand. Only then, will we have done our job well.

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DIESEL PARTICULATE EMISSIONS: COMPOSITION, CONCENTRATION, AND CONTROL

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INTRODUCTION

   The history of diesel particulate emission studies is still being written.
The time and attention of many scientists and engineers are currently being
directed at trying to understand the formation, atmospheric impact, and
health significance of diesel particulate emissions. In the past five years,
considerable information has been assembled on the chemical composition, the
atmospheric concentrations, and the prospects for controlling the fuel and
lubricant by-products of diesel combustion. The investigators of the last
five years have surely benefitted from older studies which defined the problem
and developed the vocabulary and the concepts for most of the current work.
However, new techniques and approaches to study diesel emissions coupled with
changes in diesel-engine technology guarantee new findings which must be re-
ported, digested, and occasionally reviewed.

   This paper will attempt to review recent work on the composition of diesel
particulate and compare it with particulate from other combustion sources.
Likewise, the current and projected concentration of diesel particulate in
urban areas and in other situations will be considered relative to the concen-
tration and composition of other airborne particles. Finally, the limited in-
formation available on the effects of experimental control systems on particu-
late composition will be discussed.

COMPOSITION

Particle Size

   The size distribution of diesel particles has been studied by a variety of
techniques. The most straight-forward approach has used electron microscopy
to look at diesel particles deposited on various collection surfaces.[1-3] The
chain-like configuration of diesel particles is often cited as a characteristic
feature. Variations in both size and substructure have been studied using
different engines, fuels, and operating conditions[1] to obtain clues concerning
the details of particle formation mechanisms.[4] The electrical aerosol analyzer
has been used for rapid accumulation of particle size information.[5,6] Inertial
impactors and dichotomous samplers have been used to fractionate diesel

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particles by aerodynamic size.[1,2,7] However, the proliferation of particle size studies was truncated by the common observation that the mass median diameter of diesel particles is a few tenths of a micrometer. For inhalation studies, it is important to note that the number median diameter is considerably smaller,[2] i.e., fewer than 1% of the particles are larger than 0.05 micrometer. Consequently, diesel particles are easily transported by airstreams and are readily removed from air only by high-efficiency dust filters. Likewise, the submicrometer diesel particles have low settling rates and their deposition velocities are difficult to quantify.[8] The impact of small carbonaceous particles on visibility and atmospheric chemistry and the mechanisms which remove such material from the atmosphere have been discussed recently.[9]

Black carbon

   The analysis of diesel exhaust particulate has stimulated the development of carbon analysis methods which are useful for studying other carbonaceous particles as well. The most common approach is solvent (Soxhlet) extraction which separates the particulate into a soluble and an insoluble fraction.[10] Similar mass fractionations have been made by thermogravimetry in several laboratories.[10-12] Cadle and others in our laboratories have developed a carbon analyzer which thermally distinguishes organic carbon from elemental carbon.[13] Generally, the nonextractable carbon is likewise not volatile, which is consistent with the low hydrogen content of the nonextractable material.[10] The degree of crystallinity of this black carbon may be important in understanding the formation of these particles in diesel engines.[4] This material is chemically unreactive and presumably not toxic. Its accumulation in and clearance from animal lungs will be discussed in this symposium.

Organic materials

   The molecular weight distribution and the carbon number distribution of the organic (extractable) fraction have been reported.[10,14] This material resembles slightly oxidized engine oil. The carbon number distribution determined by gas chromatography begins at about 15 carbon atoms, peaks near C_{20}, and tails to C_{40}, which is roughly the practical limit for gas chromatographic analysis.[14] Cadle et al.[12] have found that carbonaceous material continues to volatilize to $700^{\circ}C$. This observation is consistent with gel permeation results which show molecular weights as high as 5000, about 400 carbon atoms.[10] However, the vast majority of the extractables are lighter paraffinic hydrocarbons which derive from the diesel fuel and engine oil.[15] These compounds

are generally not expected to be toxic. Because of their low vapor pressures, the extractables readily condense or adsorb on small carbon particles. This means they are not likely to be involved in gas-phase photochemical reactions so they do not contribute to smog chemistry. Similar organic materials are found as 10 to 30% of the mass of ambient particulate collected in urban areas.

Some subfractions of the extractables have been subjected to intense study. Fractionation on silica and alumina columns was conducted on the first diesel extract for which Ames mutagenicity was reported.[16] Operational names for the fractions were assigned to aid interlaboratory comparisons. One of the most widely used methods of fractionation today is the high-performance liquid chromatography (HPLC) separation using a Biosil A column. The time trace of the fluorescence of the eluent has been subdivided into regions which roughly correspond to classes of compounds with increasing polarity, from aromatics to highly oxygenated compounds. This method was examined by several laboratories in a recent interlaboratory comparison conducted by the Chemical Characterization Panel of the Coordinating Research Council's air pollution research program.[17]

The polynuclear aromatic hydrocarbon (PNA) fraction of the extract has probably received the most attention. Methods for the measurement of PNA in diesel particulate have greatly improved since the early work of Falk and co-workers.[18,19] In particular, benzo(a)pyrene (BaP) can now be measured in diesel extract at the picogram-per-milligram level using high-performance liquid chromatography[20] or thin-layer chromatography with fluorescence detection.[21] BaP has been determined in the particulate from all types of vehicles[22] and from other combustion sources.[23] BaP continues to be used as an indicator of combustion particulate and of potential carcinogenicity. Efforts to understand the biological activity of BaP are unequalled and the data base of BaP measurements in ambient air is one of the most comprehensive among air pollutants.

Another subfraction of diesel extract which contains the strongly mutagenic nitro-PNA derivatives has received considerable attention in the past two years.[24-26] Analysis methods for the nitro-PNA in carbon black, in combustion particulate, and in ambient air will be discussed by several investigators at this symposium. Modifications of the Ames bioassay will be described which reveal the pathways by which nitro-PNA display mutagenicity even when the nitro-PNA are present at extremely low concentrations in the extract.[27]

Attempts at complete analysis using powerful mass spectrometric methods have revealed the chemical complexity of the extract fractions. The variety of individual compounds detectable in diesel extract was most recently shown in a

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study by Schuetzle and coworkers.[28] This variety indicates that diesel combustion processes can arrange atoms of C, H, O, N, and S in almost every chemically allowed configuration, albeit at extremely low concentrations.

The detailed chemical analysis of diesel emissions holds challenges for several more generations of researchers. However, the methods available today provide the tools to evaluate sampling methods and sampling conditions, as well as the potential formation of artifactual materials during the sampling process. The work in this latter area has been described by Bradow in this symposium and by others.[24]

Distinguishing features

Despite the application of the best analytical methods, no unique feature of diesel particulate has been identified which clearly distinguishes it from the particulate from gasoline-burning engines or, for that matter, from other combustion sources. For example, Figure 1 shows that the relative amounts of organic and elemental carbon in the exhaust particulate of gasoline-powered vehicles depends on the vehicle type and the operating conditions. The ratio of elemental carbon to total carbon in the particulate emitted from wood and natural gas shows a similar range of values. The per-mile emissions of elemental carbon from vehicles appears to depend more on the air-to-fuel ratio than they do on the fuel or engine type. Diesel particulate is also not distinguishable from particulate emitted from gasoline engines of the stratified-charge type[29] or from homogeneous-charge engines operated in a fuel-rich mode.[23]

The emission rate of BaP from a variety of passenger cars likewise shows that diesels are not unique.[22] On a per-mile basis, noncatalyst, gasoline-powered cars commonly emit more BaP than diesels. Even the relative amounts of the individual PNA from diesel engines are not distinguishable from the relative amounts of individual PNA from gasoline engines.[30]

Likewise, the presence of direct-acting mutagens in diesel extracts and the association of this activity with nitro-PNA are not unique to diesel engines.[25] A study will be reported later in this symposium which has found measurable concentrations of nitropyrene in the exhaust particulate from noncatalyst and catalyst vehicles. And in the same study, the presence of nitropyrene in ambient air samples suggests that nitro-PNA derivatives are formed in many combustion processes and perhaps in atmospheric processes and, therefore, have always been present in ambient air. It is thus clear that diesel exhaust particulate is very similar to particulate from other combustion sources.

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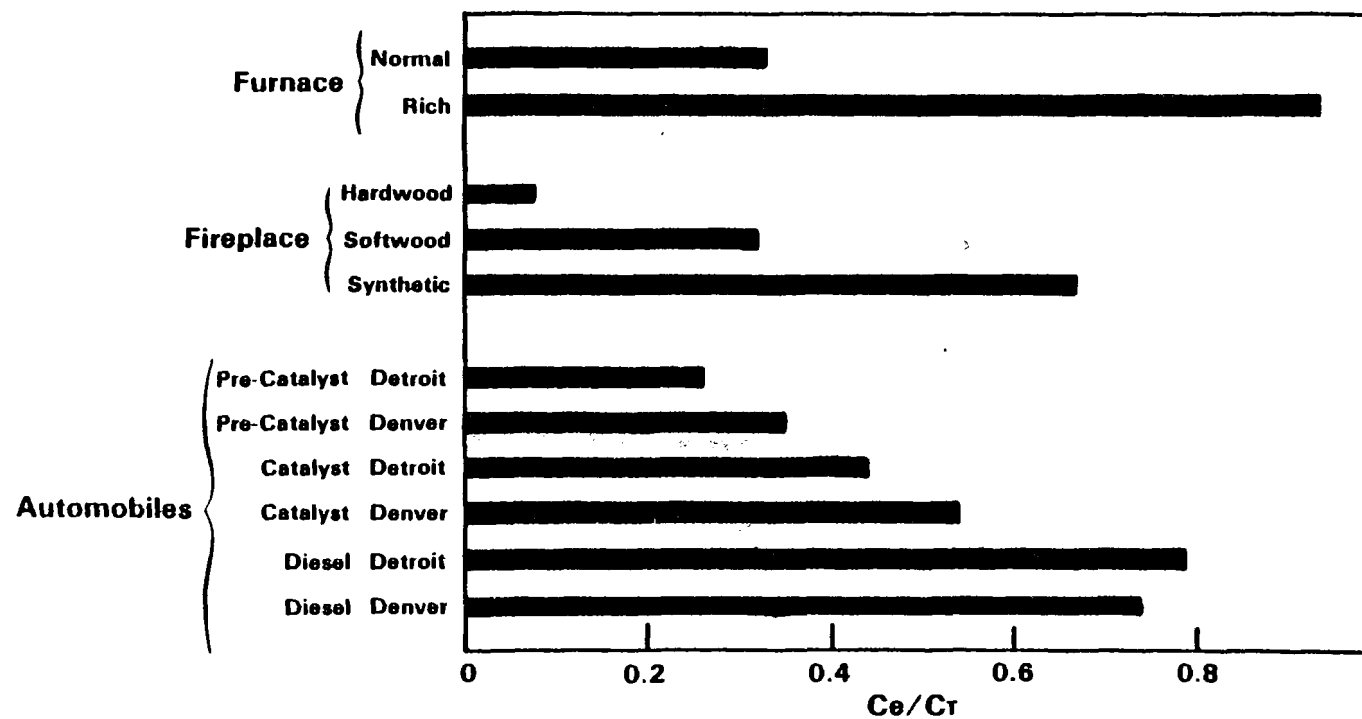
Figure 1. The ratio of elemental carbon to total carbon from selected sources.

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CONCENTRATION

Particulate carbon is an atmospheric pollutant which has not been studied extensively, but it has been suggested that carbon can cause visibility reduction, perhaps promote chemical reactions, and possibly disturb the global heat balance.[9] In the preceding section, I tried to demonstrate that diesel particulate has no unique properties which distinguish it from combustion particulate from other sources. However, since diesel vehicles emit larger amounts of particulate carbon on a per-mile basis than gasoline vehicles, it is important to project the increase in the ambient concentration of particulate carbon from expanded use of diesel-powered cars and trucks.

Modeling approaches

The most commonly used approach for predicting the concentration of diesel particulate for a given time and place has been simple modeling based on carbon monoxide and/or lead as surrogate exhaust components.[31] The data base for CO and lead is very broad because they have been measured at many air monitoring stations and in several special studies around the country. The simplicity of this approach and its direct tie to measured concentrations of vehicle emissions makes it the most reliable predictor because it automatically takes dispersion and source distribution into account.

The input information for predicting the concentration of diesel particulate at any location can simply be scaled to the surrogate emissions. One must define the per-mile emission rates and the vehicle miles traveled by all vehicle classes. For the location of interest, the predicted diesel particulate concentration can be calculated from the lead emission rates, the vehicle miles traveled, and the measured lead concentration using current or historical data. The critical assumption for the surrogate model is that lead particles and diesel particles disperse and transport identically in the atmosphere.

Current and projected diesel particulate concentrations

Estimates of the current input of diesel particulate to the nation's atmosphere range from 80,000 to 120,000 metric tons per year (MT/y).[23,32,33] More than 90% of the diesel particulate currently is from heavy-duty diesel engines in trucks and buses. Based on the urban versus rural miles traveled by heavy-duty diesel vehicles, about 25% of the total national diesel particulate is emitted in urban areas.[34] Of the 20 to 30 x 10^3 MT/y of diesel particulate emitted in urban areas, we estimate that about 75% of it is elemental carbon, which occurs in submicrometer particles.

On a national basis, residential wood burning releases a quantity of elemental carbon roughly equal to that from diesels.[23] Since residential wood burning is concentrated in heavily populated areas, elemental carbon from diesel engines accounts for less than half the total elemental carbon currently emitted in urban areas. This estimate has been confirmed using a broad set of sources of elemental carbon. Wolff and coworkers[35] found that diesel emissions accounted for 24% of the elemental carbon found in Denver's air during Nov-Dec, 1978. The average concentration of elemental carbon in Denver at that time was 5.4 micrograms per cubic meter, which means that the diesel particulate concentration in Denver was 1.7 micrograms per cubic meter (24% times 5.4 micrograms per cubic meter divided by 75% elemental carbon). Similar concentrations (in micrograms per cubic meter) of elemental carbon were found in other cities, such as 13.3 in New York, New York, 4.1 in Downey, California, 3.2 in Pleasanton, California, 3.6 in Pomona, California, 1.7 in Abbeville, Louisiana, and 1.1 in Pierre, South Dakota.[35] Assuming diesels contribute 25% of the elemental carbon in urban areas and that the elemental carbon content of diesel particulate is 75%, the concentrations of diesel particulate in these cities were New York, 4.4, Downey, 1.4, Pleasanton, 1.0, Pomona, 1.2, Abbeville, 0.6, and Pierre, 0.4. Strictly speaking, these estimates apply to specific sampling periods in several different years[35] during which diesel emissions didn't change appreciably.

Using the current data as baseline information, we can estimate the numerical relationship between the total urban emissions of diesel particulate and measured values in several cities. Total urban emissions of 20 to 30 x 10^3 MT/y result in annual-average concentrations of 4 to 6 micrograms per cubic meter in cities with the highest vehicle populations, such as New York and Los Angeles. Correspondingly, ambient concentrations are 1 to 2 micrograms per cubic meter in most other urban areas.

Estimates of diesel particulate concentrations for the future depend on the total amount of diesel particulate emitted and the distribution of diesel vehicles geographically. Particulate emissions from light-duty diesels result in proportionately larger increases in urban particulate concentrations compared with particulate from heavy-duty diesels which travel a smaller percentage of their total miles in urban areas.[34] On a national basis, passenger cars would emit 80 to 120 x 10^3 MT/y (which equals the total mass of heavy-duty particulate emissions in 1981) if the passenger car fleet contained 12 to 18 million diesel cars (10 to 15%) with an average emission rate of 0.6 g/mile. About 60% of the total would be emitted in urban areas and passenger car

diesels would contribute 10 to 15 micrograms per cubic meter of particulate in New York and Los Angeles, and 2 to 5 micrograms per cubic meter in many other urban areas. Because only 10 to 15% of the cars would emit diesel particulate, the fleet-average emissions would be 0.06 to 0.09 g/mile.

These estimates are consistent with the values reported by Bradow and co-workers[36] which were calculated for St. Louis, Missouri, using a more complex dispersion model. For a fleet-average emission rate of 0.195 g/mile, that estimate was 8 to 10 micrograms per cubic meter in the center city with a local maximum value of 13 micrograms per cubic meter. Our estimates are likewise not inconsistent with projected particulate concentrations we have published previously, which assumed a stablized fleet of 25% light-duty diesels with an emission rate of 0.2 g/mile.[31] The equivalent fleet-average emission rate was therefore 0.05 g/mile. In that work, the direct application of the lead surrogate model gave estimates of 6 to 10 micrograms per cubic meter for worst-case cities and 2 to 4 micrograms per cubic meter in other major urban areas.

It is clear, then, that urban concentrations of diesel particulate in the future will depend on the growth rate in miles traveled by light- and heavy-duty vehicles in urban areas and on their emission rates. Based on current measurements of elemental carbon in urban locations, each 25×10^3 MT/y of urban emissions nationally would produce annual-average values of 1 to 2 micrograms per cubic meter of diesel particulate in major U.S. cities.

CONTROL

The prospects for increased use of diesel engines has stimulated efforts to develop new technologies for reducing their particulate emissions. In this section, recent results from two different control approaches will be examined to determine the effect they have on the composition of the particulate as well as on the total quantity. We emphasize that the experimental particulate control systems described here are selected from the broad range of possible control systems simply because they are available for particulate characterization studies at this time. Because the health-effects studies being conducted predate the availability of particulate control systems, we think it is important to make a preliminary assessment of how potential control approaches affect the composition of the particulate.

Experimental catalyzed particulate trap

Catalytic devices in several configurations have been applied to diesel exhaust systems to lower the emission rate of gaseous hydrocarbons from diesel

engines.[37,38] As early as 1979, reports appeared in the literature which showed that catalyst temperatures sufficient to oxidize hydrocarbons were generally sufficient to convert significant quantities of sulfur dioxide to sulfate.[38-40] This has been given as the reason for an increase in the particulate emission rate with the installation of a catalytic converter.[39] At the same time, one of the most common approaches to reducing the particulate emission rate of diesel engines is to use a trap to remove the particulate and to periodically burn the material which accumulates in the trap.[41-43] In order to minimize the ignition temperature of the trapped material, trap surfaces are sometimes coated with noble-metal catalysts.

The composition of the particulate from one system of this type has been determined. The particulate trap was a single underfloor trap of metal mesh with a coating of alumina impregnated with precious metals. The car which was equipped with a 5.7-L diesel engine had been driven about 1000 miles and the trap had been regenerated about 10 times before we tested it. Ignition of the trapped particulate was initiated by throttling the engine manually.

A brief program was run to determine the chemical composition of the particulate emitted during several driving modes and during trap regeneration. The mass emission rate and composition of the particulate emitted in the EPA-specified (FTP) driving cycle were determined. Higher speed driving cycles were also used to determine the sulfate emission rate at higher exhaust temperatures. Steady-state tests of 64 km/hour were run to characterize the emissions during storage and during regeneration of the trap. This speed, i.e., 64 km/hour, was a convenient driving condition for monitoring the regeneration.

Particulate samples were collected from our large dilution tunnel on Dexiglas filters.[10] The filters used for sulfate determination were pretreated with hydrochloric acid to pacify the basic sites normally found on fiberglass filters. The determination of sulfate, extractables, benzo(a)pyrene, and organic and elemental carbon in diesel particulate have all been described previously.[10,12,20,31] In each of the trap regeneration tests, a white cloud was observed through the polyacrylate walls of the dilution tunnel. At the same time, the white cloud was visible at the outlet of the tunnel system on the roof of the laboratory. The white cloud was also observable during regeneration on the road.

The results of the chemical characterization for various conditions, including regeneration, are summarized in Table 1. Because no tests were run on this car without the catalyzed trap, direct measurements of removal efficiencies for the individual components of the particulate are not available.

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TABLE 1

COMPOSITION OF THE EMITTED PARTICULATE (in mg/mile)

Test Mode	Total Par-ticulate	Extract-ables	BaP	Organic Carbon	Elemental Carbon	Sulfate
Federal Test Procedure cycle[a]	155	8.0	0.0003	7.6	109	4.6
Highway Fuel Economy Test cycle	181	71	<0.00001	3.4	53.9	50
Sulfate Emission Test cycle	229	76	<0.000008	5.1	73.8	63
64-km/h-storage	55	6.2	0.00017	4.2	35.2	3.1
64-km/h regeneration	5360	4000	0.0002	30	40	2530

[a] Calculated from cold-start and hot-start 18-cycle results; weighted 43% cold-start and 57% hot-start.

However, I will assume the composition of the particulate emissions from this engine was typical of other 5.7-L diesel engines.[23,44] Normally, organic carbon is about 15% of the total carbon in the FTP cycle while, with the catalyzed trap, organic carbon was only 6%. In the higher speed cycles, organic carbon is typically 25%, while here it was 6%. The organic carbon emission rate was consequently lowered from normal values of 50 to 70 mg/mile down to 3 to 8 mg/mile. In addition, the BaP reduction was about 90% in the FTP cycle and was greater than 99% in the higher speed cycles.

Correspondingly, the amount of conversion of sulfur dioxide to sulfate increased from the FTP cycle to the higher speed cycles. The sulfate emission rates of 50 and 63 mg/mile in the latter cycles represent conversions of 5.7 and 7.8% of the fuel sulfur, whereas typical diesels emit 1 to 2% of the fuel sulfur as sulfate.[45] These sulfate conversions are considerably lower than those observed for similar precious-metal catalysts on gasoline-powered cars, presumably because of the differences in the temperatures.[46,47]

Throttle-initiated regeneration of this experimental catalyzed trap gave a particulate emission rate of 5360 ± 1020 mg/mile. In each case, a white cloud of emissions was observed. The variability of the regeneration emission rate presumably reflects the difficulty in igniting the stored particulate and reproducing the combustion conditions during the regeneration which lasts from 5 to 8 minutes. The total carbon was only about 1% of the total mass of particulate emitted during regeneration.

The total particulate emissions, as shown in Table 1, are not simply the sum of the components listed. A better estimate of the composition of the particulate can be obtained by adjusting the chemical components of the particulate to the mass they represent in the gravimetric determination of total particulate. For example, the carbon analysis ignores the hydrogen, oxygen, and other noncarbon elements which actually contribute to the mass of the two carbonaceous fractions.[10] Mass adjustment factors have been determined using several diesel engines which were not equipped with exhaust-trapping equipment. These results showed that the organic carbon is only 70% of the mass of the organic material volatilized from the sample in the first step of the carbon analysis, and that elemental carbon is only 90% of the residual black carbon which is oxidized in the second step.[12] Therefore, the emission rates of the carbonaceous fractions must be increased appropriately to account for the total mass. Likewise, the sulfate determined analytically is only 45% of the mass of the particulate sulfate actually weighed under the balance room conditions. The remainder of the particulate sulfate mass is water, which associates with the sulfate in the balance room as it also does in ambient air. Using these mass-adjusted components, Figure 2 shows the composition of the exhaust particulate in five different test modes. These three components account for 96 ± 4% of the total mass, which supports the use of the mass adjustment factors.

The most dramatic change in the particulate composition occurs during regeneration. At 64 km/hour, the emission rate of black carbon during regeneration is identical with its emission rate during storage. However, the total mass emission rate during regeneration is 100 times the emission rate during storage. During regeneration, the sulfate material accounts for more than 98% of the total particulate emitted. During storage, it appears that a small amount of diesel particulate passes through the trap and carries with it the hydrocarbons normally associated with diesel particles. However, during regeneration the temperatures are apparently sufficient to volatilize some organic carbon and to selectively burn high-molecular-weight organic compounds such as BaP.

These results suggest that the biological and health effects studies on typical diesel particulate may not be applicable to this diesel car. In addition, the catalyzed conversion of sulfur dioxide to sulfate must be avoided because of the high sulfur content of diesel fuel. Particularly, traps which store sulfur and release it as sulfate during regeneration may cause unacceptable sulfate concentrations in localized situations.[48]

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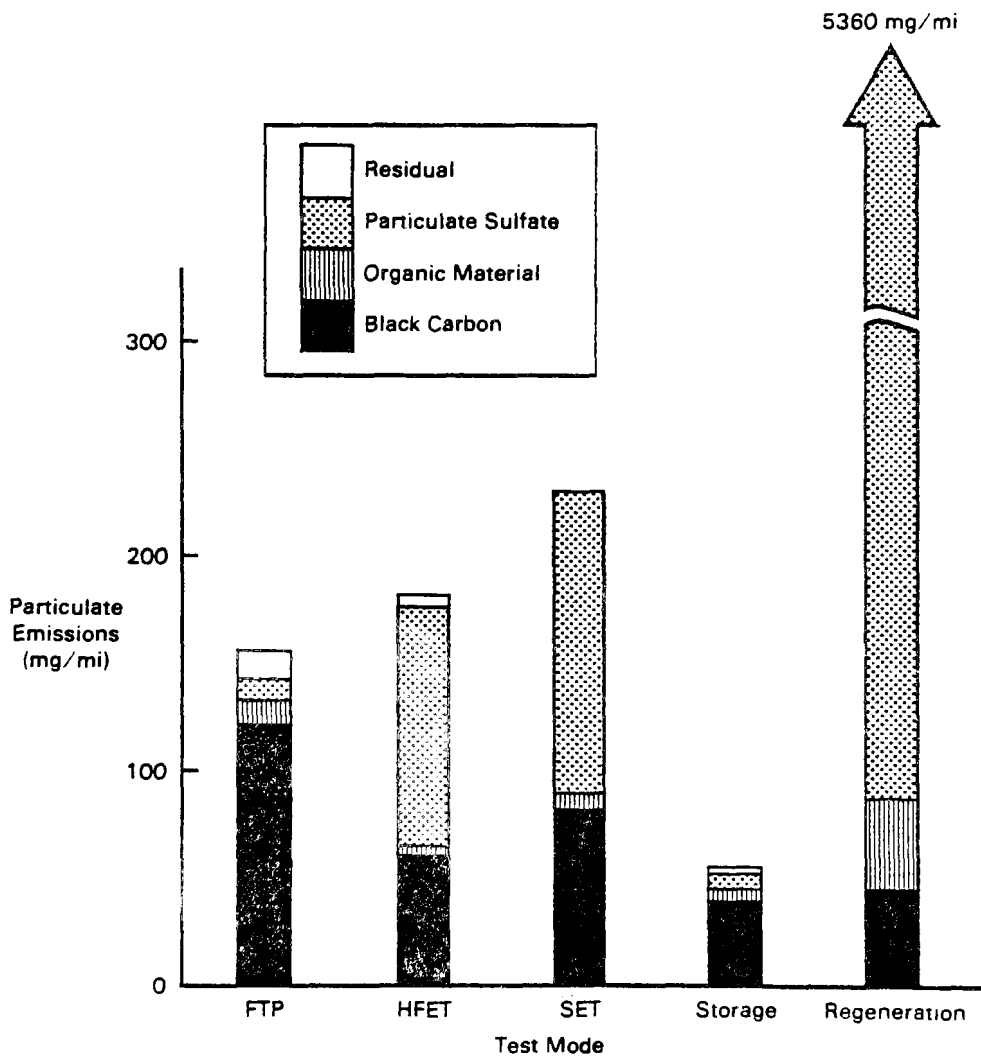
Figure 2. Composition of Exhaust Particulates from Diesel Car Equipped with a Catalyzed Trap.

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Coppered-fuel with experimental fiber trap

The second particulate control system had the following key features:
(a) the addition of copper to the diesel fuel, (b) the use of a tube-type trap
coated with small ceramic fibers which filter the particles out of the exhaust,
and (c) the spontaneous auto-regeneration of this trap under normal driving
conditions. Based on these features, the objective of our testing was to de-
termine the carbon, sulfate, extractable, and BaP content of the resulting par-
ticulate for several test modes as well as to measure the emission rate of par-
ticulate copper.

The coppered fuel and experimental particulate trap had been used on the
vehicle for about 1000 miles before we tested it. Cold- and hot-start urban
test cycles were used since these cycles produce a wide variety of exhaust
temperatures and exhaust flow rates and since these cycles are the most common
basis of comparison between diesel cars. The highway fuel economy cycle was
used for higher but variable exhaust flow rates. A series of steady-state tests
was run at 88 km/hour to allow for accurate monitoring of the inlet pressure of
the trap. In the cyclic test modes, the inlet pressure differences which re-
sulted from changes in the exhaust flow rate obscured the inlet pressure differ-
ences caused by accumulation of particulate in the trap. At 88 km/hour, we were
able to observe a progressive increase in inlet pressure and its rapid return to
baseline values during the regeneration periods. In this way, we were able to
collect separate filter samples during storage and regeneration and to determine
any composition differences. The extent to which continuous combustion occurs
in the trap cannot be determined by our tests.

Elemental carbon. Elemental carbon normally dominates the mass of total
particulate emitted from a diesel engine. The fuel-additive/trap system re-
duced the FTP emission rate of elemental carbon 99%, i.e., from 398 mg/mile to
2.0 mg/mile, as shown in Table 2, which demonstrates the high efficiency of
this trap system for removing solid particles from the exhaust. The emission
rate of elemental carbon was less than 3 mg/mile in all the tests with the
experimental trap.

Particulate organic carbon. Particulate organic carbon is normally emit-
ted at a relatively constant emission rate which appears to be independent of
driving mode and the emission rate of elemental carbon.[10] In the absence of
the particulate trap, this vehicle displayed normal emission behavior. The
emission rate of organic carbon ranged only from 50 to 75 mg/mile while ele-
mental carbon varied 6-fold from FTP to 88 km/hour steady-state driving. The
trap reduced the FTP emission rate of organic carbon 77%, i.e., from 67 mg/mi

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TABLE 2.

SUMMARY OF EMISSION RATES OF PARTICULATE MATERIALS

| Driving Mode | Material | Emissions (mg/mile) | | % Reduction |
		without trap	with trap	
FTP	Total Particulate	649	36	94
	Elemental Carbon	398	2.0	99
	Organic Carbon	67.4	15.2	77
	Extractables	103	28	73
	Benzo(a)pyrene	0.00360	0.00002	99
	Sulfate	30	3.1	90
	Copper	8.4	0.14	98
Highway Fuel	Total Particulate	351	60	83
Economy	Elemental Carbon	172	2.7	98
	Organic Carbon	56.8	35.9	37
	Extractables	86	50	42
	Benzo(a)pyrene	0.00200	0.00006	97
	Sulfate	17	2.1	88
	Copper	5.6	0.16	97
88 km/h	Total Particulate	217	27	88
store[a]	Elemental Carbon	75.1	0.7	99
	Organic Carbon	47.6	13.7	71
	Extractables	68	–	–
	Benzo(a)pyrene	0.0012	–	–
	Sulfate	15	1.5	90
	Copper	4.8	0.18	96
88 km/h	Total Particulate	217	42	81
regenerate[a]	Elemental Carbon	75.1	0.5	99
	Organic Carbon	47.6	19.2	60
	Extractables	68	–	–
	Benzo(a)pyrene	0.0012	–	–
	Sulfate	15	4.2	72
	Copper	4.8	0.25	95

[a] In the without-trap configuration the engine emissions for comparison with storage and regeneration are assumed identical.

to 15 mg/mile. At the same time, more than 99% of the BaP was apparently burned, effectively eliminating BaP from the emissions of this diesel car. Similar trapping efficiencies were observed for organic carbon in the other test modes. The low emission rate of organic carbon during the trap regeneration suggests that most of the stored organic material burns up with the elemental carbon.

Sulfate. In the no-trap configuration, sulfate emissions from this car were similar to those of other diesels, ranging from 30 mg/mile in the FTP to 15 mg/mile in the 88 km/hour cruise mode. The sulfate was 4.6% of the total particulate in the FTP, 4.8% in the highway fuel-economy cycle, and 6.9% in the cruise mode. However, with the trap in place, the sulfate emissions were about 90% lower in all the test modes. As a result, sulfate emissions were only 4 mg/mile during regeneration.

Particulate copper. The rate of injection of copper into the engine under any test condition can be calculated from the copper content of the fuel and the fuel consumption. In the no-trap configuration, the emission rate of particulate copper ranged from 4.8 mg/mile at 88 km/hour to 9.5 mg/mile in the cold-start portion of the FTP. The copper emitted accounts for 62 to 73% of the copper in the fuel consumed. Thus, it appears that this metal-additive system is reasonably self-scavenged and that only a small part of the copper consumed remains in the engine as deposits or accumulates in the engine oil. The emission rate of total particulate from this car in the no-trap configuration (0.65 g/mile) is typical of 5.7-L diesel cars which indicates that copper in the fuel does not appreciably affect the engine-out emission rate of carbonaceous particulate.

With the experimental trap in place, the copper emission rate was reduced by more than 95%, as shown in Table 2. Under trap-storage conditions, the copper emission rate was 0.14 mg/mile in the FTP, 0.16 in the highway fuel economy cycle, and 0.18 at 88 km/hour cruise. Under all these operating conditions, the trap was presumably accumulating copper at a rate of 5 to 10 mg/mile. During trap regeneration, the copper emission rate was only 0.25 mg/mile. Even during oxidation of the carbonaceous particulate on the trap (regeneration), most of the copper in the particulate was retained by the trap. The net result was that only 0.8 to 3.2% of the copper consumed in the fuel was emitted. No attempt was made to determine the chemical form of the emitted copper under any of the test conditions.

Composition of carbonaceous emissions. These emission results show that the efficiency of this control system for removing elemental carbon from the

exhaust is greater than 99%. At the same time, the efficiency for removing organic carbon is about 70%. The emission rate of particulate organic carbon ranged from 15 to 35 mg/mile, but organic carbon accounts for 88 to 97% of the total carbon as particulate. It is important to note that the emission rate of BaP from this fuel-additive/trap system was also very low. This particulate control system markedly changed the composition of the particulate emissions from the composition of typical diesel particulate. However, the use of copper in diesel fuel raises questions about compatibility with current engines and fuel systems as well as questions about the potential environmental and bio-logical effects of particulate emissions from such vehicles. Its application to real-world diesel vehicles is uncertain.

Clearly, the history of diesel particulate emission studies is still being written.

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DIESEL PARTICLE AND ORGANIC EMISSIONS:
ENGINE SIMULATION, SAMPLING, AND ARTIFACTS

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INTRODUCTION

Research into the emission of diesel particles is being conducted by government, industry, and academia. While each group is examining specific aspects, the underlying concern is the potential human health hazard of the emitted particles. This paper will discuss many of the mechanical details of testing vehicles and measuring emissions. In particular it will summarize the current techniques for simulating road operation, discuss the various diesel sampling systems for exhaust particles and organics, and consider artifactual generation of mutating agents. These details, while appearing at present to be relatively unimportant in the determination of effects, are necessary to develop methods of emissions regulation.

SIMULATION OF ROAD OPERATION

Due to the scale of the test equipment involved, it is impractical to measure pollutant emissions from a vehicle operated in traffic. Therefore, considerable effort has been expended in creating an engineering test system capable of physically simulating the emissions of a vehicle operated in traffic while physically stationary in a laboratory.

The road-simulation system used for this purpose is called a chassis dynamometer, a roll-test machine capable of producing resistive forces at the drive wheels of a car or truck. These forces can reasonably match those experienced on the road. Roll-test dynamometers with computer controls are now available to match most of the important road force conditions experienced.

Figure 1 depicts such a dynamometer installed in the U.S. Environmental Protection Agency (EPA) laboratory located in the Research Triangle Park (RTP), North Carolina. Dynamometers with similar capabilities are available at a few other institutions, notably General Motors (GM), Ford, Volkswagen (VW), and the New York State Department of Environmental Quality. Basically, these systems are capable of simulating the aerodynamic drag, inertial forces, and tire rolling resistance of a vehicle operated on a level road. The simulation equations are as follows:

RTP – CELL 1

DYNAMOMETER ROAD SIMULATOR

BELT DRIVE ROLL COUPLER

SPEED TRANSDUCER

FLYWHEEL

150 hp.

TORQUE TRANSDUCER

ACCELERATION

CAT DISPLAY

TORQUE

ROLL SPEEDS

μP CONTROLLER

FORCE = a + bV + cVd + m$\frac{dV}{dt}$

POWER RANGE: –150 TO 150 hp.
INERTIA SIMULATION:
1500 TO 10,000 lb.
IN 1 lb. INCREMENTS
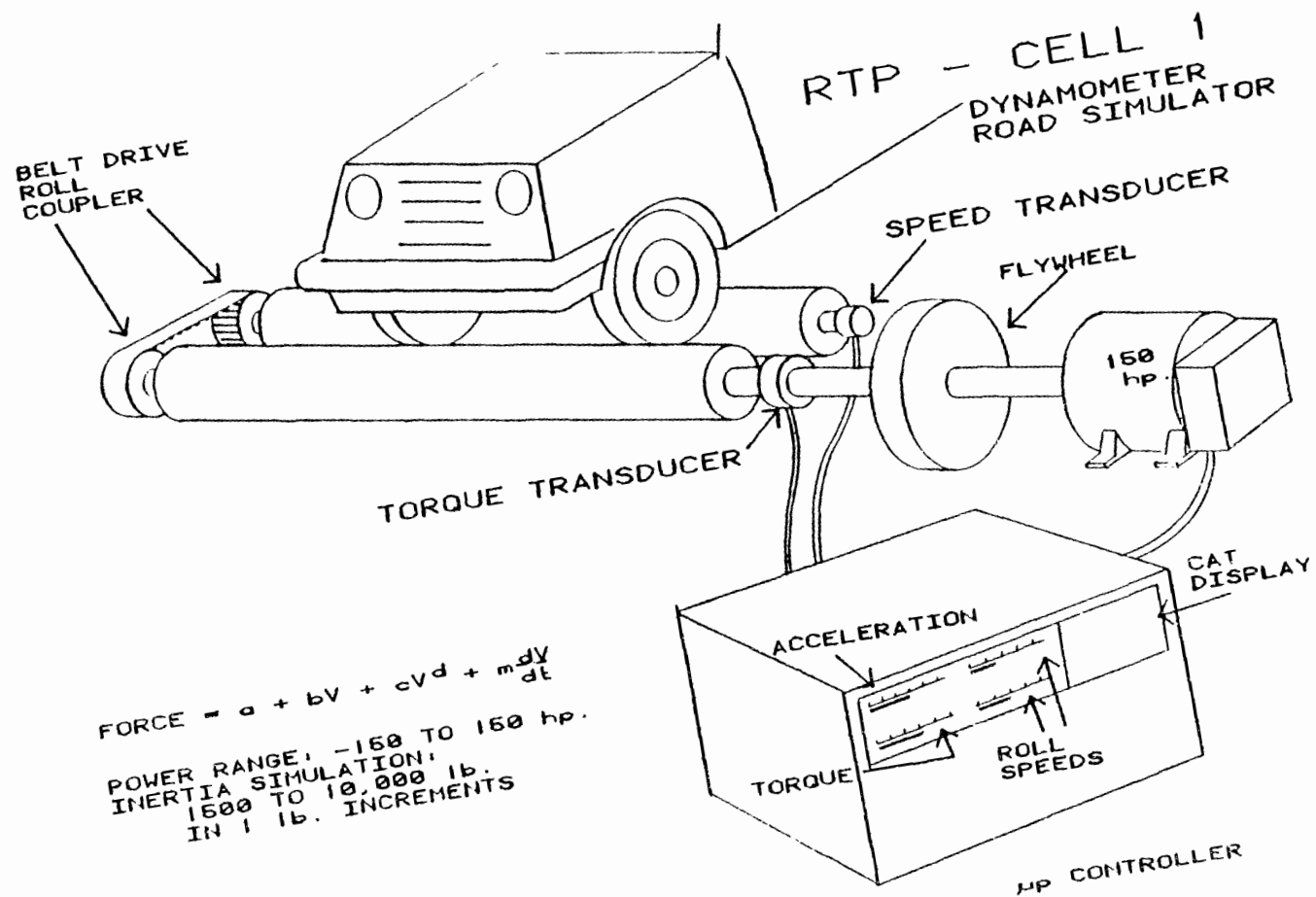
Fig. 1. Layout of EPA-RTP dynamometer.

1. Aerodynamic drag: $\text{Force}_a = C_1 V^2$

2. Interital forces: $\text{Force}_I = (\text{Mass})\dfrac{dv}{dt}$

3. Tire rolling resistance: $\text{Force}_t = C_2 - C_3 V$; where $C_2 \gg C_3 V$

4. Total force: $\text{Force}_a + \text{Force}_I + \text{Force}_t = C_1 V^2 + C_2 (\text{Mass})\dfrac{dv}{dt}$

They are graphically represented in Figure 2. The variations in resisting
force applied to the drive wheels of a passenger car can be seen as a func-
tion of steady-state forward vehicle speed caused by aerodynamic drag and
tire resistance. In addition to these forces, inertial force equivalent to
the mass times acceleration rate is applied either by the use of flywheels,
i.e., physical mass, or electrically by sensing acceleration rate instantane-
ously and controlling applied torque from the electric motor. Next, the
dynamometer is capable of controlling resistive forces to the drive train by
any simulation equation of the form:

$$\text{Force} = a + bV + CV^d + \text{Mass}\left(\dfrac{dv}{dt}\right)$$

Thus, most conditions experienced by real vehicles operated on roadways can be
simulated. There are some real limitations in the availability of road data,
particularly dealing with transient driving, to insure that the road simulation
is nearly perfect. In the next few years, adequate road data will probably be
available to meet this need.

In the meantime, we have several road driving cycles available, generated
principally by using Monte Carlo methods and screening procedures on large
speed-time data bases. The available passenger car cycles are shown in Table 1.
Each of these is a speed-time route representing some facet of normal operation
in which emissions could be a problem. Table 1 shows a few of the pertinent
features of these cycles, including average speed, distance, and number of
complete stops. The routes currently used span a wide range of driving
conditions from cross-town driving in lower Manhattan to driving on a two-lane
country highway.

In dealing with the forces applied, an automobile engine experiences a
variety of speed-load conditions. Figure 3 shows what happens to a VW Rabbit
passenger car as it operates on the highway fuel economy test cycle (HWFET)

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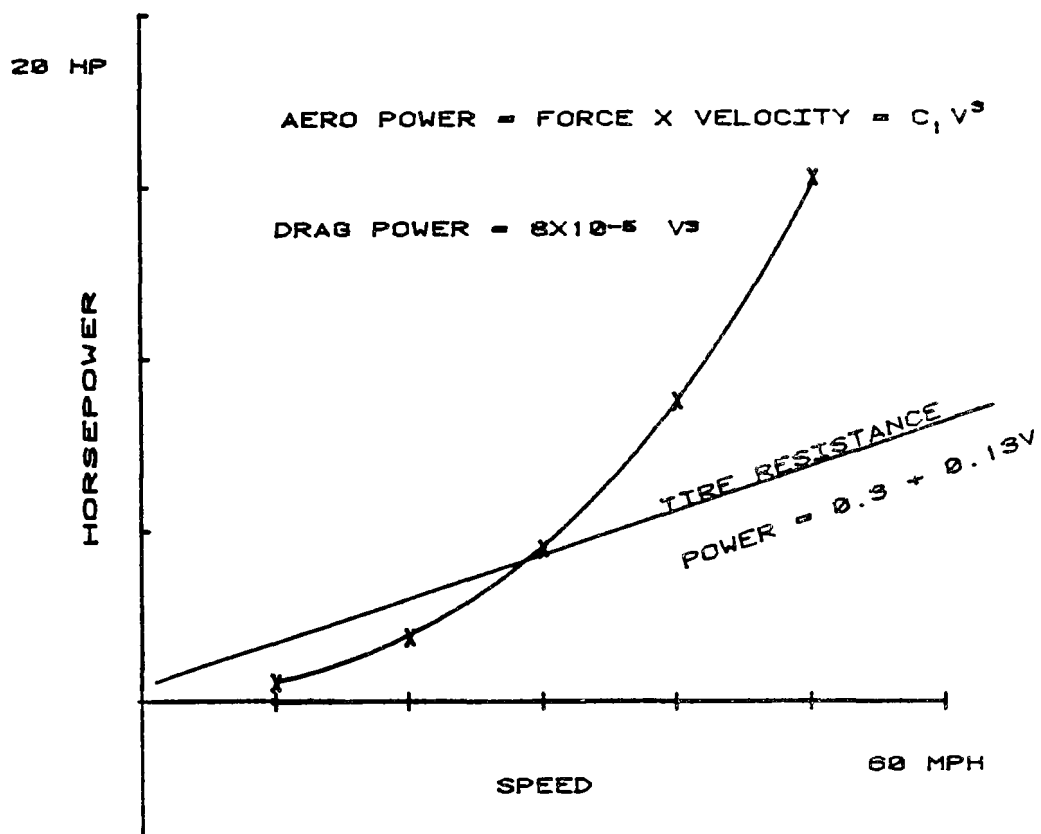
Fig. 2. Graph of VW Rabbit aerodynamic drag and tire factors.

TABLE 1

STANDARD DRIVING CYCLES USED IN VEHICLE TESTING

Driving Cycle	Average Speed (km/h)	(mph)	Distance (km)	Number of Stops
New York City Cycle (NYCC)	11.4	7.1	1.9	10
Morning Commuter: Federal Test Procedure (FTP), Los Angeles Cycle No. 4 (LA-4)	31.5	19.5	12	18
Crowded Urban Expressway (CUE)	56	34.8	21.7	3
Highway Fuel Economy Test Cycle (HWFET)	77.5	48.2	16.5	1

test just described. These traces were obtained with the real-time system described by Gabele[1] and constitute actual dynamometer test data. The middle speed trace (in mph) shows that this cycle is basically a steady-state route with an initial acceleration, a final stop, and small speed variations associated with vehicle control. The upper trace shows engine intake air flow (in SCFM) on the same scale; after this initial acceleration, there is relatively little variation in this parameter. This air rate is a rough indication of how hard the engine is working. The bottom trace shows one of the important sampling parameters, dilution ratio on the same scale, for a dilution air flow rate of 350 CFM. For this condition, the idle dilution ratio is about 15 to 1, while the steady-state is about 5 to 1. Increasing the blower rate to about 1000 CFM would increase the dilution ratio to about 45 at idle and 15 at steady-state. To achieve a dilution ratio of near atmospheric conditions with this car, say 500:1 at steady-state, one would need a blower of about 10,000 CFM flow rate. Work is in progress to install such a system in cell #2 in the RTP facility.
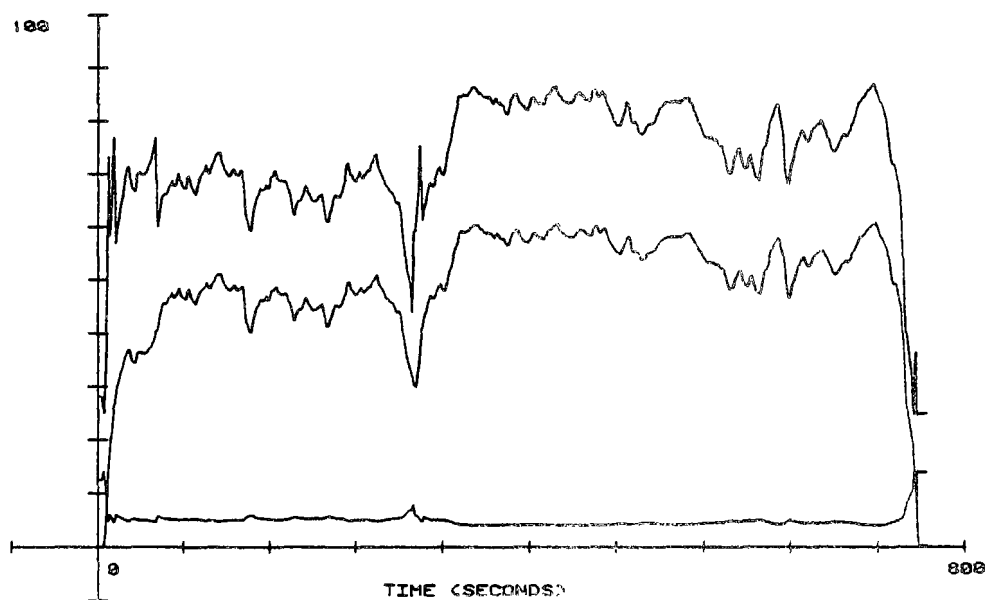
Fig. 3. Speed traces showing VW Rabbit operation under the highway fuel economy test cycle (HWFET). The upper trace is engine intake inflow in standard cubic feet per minute (CFM); the middle trace is speed in miles per hour (mph); and the bottom trace is the dilution ratio.

Figure 4 shows a similar set of traces for the New York City Cycle (NYCC). Here the speed trace shows the typical large number of stops incurred driving across lower Manhattan from about 10th Avenue to 1st Avenue. Random slow-down portions are made as if this car were caught in traffic and stopping for traffic lights, spending about 4% of its time in idle. This car spends a fair amount of time accelerating to mid-block speeds, and rather high intake air flows periodically occur. The dilution ratios are again between about 15 to 1 and 5 to 1, but a greater portion of the time is spent in the high dilution ratio condition.
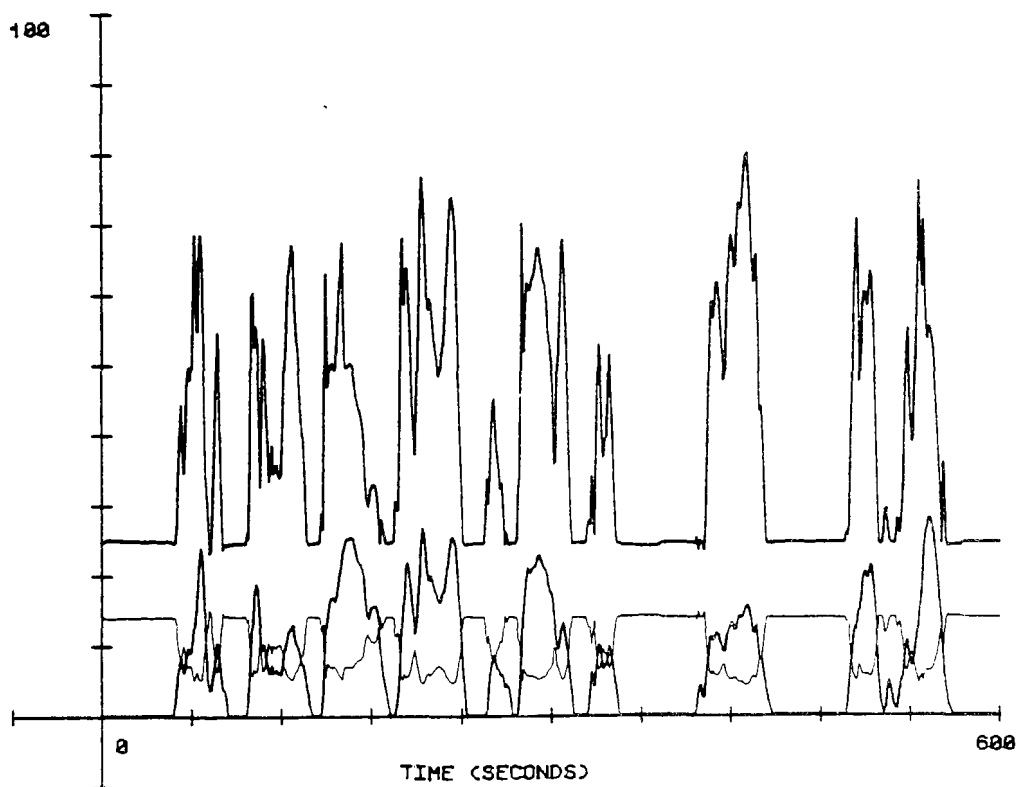
Fig. 4. Speed traces of a VW Rabbit on the New York City Cycle (NYCC). The upper trace is engine intake inflow in standard cubic feet per minute (CFM); the middle trace is speed in miles per hour (mph); and the bottom trace is the dilution ratio.

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The final figure in this series, Figure 5, shows what happens on the Federal Test Procedure (FTP), a Los Angeles commuter route simulation. Here, all the curves are very spikey and a bit hard to follow. Still the intake air flow rates and dilution factors are within the ranges of the other cycles.
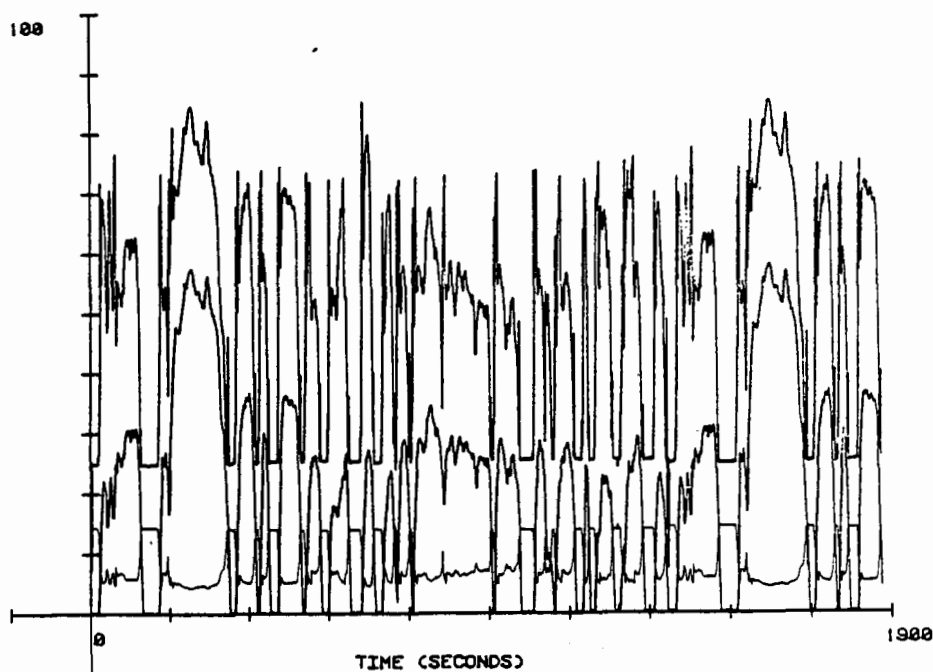
Fig. 5. Speed traces of a VW Rabbit on the Federal Testing Procedure (FTP) commuter route. The upper trace is engine intake inflow in standard cubic feet per minutes (CFM); the middle trace is speed in miles per hour (mph); and the bottom trace is the dilution ratio.

So, despite prominent differences among these cycles, the engine experiences at least some high load and some idle operation in every one of them, and only the percentage of the time spent in each mode changes. Therefore, cycle-to-cycle differences are expected only for those engine-produced pollutants that experience a substantial difference in emission rate as a function of driving mode or work required. Ample evidence exists that for carbon monoxide, e.g., from gasoline-fueled spark-ignition engines, some dramatic effects of this type occur. However, for current generation diesel passenger car engines, there appear to be only minor variations in particle emissions with change in driving cycle.

Figure 6 is a chart of emissions values for four driving cycles using the
same VW Rabbit. Total hydrocarbons (THC), particle mass, and the Ames activity
in revertants/mile for TA98 -S9 show relatively little variation with driving
pattern, especially for particle mass and mutagen emissions.[2] Therefore, at
least with this car, driving patterns do not greatly influence the important
emission rates. Gibbs et al.,[3] Hare and Baines,[4] and Naman et al.[5] have all
come to the same conclusion. From a data base encompassing about 30 in-use
diesel passenger cars, it appears that driving cycles and load simulation are
relatively unimportant matters within reasonable limits.   Lang et al.[6] have
recently conducted a study of the particle emissions of 20 in-use gasoline
cars. These authors have also concluded that mutagen emission rates are only
weakly dependent on driving cycle.

NYCC   FTP
CUE   HWFET
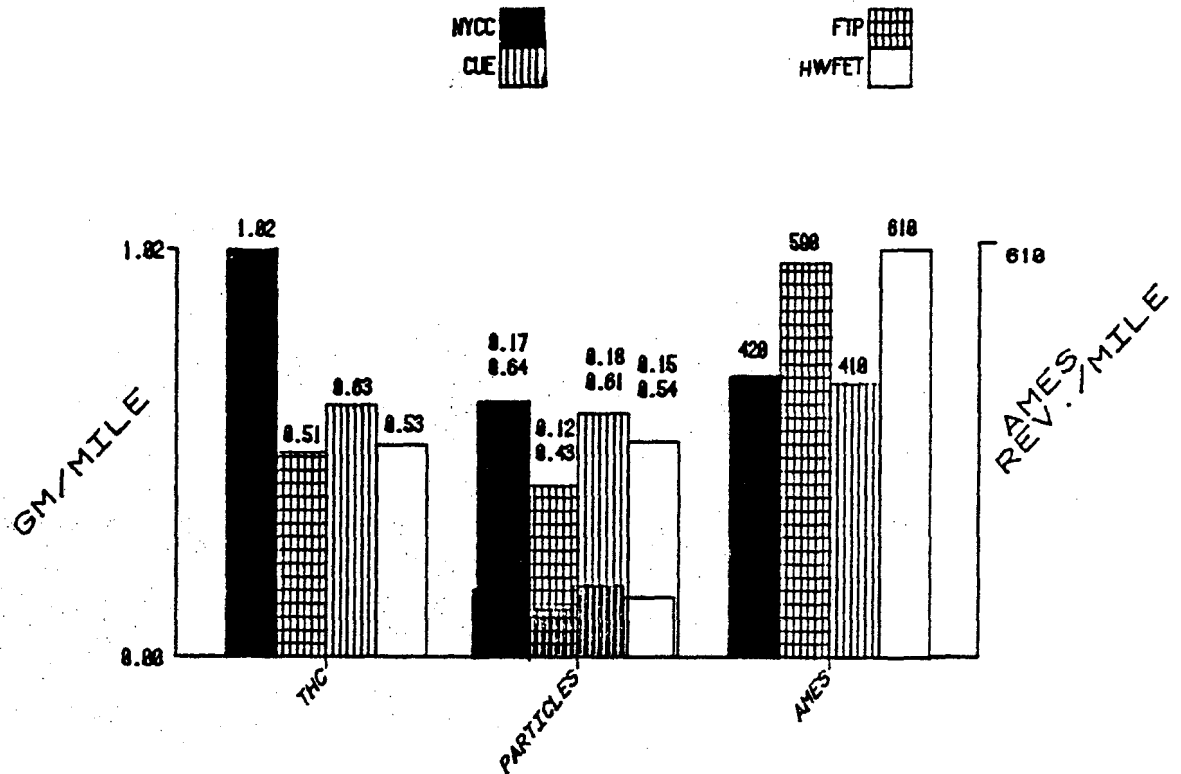
Fig. 6. Emissions data for a VW Rabbit tested under various driving conditions.

Diesel and gasoline trucks can also be tested on a chassis dynamometer using driving cycles similar to those used for passenger cars. Dietzmann et al.[7,8] have reported results from four diesel and two gasoline trucks operated over the EPA transient driving cycle. Dietzmann et al.[8] also split the samples by mode for this driving pattern to develop particle emissions factors for trucks. Figure 7 shows particle emission rates for the six trucks on a gm/km and gm/kg of fuel basis. For comparison, data from 20 in-use diesel passenger cars[3] and 20 in-use gasoline passenger cars[6] are included.
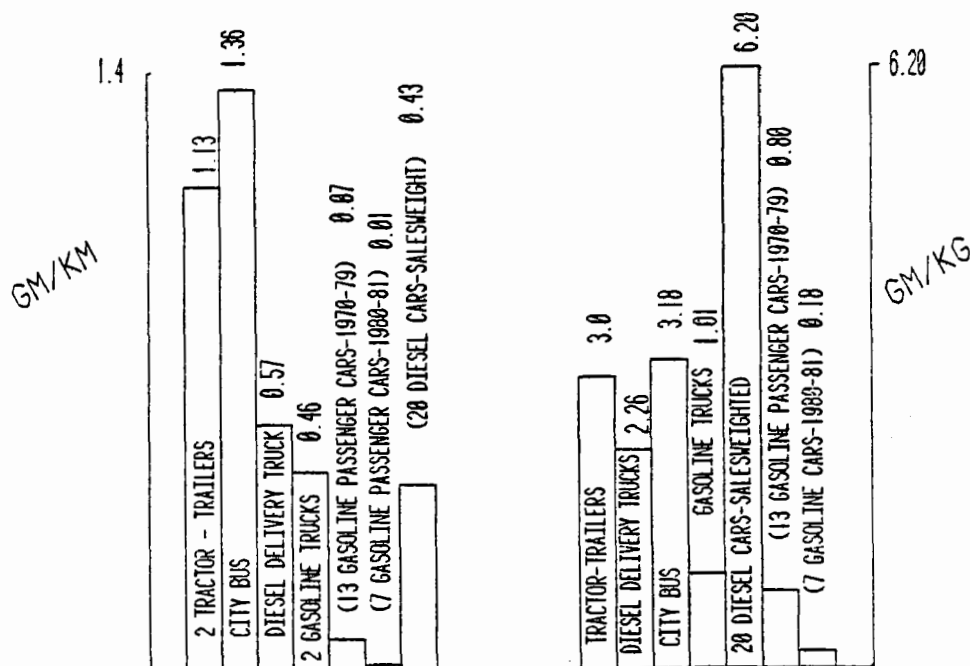
Fig. 7. Bar graph showing particle emission rates from six trucks.

Clearly, significant differences among vehicle types exist in gm/km and gm/kg of fuel emission factors. These differences will probably be reflected in the relative influence of vehicle types on atmospheric concentrations of particles. The Dietzmann papers also indicate a substantial bias among driving cycles for heavy duty trucks, both gasoline and diesel. More than half the particle mass is emitted in the Los Angeles freeway mode for both types of trucks. These differences in emissions factors between classes of trucks and passenger cars are important in that they influence the need for and cost of control.

SAMPLING OF DIESEL EXHAUST PARTICLES AND ORGANICS

A variety of sampling systems have been used for diesel exhaust particles. Figure 8 shows a rather elaborate system currently in use at the Mobile Source Emissions Research Branch at EPA-RTP. Actually, two dynamometer cells are similarly equipped in this facility with cell #1 devoted to gasoline spark-ignition engine work and cell #2 devoted to diesel.

It is difficult to say that any one of these is "typical," since a variety of shapes and sizes of equipment are used. The equipment shown in Figure 8 is especially interesting, however, since this cell was used to generate the large samples for biological testing from several cars. In this case, room air is drawn through a filter and charcoal bed, then mixed with exhaust to produce a diluted stream in which the exhaust stream is cooled to near room temperature. Using typical gaseous emissions test conditions, e.g., a blower speed of 350 CFM, dilution ratios of about 5 parts of air to 1 part of exhaust are typical for a small car like the VW Rabbit, as shown previously. For larger cars or trucks greater quantities of dilution air are needed, and it is rather common to use bigger constant volume samplers (CVS) blowers for sampling. In this case, flow rates of up to about 1200 CFM are available with two parallel positive displacement pumps; a still larger heavy-duty CVS with a capacity of 10,000 CFM is currently being installed in this facility. Dietzmann et al.[7] have used multiple pumps to achieve flow rates as high as 12,000 CFM in testing diesel tractors.

It is interesting to note that very similar results are obtained from a number of different systems. Tai Chan et al.[9] have recently reported a study of raw exhaust electrostatic precipitation compound with air dilution. These authors find very similar results, both in terms of particle mass and in Ames mutagenicity values for both methods. The cooled raw exhaust collector samples produced somewhat larger amounts of organics, mainly acidic-salt components, but even chemical fractions had similar mutagenic activity with the exception of acidic fractions.

In the past, condensation and filtering of raw exhaust have been used to acquire samples for analysis of polynuclear aromatics (PNA). This technique is still very common in Europe.[10] Recently, some studies have been reported involving Ames mutagenicity results from the University of Stockholm[11,12] and comparative studies of polynuclear aromatic hydrocarbon (PAH) emissions from Volkswagen.[13] These works have shown similar levels of mutagenicity and PH for gasoline and diesel vehicles. A report by Stump et al.[14] strongly suggests that condensation procedures produce results very similar to those obtained by
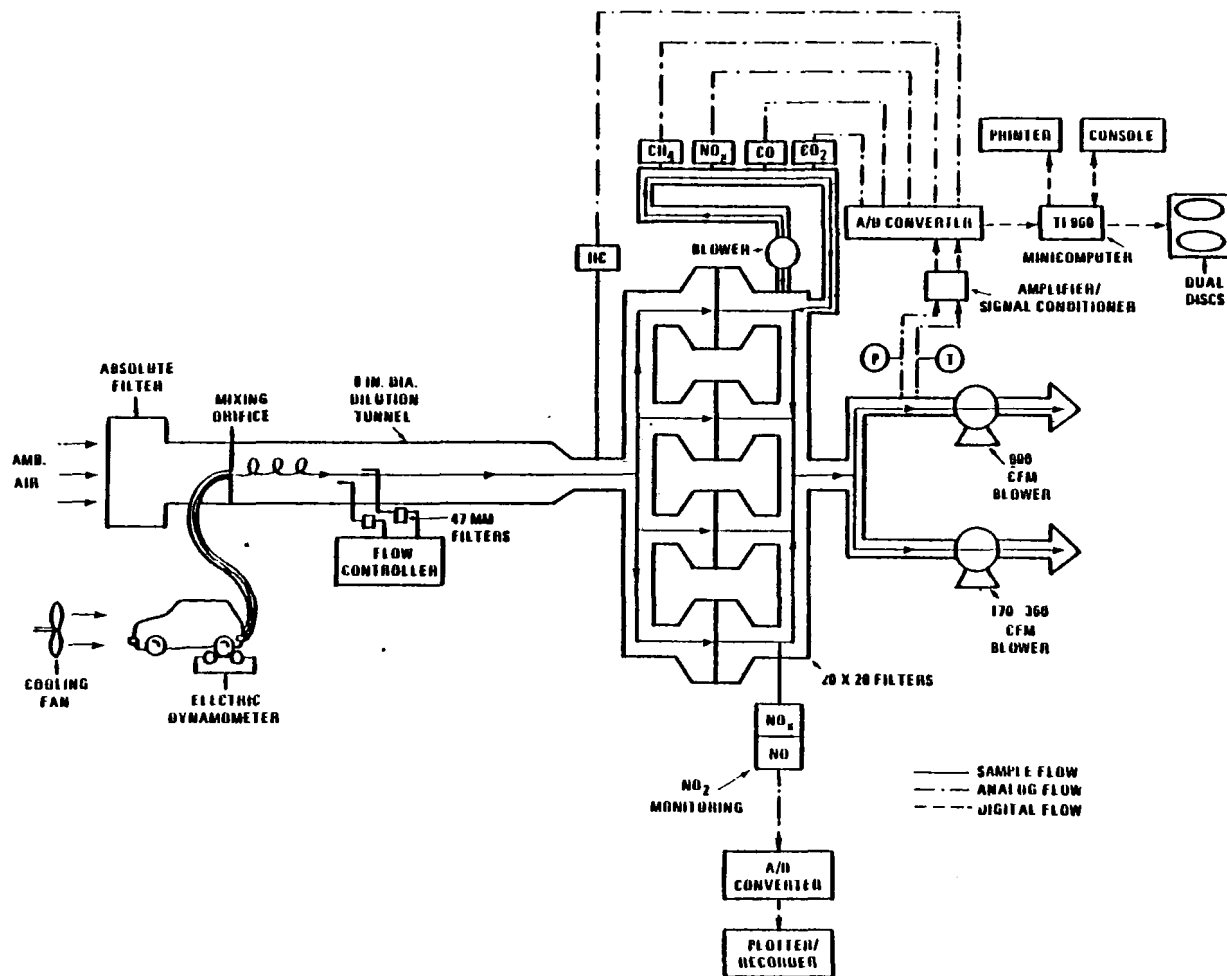
Fig. 8. Diagram of EPA-RTP diesel sampling system.

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air-dilution techniques. Therefore, it appears that different sampling procedures result in samples with similar chemical characteristics and mutagenic activity.

GENERATION OF ARTIFACTS

Several phenomena have been proposed as mechanisms by which artifactually high levels of organics or mutagens could exist in air-dilution samples of diesel particles. Dolan and Kittleson[15] suggested that air-dilution procedures could produce particle samples containing greater organics levels than exist in the ambient and postulated that a condensation mechanism might be possible in the typical range of dilution ratios used in sample collection. However, Plee and MacDonald[16] conducted a series of experiments and developed a model of air dilution processes that argues against this postulate. These authors calculate a particle-organic association energy of about 7K cal/mole, which is somewhat in excess of latent heat values. Ross et al.[17] have studied the physical chemistry of this association process and found it to be somewhat more complicated. Nevertheless, the global energy of dissociation appears to be near the 7K cal/mole calculated by Plee and MacDonald.[16] Pierson et al.[18] suggest that air samples are similar to dilution tube samples in activity and composition.

In either case, the principal argument deals with the importance of particles. Gaseous organics, however, appear to be readily absorbed in the human lung.[19] Studies have now been done with toluene, benzene, methylene chloride, nitrobenzene, and other gaseous hydrocarbons at low level and all seem to be absorbed very readily; usually, uptake is 30 to 70% of the dose.[19] So, whether the mutagenic material is in the particle or gas phase may be immaterial. The only significant issue is the relative amounts of active material in the two phases. Kraft and Lies[13] report that about 70% of the PAH in condenser experiments is trapped on a filter and only 10% is in the conden- sate. Stump et al.[14] find virtually no mutagenic activity in gasoline car porous polymer gas trap samples. From diesel cars, the picture is more complicated, but even there at least two-thirds of the activity is in particles. Therefore, it appears little if any activity is lost in the gas phase.

The final artifact mechanism proposed deals with nitrogen dioxide (NO_2). Gibson et al.[20] have shown that re-exposure of filter samples of diesel particles to the gas phase of diesel exhaust can elevate mutagenic activity. Bradow[21] described some diesel experiments in which dilution-air NO_2 levels of about 100 ppm roughly tripled mutagenic activity. However, more recent work

has revealed that NO_2 levels above about 5 ppm are needed to produce this effect. At blower speeds of 350 CFM with a VW Rabbit-size vehicle, the NO_2 levels in any cycle never exceed 5 ppm. With a larger Oldsmobile-size car, a blower speed of 600 to 800 CFM is needed to maintain NO_2 concentrations below 5 ppm. For gasoline cars, NO_2 is virtually undetectable. Such NO_2 effects apprently have not seriously complicated any of the exhaust samples collected so far. Very high dilution ratio experiments could be conducted to confirm these findings.

The filter media efficiency studies recently reported by Black and Dober-stein[22] (1981) have shown that several media give essentially identical mass emission rates and extractable mass in handling diluted exhaust; therefore, within reasonable limits, choice of media does not bias samples in these respects. Clark et al.[23] demonstrated that commonly used filter media all produce the same sample mutagenic activity; therefore, this property is also not biased by filter media.

SUMMARY

Several working hypotheses can be drawn relative to measuring diesel exhaust particles and organics. They are:

1. Passenger car dynamometer simulation and driving cycles are adequate for determining mass emission rates. The choice of a simulation condition appears to make little difference. Evidence for trucks is fragmentary, but high load freeway driving produces especially high particle emissions.

2. Sampling of particles and organics can be done in a variety of ways. Since most sampling systems yield similar results, the choice is not critical. If measuring mutagenic agents is the issue, then the distribution between gas and particle phase is also not critical.

3. Artifactual generation of mutating agents in sampling seems to be relatively unimportant. Filter media studies are negative, with respect to artifacts. Although there is evidence of artifact formation from NO_2 concentrations above 5 ppm, this condition is not generally present under normal sampling conditions.

Consequently, in the context of laboratory studies of diesel emissions, it appears difficult to choose conditions that will produce totally invalid samples. Differences in particle emission factors from various vehicle types are substantial, however, and these influences on air quality are important.

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PARTICULATE EMISSIONS FROM SPARK-IGNITION ENGINES

by

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Experiments were conducted at the U.S. Department of Energy's Bartlesville (Okla.) Energy Technology Center to quantify particulate and gaseous emissions from current-production vehicles equipped with spark-ignition engines, to determine the influence of fuel and ambient temperature on particulate emissions, and to characterize particulates in terms of their carbon content, soluble organic fractions, and biological activity.

Four 1980-81 model-year vehicles equipped with oxidation and three-way catalysts and spark-ignition engines ranging from 1.6 liter, 4-cylinder to 4.3 liter, V8 (see table 1) were tested on a climate-controlled chassis dynamometer using the driving cycles of the 1975 Federal Test Procedure. The vehicles were operated at 20°, 50°, 75°, and 100° F (-7°, 10°, 24°, and 38° C, respectively) ambients on gasoline and at 75° F ambient on four fuel blends: 90 percent gasoline/10 percent ethanol, 90 percent gasoline/10 percent methanol, 93 percent gasoline/7 percent methyl tertiary butyl ether, and a commercial gasohol.

Particulate matter was collected on 40- by 40-inch filters using the total volume of the exhaust, and on conventional 47 mm filters using a sampling probe and a portion of the exhaust. Bioassays of dichloromethane extracts of the samples were carried out using the Salmonella mutagenicity (Ames) test at the Lovelace Inhalation Toxicology Research Institute.

The results from the Federal Test Procedure (figure 1) showed a significant reduction in particulate emissions with the alcohol/gasoline fuel blends when compared to gasoline alone. The methyl tertiary butyl ether/gasoline fuel blend showed a slight reduction in particulate emissions. The carbon monoxide emissions were slightly reduced with the alcohol/gasoline fuel blends. Hydrocarbon emissions remained relatively unchanged, oxides of nitrogen emissions were slightly increased, and fuel economy was 2 to 3 percent lower (data not shown). Ambient temperature seemed to have a slight effect on particulate emissions. Overall, particulates emitted from the vehicles with spark-ignition engines were 90 to 100 times lower than particulates emitted from current-production diesel vehicles.

Samples of particulate extracts from vehicles fueled with gasoline and gasohol were separated using a silica column. The results obtained from the separation showed a higher percentage of polar compounds in the gasohol than in the gasoline samples (figure 2).

Benzo(a)pyrene and nitropyrene levels in the gasohol particulate extracts were at least 50 percent lower than the measured levels in particulate extracts from gasoline-fueled vehicles.

Dichloromethane extracts of the particulate exhaust from vehicles operating on gasoline were evaluated in _Salmonella_ strain TA-100, with and without the addition of a liver enzyme preparation (S-9) as a source of metabolic enzymes. Extracts of particulate exhaust from the four vehicles operated on gasoline and/or on alcohol/gasoline fuel blends produced direct, dose-related increases in mutagenicity (see table 2). The addition of S-9 either decreased or did not alter the observed direct mutagenicity (data not shown).

The addition of 10 percent ethanol to gasoline either decreased or did not significantly change the mutagenicity of the resultant exhaust particulate extracts (table 2). Operation of the Mercury Monarch on the methanol blend increased the mutagenicity of the exhaust particulate extracts but decreased mutagenicity in the Chevrolet Citation. Commercially available gasohol resulted in particulate extracts that were less mutagenic in the Ford Escort but not significantly different in the other cars.

The mutagenic potencies of the extracts do not reflect differences among cars in the mass of mutagenic material associated with the particulate emission rates. Therefore, the mutagenicity data were normalized so that comparisons of the amount of mutagenicity could be made among cars. This was done by dividing the mass of dichloromethane extractable material from each filter by the number of miles of vehicle operation (26), to yield mg/mile of particulate associated organic material. This number was multiplied by revertants per microgram (µg) to estimate the amount of mutagenicity emitted from each car (revertants per mile).

When compared to gasoline, the addition of 10 percent ethanol or methanol to gasoline, or operating the vehicles on the commercial gasohol, reduced the mass emission rate (mg/mi) of organic materials associated with the particulates. This resulted in significant reductions in revertants per mile for all of the alcohol fuel blends tested (table 2).

Table 1. Test vehicles

	Ford Escort	Oldsmobile Cutlass	Chevrolet Citation	Mercury Monarch
Engine displacement, CID (liters)	98 (1.6)	263 (4.3)	151 (2.5)	250 (4.1)
Carburetion	2 bbl	2 bbl	2 bbl	1 bbl
Compression ratio	8.8	7.5	8.2	8.6
Transmission	Manual 4-spd	Auto	Auto	Auto
Emission Control System:				
EGR	Yes	Yes	Yes	Yes
Air pump	Yes	Yes	No	Yes
Air injection	No	No	Yes	No
Oxidation catalyst	No	No	Yes	Yes
Three-way catalyst	Yes	Yes	No	No
Charcoal canister	Yes	Yes	Yes	Yes
Axle ratio	3.59	2.29	2.84	2.79
Inertia weight, lb	2375	3750	2875	3625
Actual dyno load, hp	6.4	11.5	6.6	11.1

Table 2. Influence of alcohol fuel blends on mutagenicity of spark-ignition engine exhaust particulate extracts

Vehicle and Fuel	Revertants/μg Extract TA-100[a]	Emission of Particulate Associated Organic Material (mg/mi)	Revertants per Mile
Ford Escort			
Gasoline	10	1.5	15,000
Ethanol blend	9	1.1	9,900
Commercial gasohol	4	1.2	4,800
Oldsmobile Cutlass			
Gasoline	10	1.7	17,000
Ethanol blend	5	0.6	3,000
Commercial gasohol	13	0.6	7,800
Chevrolet Citation			
Gasoline	17	1.9	32,300
Ethanol blend	14	0.9	12,600
Methanol blend	11	0.8	8,800
Commercial gasohol	10	1.0	10,000
Mercury Monarch			
Gasoline	16	7.1	114,000
Ethanol blend	12	2.8	34,000
Methanol blend	26	3.3	86,000
Commercial gasohol	20	2.2	44,000

[a]Slope of linear portion of dose-response curve, without S-9.
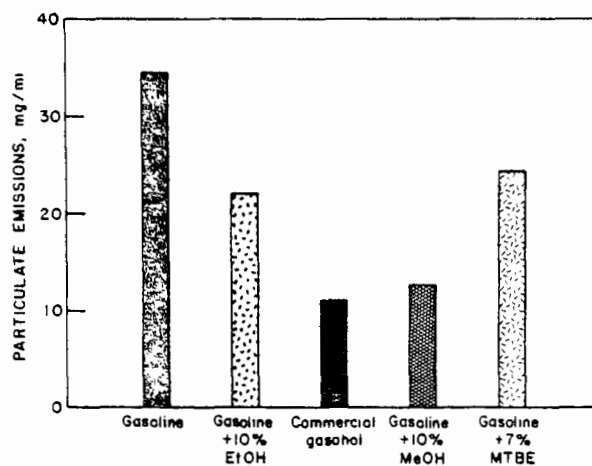
Figure 1.   Influence of fuel extenders
on particulate emissions.
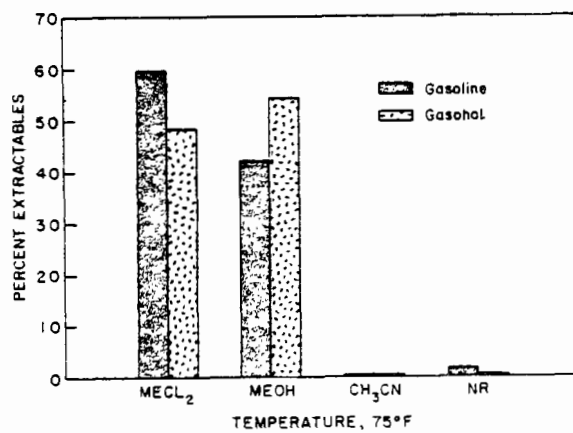
Figure 2.   Particulate extracts from vehicles
operating on gasoline and gasohol.

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PARTICULATE EMISSION CHARACTERIZATION STUDIES
OF
IN-USE DIESEL AUTOMOBILES

by

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A sample of 20 in-use diesel automobiles has been repeatedly tested over a two-year period to accumulate emissions characterization data with major emphasis on particulate. Each vehicle test included replicate sample collection for "as received" and "control fuel, control oil" vehicle conditions. Driving cycles tested at each vehicle condition included: FTP, CFDS (CUE), HFET, 50 mph cruise, NYCC, and Idle. Measurements in each test cycle included gaseous emissions, fuel economy, and particulate emissions; also, individual particulate samples were collected for each individual test cycle in sufficient quantity for subsequent chemical and bioassay analyses. These particulate samples were quantified for soluble organic fraction (SOF) and Ames test direct-acting mutagenic response by TA98 (-). These parameters are examined for 60 vehicle tests to indicate particulate character effects for vehicle types and test cycles. Results will be presented for three vehicles in the sample group which have been tested over sufficient mileage accumulation intervals to provide limited insight into vehicle aging effects.

Table I gives average values of the mutagenic activity of the SOF directly. In general, FTP samples exhibit a higher specific activity than samples from the same vehicle for other driving cycles. Expressed as a percent of total particulate, however, the SOF (%) is generally less for the FTP than other cycles as seen in Table II.

When SOF mutagenic response and SOF (%) data are combined to express mutagenic response on a particulate mass basis, these differences are mostly indiscernable as shown in Table III.

From Table III, with some exceptions, it can be seen that the bio-activity on a total particulate basis is quite uniform within a vehicle type. This is in spite of rather large variations in activity among the various vehicles tested to generate the averages. Thus, we conclude that, as a first

approximation, for a given vehicle test a "gram of particulate is a gram of particulate" regardless of driving cycle. When variations in vehicle particulate emission rate per distance travelled are incorporated to the data of Table III, the per-mile emission of bio-active material can be calculated as given in Table IV.

Since all test cycles except the FTP begin with a warm vehicle, the foregoing conclusions may not include parameters related to vehicle start-up. A battery of tests were performed on a VW diesel in the Winter of 1981 to preliminarily investigate cold-start effects on particulate, SOF, and mutagenic activity. Overnight vehicle soak at laboratory and outdoor ambient conditions were followed by: FTP, Bag I, 10 min. pause, FTP, Bag III to give separate particulate samples for each bag at each condition. The cold-ambient tests were repeated, and average values from two runs are reported. Continuous temperature recordings of ambient temperature, crankcase lubricating oil, and fuel temperature (between pump and injectors) were obtained. When results for the normal FTP Bag III were used as a basis for comparison, the other three test conditions yield ratios as given in Table V.

Comparison of the 0°C Bag I results to the 20°C base condition showed a 74% increase in particulate emission, 11% increase in SOF emission corresponding to a 36% decrease in SOF expressed as a percentage of particulate. SOF mutagenic response was increased by a factor of 3.6 and 4.0 when expressed as revertants/mile. These results are in general agreement with those presented above in comparison of FTP and other driving cycles for the in-use sample group.

Table I. Mutagenic Activity Per Microgram SOF
(Revertants/μg SOF)

	FTP	HFET	50mph Cruise	CFDS	NYCC	IDLE	Sample n
GM	3.8	2.4	2.1	2.7	1.4	2.4	57
VW	11.3	10.9	8.1	11.0	16.8	3.4	36
MB	4.8	4.4	3.5	5.9	1.8	2.7	18

Table II. SOF (%)

	FTP	HFET	50mph Cruise	CFDS	NYCC	IDLE	Sample n
GM	25.1	34.1	39.3	31.2	31.7	24.6	57
VW	20.0	21.5	20.7	22.7	33.4	55.0	36
MB	14.0	13.6	15.2	14.6	14.8	14.6	18

Table III. Mutagenic Activity Per Microgram Particulate
(Revertants/μg Particulate)

	FTP	HFET	50mph Cruise	CFDS	NYCC	IDLE	Sample n
GM	0.77	0.75	0.72	0.74	0.36	0.43	57
VW	1.95	2.06	1.53	2.26	5.82	1.94	36
MB	0.53	0.45	0.40	0.68	0.27	0.39	18

Table IV. Mutagenic Activity Per Vehicle Mile
(10^5 Revertants/Mile)

	FTP	HFET	50mph Cruise	CFDS	NYCC	IDLE*	Sample n
GM	6.5	2.7	2.2	3.8	8.6	0.74	57
VW	6.9	6.1	4.4	7.4	27.0	0.47	36
MB	2.8	1.7	1.3	3.0	6.2	0.17	18

*10^5 Revertants/Minute

Table V. VW Cold Start Particulate Comparisons

Vehicle Test Condition	Mean Temperatures °C			Particulate (g/m)	SOF (%)	SOF (g/m)	TA98 (-) Revertants		
	Overnight Soak	Injector Fuel Line	Crankcase Lube				R/μg SOF	R/μg Part.	10^5 R/mi
Base Condition = (FTP Bag III)	20	25	90	0.34	24.8	0.084	5.8	1.44	4.9
Note: Results below shaded areas are ratios to base condition									
Bag III after ambient Cold Soak Bag I	0	18	98	1.0	0.87	0.87	1.5	1.3	1.3
Normal FTP Bag I	20	21	48	1.18	0.79	0.93	1.8	1.4	1.7
Cold Ambient Soak FTP Bag I	0	5	38	1.74	0.64	1.11	3.6	2.3	4.0

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DIESEL EXHAUST TREATMENT DEVICES: EFFECTS ON GASEOUS AND PARTICULATE EMISSIONS AND ON MUTAGENIC ACTIVITY

by

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In conjunction with the Ford research effort on the control of diesel exhaust particulate emissions we have investigated and characterized the emissions from four diesel particulate emission control devices. These include two ceramic honeycomb monolithic filter traps, one of which is coated with a   catalyst, a compacted wire-mesh particulate trap with a precious metal (PM) catalyst coating, and a free-flow monolithic catalyst with a PM coating. The   four devices and the test vehicle are described in Table I.

Gaseous and particulate emissions have been compared to those from the uncontrolled baseline vehicle. The particulate material has been fractionated into the sulfate, soluble organic and inorganic fractions by Soxhlet extraction using dichloromethane. The extract was analyzed by high performance liquid chromatography (HPLC) analysis with fluorescence and ultraviolet absorption detectors and subsequently characterized by the Salmonella typhimurium plate incorporation assay (Ames) using strains TA-98, TA-100 and TA-1538, without metabolic activation. The results are summarized in Table II.

The ceramic honeycomb traps are extremely efficient filters for removal of the inorganic fraction (mostly elemental carbon) and are less efficient for removal of the soluble organic fraction. The catalyst coated honeycomb trap shows some activity for CO and for gaseous hydrocarbon but is also capable of producing sulfate at 50% above the baseline emission value.

The catalyst coated wire-mesh trap shows high activity for CO, HC and also for the soluble organic fraction but in addition produces large yields of sulfate. It is not an extremely efficient filter for the inorganic fraction of the particulate material.

The catalyst coated ceramic honeycomb trap and the catalyst coated wire-mesh trap significantly reduce the number of _Salmonella_ revertants per mile travelled. Provided that the wire-mesh trap could be modified to minimize sulfate formation, both of the above traps have the potential for reducing the total particulate emission rates to values near or below the 0.2 g/mi level.

The HPLC results show that the extracts from the trap systems in general are qualitatively similar to those from the baseline vehicle. The traps seem to show the highest collection efficiency for the aliphatic type compounds in the soluble organic fraction.

Regeneration and durability of the trap systems studied remain as areas for further research and will not be addressed in this report.

Table I. Description of Diesel Exhaust Treatment Devices[a,b]

Designation	Type	Catalyst	Volume in^3	Cell Density cells/in^2	Filter Area in^2
A	Ceramic Honeycomb Filter Trap	None	119	100	1970
B	Ceramic Honeycomb Filter Trap	PM	119	100	1970
C	Compacted Knitted Wire-Mesh Trap	PM	186	NA[c]	8370
D	Free-Flow Monolithic	PM	80	100	NA[c]

[a]The control devices used in this study represent early experimental versions of potential particulate control devices and hence the results do not reflect future design modifications.

[b]Vehicle = 2.3 1 Opel (European) Diesel, 3000 lbs IW, 10.3 hp PAU, 3.89 Axle Ratio, 4 Speed Manual Transmission, CVS Driving Cycle. During the course of this study the original engine (A) was replaced with a new engine (B) due to excessive HC emissions.

[c]NA = Not Applicable

Table II. Gaseous and Particulate Emisions from Diesel Particulate Control Devices

Device	Engine	HC	CO	Total Particulate	Sulfate	Organic	Inorganic	Ames	
		g/mi	g/mi	mg/mi	mg/mi	mg/mi	mg/mi	rev/ug	rev/mi x10^{-5}
Baseline Vehicle	A[a]	0.99	1.65	436	13	309	127	1.9	5.9
	B	0.61	1.50	303	23	138	142	2.0	2.9
				Ratio to Baseline Emission Rate					
A, Clean[b]	A	0.91	1.03	0.44	0.31	0.56	0.02	1.9	1.1
	B	0.98	0.93	0.21	0.61	0.41	0.00	2.6	1.1
A, Loaded[c]	A	0.64	1.05	0.21	0.31	0.27	0.05	1.9	0.51
	B	0.77	0.93	0.25	0.52	0.46	0.05	2.2	1.0
B, Loaded[c]	A	0.52	0.56	0.24	1.54	0.25	0.07	0.42	0.10
C	A	0.05	0.08	0.68	12.5	0.06	0.87	1.2	0.07
D	A	0.89	0.72	0.89	1.08	0.84	0.99	1.2	1.2

[a]Engine A replaced by Engine B due to excessively high HC emission rate.
[b]Trap had just been regenerated.
[c]Trap loaded with soot by mileage accumalation.

CHARACTERIZATION AND OXIDATION
OF DIESEL PARTICULATE

by

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INTRODUCTION

A study was recently completed by Battelle-Columbus Laboratories for the U.S. Environmental Protection Agency to evaluate emissions control on light-duty diesel vehicles by postcylinder oxidation. The primary objective of this program was to determine the feasibility of thermal or catalytic oxidation as a means of diesel particulate emissions control.

Two major efforts in this program involved characterization of the particulate for chemical and physical parameters relating to ignition and oxidation, and development of a catalytic ignition concept for reducing the ignition temperature of the particulate in the exhaust system.

CHARACTERIZATION OF THE PARTICULATE

Particulate characteristics were measured using samples collected directly from the surface of the exhaust pipe and samples collected by filter from a dilution system.

Physical Properties

The particulate physical properties included surface area, size distribution, and mass concentration. These were measured using dilution system samples, hence, representing the particulate after entering the atmosphere. The surface area was approximately 100 m^2/g, the mass median particle diameter was in the range of 0.1 to 0.3 μm and was observed to increase in size as engine speed and load increased, and the mass concentration in the engine tail pipe was observed also to vary with engine speed and load. Mass concentrations of particulate in the exhaust gas varied from 20 mg/m^3 at the lowest speed and lightest load to 500 mg/m^3 at maximum speed and load. These values translate to 0.1 to 3.8 grams per mile, respectively, a fairly "dirty" exhaust for an automotive diesel (and not representative of current diesel engines).

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Chemical Properties

The chemical properties were measured using the exhaust-pipe particulate samples and included soluble organics (using toluene), carbon, hydrogen, oxygen, ash, and trace minerals. The soluble organics ranged from 2 to 10 percent, with no evident correlation with engine speed/load conditions. By comparison, the soluble organic content measured in particulate collected in the dilution system, for the same range of engine speed and load conditions, varied from 3 to 25 percent, increasing as speed and load decreased.

Carbon content ranged between 73 and 93 percent, hydrogen content varied from 0.5 to 1.8 percent, and ash content ranged from 0.1 to 2.2 percent. Again, no correlation with engine conditions was noted for any of these parameters. About 7 percent oxygen was found in two particulate samples, and 23 percent in a third sample. Part of the oxygen is present in the particulate as SO_4 and part may represent partial oxidation (to CO_2, perhaps) but with the products still bound in the particulate.

The trace mineral analyses revealed considerable calcium, iron, phosphorus, and magnesium in the particulate. Similar analyses of samples of the fuel and lube oil indicated that the lube oil was most likely the source of these elements as well as of chromium, copper, manganese, and lead, found in lesser quantities in the particulate. These results imply that a substantial portion of the particulate may derive from the lube oil.

Ignition Properties

A Differential Thermal Analyzer technique was used to measure ignition temperatures and maximum oxidation temperatures for the particulate samples obtained in the engine exhaust pipe. The mean ignition temperature for the samples evaluated was 594 C, with a variation range of 583 to 604 C. The only significant correlations with engine operating conditions and with physical and chemical properties appeared to be with the presence of hydrocarbons in the exhaust and with aluminum and lead in the particulate. Lower ignition temperatures occurred with higher exhaust gas hydrocarbons and higher particulate aluminum and lead.

More significant changes in ignition temperature were observed when the bulk density and the amount of the particulate sample in the DTA were altered. Increasing the sample size and the bulk density by factors of 10 resulted in a decrease in ignition temperature of about 150 C. This result suggests that if a method could be devised for compacting the particulate in a trap, it would be more easily ignited under normal exhaust-gas conditions.

CATALYTIC IGNITION

In this study the catalytic ignition of diesel particulate was initially developed in bench-scale experiments. Final experiments were conducted in the exhaust system of an engine to verify the applicability of the concept to the actual engine environment.

Bench-Scale Experiments

The bench-scale experiments were carried out using a hot-tube reactor in which small samples of the diesel particulate were subjected to a gradually increasing temperature until ignition and oxidation occurred. Experiments were conducted to identify potentially catalytic materials, to explore methods of catalyst application, and to determine the magnitude of the catalytic effect in relation to the amount of catalytic material used.

Metal salts such as copper chloride, manganese chloride, and cobalt chloride were found to be capable of reducing the ignition temperature of the particulate by as much as 200 C. The addition of sodium or ammonium salts to the metal salt reduced the ignition temperature another 50 C, for a total reduction of 250 C (resulting in an ignition temperature of about 350 C for the catalyzed particulate). For most of this work, copper chloride equivalent to 7.5 mg Cu/g soot was used to achieve the catalyzed ignition.

Exhaust System Experiments

Final experiments were conducted in the exhaust pipe of a production diesel engine, where the results of the bench-scale experiments were confirmed. Various particulate trap materials and configurations were tried, including quartz wool, stainless steel wool, foamed alumina, and porous ceramic honeycomb. The porous ceramic honeycomb traps proved to be the most suitable, providing high surface area in a small volume, good collection efficiency at low pressure loss, and tolerance to the high temperatures reached during oxidation of the particulate.

Successful regeneration of the trap in the engine exhaust was achieved by intermittent injection of a water solution of the copper chloride/ sodium chloride catalyst mixture into the exhaust stream immediately upstream of the trap, after a period of particulate collection on the trap. The actual ignition temperature in these tests ranged from 350 to 400 C. The amount of soot burned in each of these final trials is unknown but the copper concentration is believed to have been somewhat greater than used in most of the bench-scale tests.

CONCLUSIONS

The concept of using a catalyst material introduced into the exhaust of a diesel engine in a manner which allows the catalyst to associate with particulate collected on a trap has been successfully demonstrated. The catalyst acts to reduce the ignition temperature of the trapped particulate by about 250 C, which results in particulate burnoff and trap regeneration at significantly lower exhaust gas temperatures than would be required otherwise. This concept could provide more highly controlled burnoff of the trapped particulate, leading to lower peak oxidation temperatures in the trap (promoting longer trap life and improved trap reliability) and to minimization of the danger of unscheduled trap burnoff as a fire hazard.

Practical application of this concept requires development of suitable hardware and identification of the optimum operation cycle for the trap-oxidizer/catalyst injection system. The enclosed figure illustrates, in simplified form, a particulate control system that might be developed based on the metal-salt-catalyst concept briefly described in this paper.
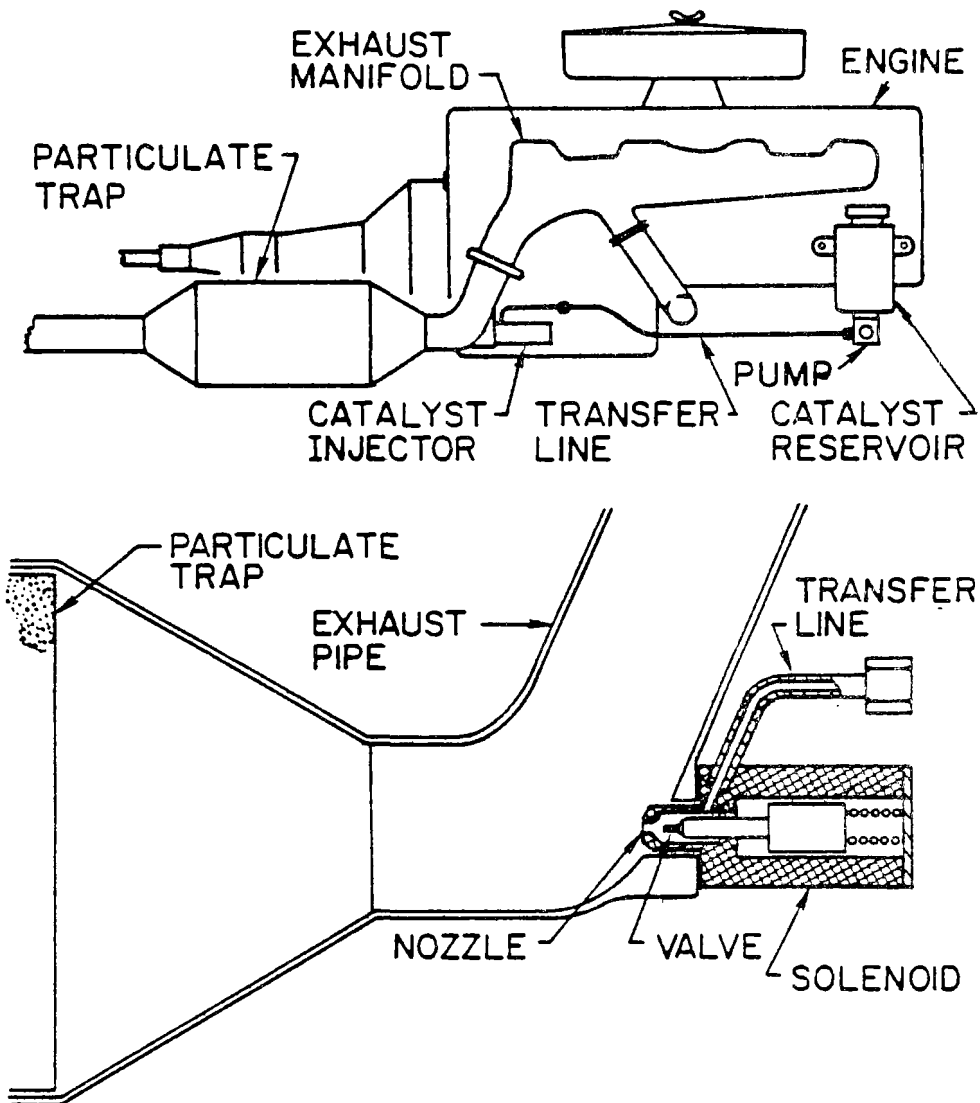
DIESEL ENGINE PARTICULATE CONTROL SYSTEM
BASED ON BATTELLE METAL-SALT-CATALYST
CONCEPT

HEAVY-DUTY DIESEL ENGINE EMISSIONS -- SOME EFFECTS OF CONTROL TECHNOLOGY

by

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Use of various control technology methods to reduce specific emissions such as NO_x or particulates usually result in changes to other constituents in the exhaust of diesel engines. The effects of control technology on EPA Advisory Circular No. 76 emissions are reported. Unregulated emission trade-offs as a result of timing, EGR, catalysts, and engine modifications are discussed. Fuel consumption increased with most changes.

Although the emission levels are changed as a result of the control technology, the emissions pose no obvious health risk based on estimated exposure levels and available health effects data.

Table 1. Engine Change Tradeoffs

↑ = Increase ↓ = Decrease ⟶ = No Change

Change	Particulates Total	SEF	HC	NO_x	ALD	BAP	Fuel Cons	Power
PG-OI	↑	↑↓	↑↓	↑	↑↓	↑	↓	⟶
EGR	↑	↓	↓	↓	↓	↓	↑	↘
Timing								
Advance	↑↓	⟶	⟶	↑	↑↓	↑	↓	⟶
Retard	↑↓	↑	↑↓	↓	⟶	↑	↑	↓
Aftercooling	↑↓	↑↓	↑↓	↑↓	↓	↓	↑↓	↑
Injector SAC Volume Increase	↑	↑	↑	↑	↑	↑	⟶	⟶
Catalyst*	↑↓	↑↓	↓	↓	↓	↓	↓	⟶
Fuel (BaP Increase)	—	—	—	—	—	↑	—	—

* SO_4 ↑↓ , NH_3 ↑

SECTION 2

CHEMICAL AND BIOASSAY CHARACTERIZATION

METHODOLOGY OF FRACTIONATION AND PARTITION OF DIESEL
EXHAUST PARTICULATE SAMPLES

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INTRODUCTION

The decision by the U.S. Environmental Protection Agency to examine the
health effects of diesel particulate emissions has resulted in a great increase
in the number of investigations to analyze their physical, chemical and bio-
logical characteristics.[1] Diesel particulate emissions are a very complex
mixture of carbonaceous matter containing adsorbed and/or condensed organic
components from the combustion of fuel and lubricants. The organic solvent
extractable fraction of these particulates is extremely complex and has been
reported to contain hundreds of individual compounds.[2] A number of studies
have reported that these solvent extracts are mutagenic as determined by the
Ames Salmonella microbial assay.[3-8] The greatest activity has been found to be
present in certain compound class fractions within these extracts which do not
require metabolic activation.[7-10] Chemical characterization studies have been
used in an attempt to identify the compound classes or specific compounds
present in these fractions.[4,10-11] In addition, polynuclear aromatic hydro-
carbons (PAH), particularly pyrene, have been reported to readily react with
nitrogen oxides to form nitrated derivatives which are powerful direct-acting
mutagens.[12,13] Both the PAHs and nitrogen oxides are present in the exhaust
of diesel engines and thus the formation of nitroaromatics may indeed be
possible. These results have prompted increased attention to the characteri-
zation of diesel particulate extracts to identify the compounds or compound
classes which are responsible for the biological activity.[4,14-17] Identifica-
tion of specific mutagens or classes of mutagens is important to determine
whether these components are present in the exhaust which is emitted to the
atmosphere or are formed as a result of the dilution particulate collection
and analysis procedures. This information must be established before potential
effects of these compounds on the environment can be addressed.

The objective of this paper is first to briefly review the general tech-
niques used for chemical characterization of diesel particulates. This review
is followed by a description of a procedure which can be used to quantitatively
characterize the chemical and direct-acting mutagenic properties of the soluble

organic fraction of these particulates. This procedure is based on a mass
fractionation and Salmonella bioassay to isolate classes of compounds which are
responsible for the mutagenic activity. Finally, applications of this pro-
cedure are presented for particulate extracts from two light duty diesel
engines and one heavy duty diesel engine.

MATERIALS AND METHODS

    Review of General Techniques. Figure 1 illustrates a typical experimental
approach used in the characterization of the soluble organic fraction of diesel
particulates. This general approach involves extraction of the organic mater-
ial from the collected particulate using an organic solvent, concentration of
the extract for subsequent analysis, fractionation into less complex non-polar
and polar sub-groups and a final concentration to facilitate chemical and
biological analysis. Ames Salmonella bioassay on the fractions requires that
the solvent be exchanged with one which is compatible with the assay, such as
dimethyl sulfoxide (DMSO).
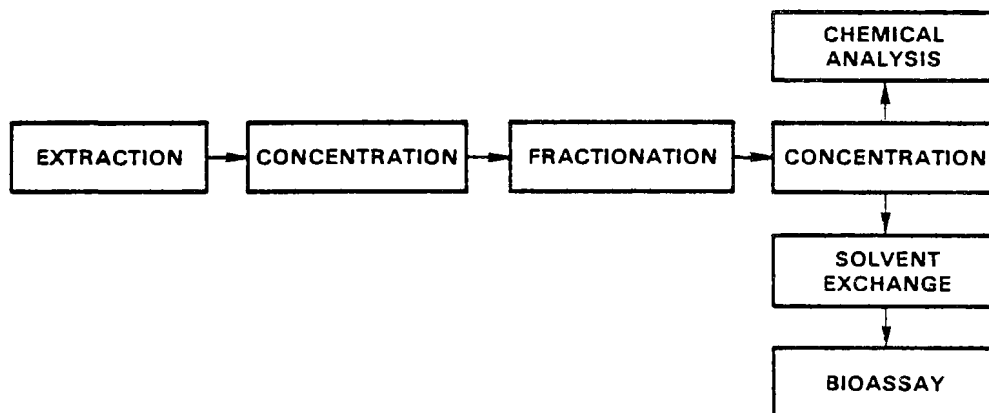
Fig. 1. General Experimental Approach

Chemical analysis of the fractions for identification of individual compounds has been carried out using a variety of chromatographic and mass spectrometric techniques. The non-polar fractions have been well characterized and consist of PAH and aliphatic hydrocarbons.[18-22] Identification of specific compounds in the polar fractions is difficult since these compounds are thermally labile, highly polar, low in volatility and very low in concentration.[2,23] Three specific techniques have been used to identify constituents in these polar fractions: (1) high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS)[2], (2) combined liquid chromatography/chemical ionization mass spectrometry (HPLC/MS)[11], and (3) high resolution gas chromatography/negative ion chemical ionization mass spectrometry (HRGC/NCI-MS) using on-column injection.[24] The latter two methods provide injection of the fraction at ambient temperature to avoid thermal degradation of the polar organics. In addition, since the energetics of chemical ionization are less than that of conventional electron impact ionization, the ionized molecules fragment less and a greater amount of the total ion current remains with the molecular ion. This feature aids the identification of the individual compounds. Negative ion chemical ionization is especially sensitive and selective for detection of several types of polar organics such as nitro or oxygenated PAH, because of the electronegativity of the polar substituent.

Within this general experimental approach, the procedures for extraction, concentration and fractionation can directly influence the integrity of the extract. To relate analysis data to the initial particulate sample, these procedures should not change the chemical and biological characteristics of the sample. An overview of these three procedures is presented in the following section.

Extraction. Two widely employed methods for removal of organics associated with diesel particulates are soxhlet extraction and ultrasonic extraction using a suitable organic solvent. The total quantity of material extracted by these techniques is influenced by the type of particulate being extracted, contact time with the solvent, temperature of the solvent during the extraction, and polarity of the solvent. Recent studies conducted by the Coordinating Research Council indicate that either technique can be used satisfactorily to measure the total soluble organic fraction.[25] Extraction by sonication is usually more rapid (0.5 to 1.5 hours) than using the Soxhlet technique (3 to 24 hours) but sonication requires several additional analytical steps to remove suspended soot and filter particles. Soxhlet extraction can be conducted unattended and many particulate samples can be extracted simultaneously in separate apparatuses.

The type of organic solvent used for extraction has received much attention recently. In general, methanol and aromatic-alcohol mixtures have been found to extract the largest quantities of material from the particulate presumably due to increased extraction of inorganic material.[7,26,27] Aromatic solvents have been shown to extract the largest quantity of PAH.[28] Methylene chloride, however, has been reported to extract the largest quantity of biologically active material from particulate, and is usually the solvent chosen for biological screening.[26]

Techniques such as vacuum sublimation and thermal desorption have been used as rapid methods for determining the organic content of particulate filter samples. Although these techniques may provide reasonable results for the volatile fraction of the organics associated with diesel particulates, the nonvolatile components are not completely removed. Collection of the organics using these techniques is difficult and chemical characterization of individual compounds and compound class is not possible.

Concentration. Concentration is necessary within various steps of the overall procedure. The solvent must be removed from the extract, or an aliquot of the extract, to determine the residue mass for calculating the total organic mass. To facilitate separation into fractions, the extract must be concentrated to a small volume, typically 50 ml or less. Finally, once fractionation is complete, the resulting fractions must be concentrated for chemical analysis by instrumental techniques.

Typical methods of concentration include removal of the solvent by rotary-film evaporation[25], vortex evaporation[26], nitrogen stream blow-down[25,27], and Kuderna-Danish concentration. The method of concentration will in many cases depend on the volume of solvent to be removed. Rotary film evaporation is a very rapid means of removing large quantities of solvent (1000 ml to 10 ml), and is probably the most widely used technique. Vortex evaporation is a very convenient method for reducing solvent volumes from 10 ml to 1 ml or less. Using this method, the solvent is transferred to a centrifuge tube, which is placed in the vortex unit. During the vortex mixing, the solvent is removed while heating (20-60 C) under vacuum (30 inches of H_2O). Nitrogen stream evaporation is used to concentrate the solvent to several hundred microliters or to dryness. Rates of evaporation are controlled by the nitrogen flow rate and the solvent temperature. Kuderna-Danish concentration has not received much attention for the concentration of particulate extracts primarily due to the overall time of concentration.

Concentration steps are generally a major source of compound loss through adsorption, evaporation, incomplete transfer between containers, and/or reaction with other constituents. Extreme care must be taken to insure minimal sample loss and maintaining sample integrity.

The total organic mass can be determined by total removal of the solvent under vacuum using a rotary-film evaporator, transfer of the residue to a tared vessel, and drying under nitrogen to constant weight. An alternative method is to reduce the volume of extract to a known value and remove a small aliquot for a residue mass measurement. The total extracted mass can be calculated by multiplying the mass concentration in the aliquot by the total extract volume. Using this alternative procedure, it is not necessary to bring the entire extract to dryness and may help preserve the chemical and biological integrity of the extract.

Fractionation. Fractionation of the particulate extract is usually required before further chemical and biological characterization can be achieved. A variety of partitioning methods and chromatographic techniques have been used to effect this fractionation on the basis of chemical functionality.[4,27-31]

Acids and bases can be removed from the neutrals by liquid-liquid partitioning sequencially with aqueous solutions at low and high pH. The neutral organic compounds can be further separated using solvent partitioning, and/or column chromatography. Pellizzari has developed a solvent partitioning scheme to separate the neutrals into non-polar, polar, and PAH fractions.[32] Column techniques using Sephadex LH-20, silica gel, and alumina have been developed by numerous workers to separate the neutrals based on compound classes.[25,27-31] Jewell has used a combination of ion-exchange coordination and adsorption chromatography (known as SARA technique) for the separation of acids, bases, neutral nitrogen-containing compounds, saturated hydrocarbons and aromatic hydrocarbons.[33]

The application of high performance liquid chromatography (HPLC) as a means of rapid fractionation of particulate extracts has been reported by Huisingh, et al.[4] This method has recently been modified by Schuetzle and co-workers to produce six specific fractions.[2] A complete fractionation is possible in approximately 40 minutes. In an interlaboratory validation study of HPLC fractionation, recovery of total mass is reported to be near 100 percent. However, biological activity of the fractions have been shown to be highly dependent on the influence of fractions containing very small mass. Although HPLC can be used to rapidly fractionate particulate extracts, it is limited in

the quantity of mass (∿15 mg) which can be fractionated during a single analysis. Thus many separations may have to be carried out to obtain the necessary quantity of material for chemical and biological characterization. There are several problem areas that are associated with the procedures for fractionation. The various steps involved with fractionation can certainly increase the preparation time, which is generally not desirable when large numbers of samples are to be analyzed. Contamination can readily occur during fractionation and can lead to discrete artifacts and interferences. Incomplete extraction from the fraction procedure can be a major source of sample loss. Liquid-liquid partitioning may cause the formation of tars, insoluble material, or stable emulsions, which result in inefficient separation.

To remove sources of contamination, all materials must be routinely demonstrated to be free of interferences under conditions of the analysis by running parallel laboratory blanks. The use of high purity solvents helps minimize interferences. Solvents should be always checked for purity. Glassware must be scruptuously cleaned usually by detergent washing, distilled water rinse, solvent rinse and heating at 450 C for several hours.

Procedure for Fractionation of a Particulate Extract into Specific Compound Classes. A fractionation method to separate chemical classes from extracts of particulate samples was developed by Battelle in 1978. Several refinements in the original procedure have been made, and considerable experience in its use has been obtained on a wide variety of particulate sample types. There are two advantages associated with this procedure which are pertinent to the analysis of the organic material extracted from diesel particulate samples:

- The method is sensitive enough to fractionate small quantities of total extracted mass. Particulate extracts containing 1 to 4 mg of total extracted mass have been sufficient for compound class separation and chemical analysis.
- The method can be conveniently scaled up for fractionation of much larger samples when biological as well as chemical characterization is required. Particulate extracts containing several grams of extracted mass have been successfully fractionated.

A total quantity of extracted mass of at least 50 mg is required for both chemical characterization and biological screening. The following description of the procedure is based on this minimum quantity. For larger or smaller samples, appropriate adjustments to the procedure can be made.

A schematic of the overall procedure is shown in Figure 2. The particulate sample is first extracted and two aliquots are removed; one for a residue mass measurement and one for a bioassay measurement. These measurements are used to calculate the total quantity of mass and biological activity in the extract. The remaining extract is separated into six compound class fractions. Two aliquots are removed from each fraction for determination of their residue mass and biological activity. A material and bioassay balance is calculated as percent recovery by summation of the fraction values, dividing by the original value and multiplying by 100 percent.
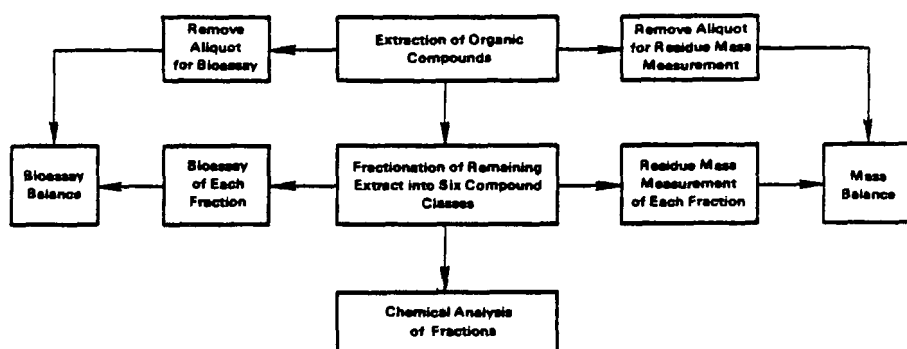
Fig. 2. Schematic of Fractionation Procedure

Extraction of Particulate Filter Samples. Particulate filter samples are extracted with 700 ml of solvent using the Soxhlet technique. An extraction time of 16 hours at a 15-minute cycle rate is normally used. Longer extraction times may be necessary if the solvent in the extractor is still colored after 16 hours. After extraction, the solvent is concentrated to exactly 50 ml by rotary evaporation. Two aliquots are removed from the extract; one (100 μl) for a residue measurement and one (500 μl) for Salmonella mutagenesis bioassay. The aliquot for residue mass measurement is deposited on a tared aluminum pan and the pan is placed under an infrared lamp to evaporate the solvent. After removal of the solvent, the pan is weighed (to constant weight) to the nearest microgram on an electronic balance such as a Mettler ME-30. This measurement is used to determine the total extractable organic mass from the particulate filter samples. The aliquot removed for bioassay is used to determine the total mutagenic activity originally present in the extract.

The Fractionation Procedure. Acids and bases are first separated by liquid-liquid partitioning. The neutrals are further partitioned by silica gel column chromatography. A schematic representation of the entire fractionation procedure is shown in Figure 3, and the generated fractions are listed in Table 1. For purposes of discussion, the fractions are referred to numerically as #1, #2, etc.

TABLE 1

COMPOUND CLASSES GENERATED BY FRACTIONATING SCHEME

Fraction	Fraction #
Bases	1
Acids and Phenols	2
Aliphatic Hydrocarbons	3
Aromatic Hydrocarbons	4
Moderately Polar Neutrals	5
Highly Polar Neutrals	6

Separation of Bases. In the fractionation scheme, the bases such as amines are first separated from the extract by liquid-liquid partition with 100 ml of 5 percent sulfuric acid solution three times. The aqueous phases are combined and the bases then back extracted into methylene chloride after adjustment of the pH of the solution to 12-13. The volume of the methylene chloride solution containing the bases is then reduced to exactly 5 ml by rotary and vortex evaporation. An aliquot of 10-100 µl (depending on the quantity of mass present) is removed to measure the organic mass in this fraction. The bases constitute Fraction #1. After an aliquot of 50-100 µl is removed for chemical analysis, the solvent is exchanged with DMSO and the fraction submitted for Ames bioassay.

Separation of Acids. The acidic (carboxylic) and phenolic compounds are extracted from the organic solution with 100 ml of 5 percent sodium hydroxide solution four times. The aqueous phases are combined and the acids then back extracted with methylene chloride after addition of Na_2SO_4 (for "salting out") and adjustment of the solution's pH to about 1. An aliquot of 100 µl is removed to measure the organic mass in this fraction. The acids and phenols constitute Fraction #2. Ames testing and chemical analysis is carried out on the acids by the same procedure as used with the bases.

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Fig. 3. Procedure for Extraction and Fractionation of Organic Material in Diesel Particulate

Diesel Particulate Filter Sample

Soxhlet extract in 700 ml solvent for 16 hours. Concentrate extract to 50 ml by rotary evaporation

Ames Mutagenesis Bioassay on Extract

Remove 500 μl For Ames Bioassay

Particulate Extract Concentrated to 50 ml

Remove 100 μl for residue wt

Measure total organic extractable mass

liquid liquid partition with 5% H₂SO₄ (100 ml, 3 times)

Bases in Aqueous Solution

Add 40% KoH till pH 13. liquid liquid partition with CH₂Cl₂ (100 ml, 3 times). Dry with anhydrous Na₂SO₄.

Bases in CH₂Cl₂ Solution

Evaporate to 5 ml remove 100 μl for residue weight

Measure Organic Mass in Fraction 1

Evaporate down to 1 ml
Add DMSO to 5 ml
Evaporate to 1 ml
Add DMSO to 5 ml

Ames Mutagenesis Bioassay

Neutrals and Acids in Organic Solution

liquid liquid partition with 5% NaOH (100 ml, 4 times)

Wash with distilled water (100 ml, once)

Neutrals in Organic Solution

Dry with anhydrous Na₂SO₄.
Evaporate down to 1 ml
Add hexane to 10 ml
Evaporate down to 1 ml
Add hexane to 10 ml
Evaporate down to 1 ml
Silica Gel Column Chromatography

Eluent, (Volume)	Fraction	Sample Code
Hexane (50 ml)	Aliphatics	3
Hexane-Benzene 1:1 (100 ml)	Aromatics	4
Methylene Chloride (100 ml)	Moderately Polar	5
Methanol (100 ml)	Highly Polar	6

Evaporate each Fraction to 5 ml remove 100 μl for residue weight

Measure Organic Mass in Fractions 3-6

Evaporate each Fraction to 1 ml
Add DMSO to 5 ml
Evaporate each Fraction to 1 ml
Add DMSO to 5 ml

Ames Mutagenesis Bioassay on Fractions 3-6

Acids and Phenols in Aqueous Solution

Add 6M H₂SO₄ till pH = 1. liquid liquid partition with CH₂Cl₂ (100 ml, 3 times). Dry with anhydrous Na₂SO₄.

Acids and Phenols in CH₂Cl₂ Solution

Evaporate to 5 ml. Remove 100 μl for residue weight

Measure Organic Mass in Fraction 2

Evaporate to 1 ml
Add DMSO to 5 ml
Evaporate to 1 ml
Add DMSO to 5 ml

Ames Mutagenesis Bioassay on Fraction 2

Separation of Neutrals. The organic solution containing the neutral com-
pounds is further partitioned into four fractions by open column chromatography
on 5 percent H_2O-deactivated silica gel. The silica gel columns (2.5 cm i.d. x
15 cm long) are packed with 20 g of the silica in a hexane slurry and the gel
retained with a glass frit. Columns are prepared for each organic solution to
be partitioned. An additional column is also prepared to check the accuracy
of the silica gel deactivation before partitioning the neutral organic solution.
This is done by measuring the volume of hexane required to elute 500 µg of
anthracene. The migration of the anthracene is monitored by a 366 nm UV lamp,
and the volume of hexane is measured during the migration. When the silica gel
is deactivated 5 percent, anthracene starts to elute from the column after the
addition of 140 ± 10 ml of hexane. For 3 percent and 7 percent deactivation,
the volume of hexane required is 270 ± 12 ml and 115 ± 8 ml, respectively.

Upon assurance that the silica gel is 5 percent H_2O deactivated, the neutral
organic compounds are further fractionated. Separation of the neutrals into
compound classes using silica gel is carried out by increasing the polarity of
the eluting solvent. Therefore, the separation scheme must begin with the most
nonpolar eluent, and the solvent must be exchanged with hexane before trans-
ferring the solution to the silica gel column. This is accomplished by
reducing the volume to 5 ml, adding 45 ml of hexane and reducing again to 5 ml.
Repeating this process will result in a complete solvent exchange. The solu-
tion is transferred to the silica column using a Pasteur pipette. Polar
neutral compounds will be present in the original vessel as insoluble material
after the solvent exchange into hexane. As the column elution solvent is
changed it should first be introduced into the vessel to dissolve the polar
material and then transferred to the silica column. Four elution solvents are
used in the following sequence: 60 ml hexane, 100 ml hexane/benzene (1:1),
100 ml methylene chloride, and finally, 200 ml methanol. The eluent is
collected individually for each solvent and corresponds to fraction numbers 3,
4, 5 and 6. These fractions are evaporated to exactly 50 ml and 100 µl removed
to measure the organic mass. After measurement, 1-4 ml is removed from each
fraction for chemical analysis and the remaining solution submitted for Ames
bioassay after solvent exchange with DMSO. Saturated and unsaturated hydro-
carbons are present in the aliphatic compound class. The aromatic fraction
will contain the polynuclear aromatic hydrocarbons, alkyl substituted benzenes,
nitrogen and sulphur heterocyclic hydrocarbons and mono nitro derivatives of
2, 3 and 4 ring PAH. Chlorinated hydrocarbons as well as silicones will also
be present in this fraction. The moderately polar neutral fraction will

contain mono nitro substituted PAH greater than 4 rings as well as all dinitro and trinitro PAH. Mono oxygenated PAH of 2, 3 and 4 rings such as fluorenone will be present in this fraction. The highly polar neutral fraction will contain substituted PAH with more than one type of functionality (i.e., nitro and keto groups such as in nitrofluorenones) and poly oxygenated PAH. Any plasticizers, such as phthalates and sebacates in the analytical system will also be present in this fraction.

Concentration of Fraction Aliquots for Chemical Analysis. The fraction aliquot is transferred to a 5 ml pyrex Chromaflex tube, and capped with a piece of aluminum foil. The tube is placed in a water bath so that the water line is even with the solvent. The temperature of the bath is maintained at 10 C above the boiling point of the solvent. A nitrogen stream (200-500 μl/min) is introduced to the Chromaflex tube using a stainless steel capillary, positioned 0.5 cm above the reflux line of the solvent. This allows a portion of the solvent to be removed slowly while the remaining solvent condenses and continuously rinses the walls. As the solvent is removed, the position of the Chromaflex tube is adjusted to maintain the solvent line even with the level of water bath. The solution is concentrated to 100 μl, which takes about 2-4 hours, depending on the volatility of the solvent. Concentrated solutions are stored at -70 C until analysis to minimize sample degradation.

Ames Salmonella Mutagenesis Bioassay. The assay is conducted by adding a 0.1 ml aliquot of an overnight broth culture of tester strain TA-98 (without metabolic activation, or S-9) to 2 ml of molten top agar supplemented with biotin and a trace of histidine. Subsequently, dose levels of the test extracts and compound class fractions are added to the appropriate tubes. Dose levels used are 25, 50 and 100 μg. The contents of these tubes are mixed thoroughly and poured over the surface of selective agar plates. Following solidification, the plates are incubated for 72 hours at 37 C and scored for the number of colonies growing on each plate.

The number of revertant colonies on the duplicate mutagenesis plates are averaged for each concentration of test material and control. The control value or number of spontaneous revertants are subtracted from the counts of the test dosages. The resulting number of revertants are then divided by the dosage to ascertain the activity of the extracts or fractions per unit weight.

RESULTS AND DISCUSSION

The application of the chemical and biological characterization of organics associated with particulates has been demonstrated for particulate filter

samples from two light-duty passanger car diesel engines (LDDI and LDDII) and
one heavy-duty truck type diesel engine (HDD). All particulate filter samples
were collected on Pallflex T60A20 PTFE impregnated glass fiber filters using an
exhaust dilution tunnel. Particulate filter samples were collected from the
test engines as they were operated under the following conditions:

> LDDI: steady-state, medium speed/medium load
> LDDII: highway fuel economy test cycle
> HDD: steady-state, rated speed/rated load

Table 2 summarizes the extraction and fractionation results from these three
engines. A discussion of these results for each engine is presented in the
following section.

TABLE 2

EXTRACTED ORGANIC MASS DATA

| | | FRACTION MASS, mg | | | | | | |
ENGINE TYPE	TOTAL ORGANIC EXTRACTABLE MASS, mg	BASE	ACID	ALIPHATIC	AROMATIC	MODERATELY POLAR NEUTRAL	HIGHLY POLAR NEUTRAL	TOTAL FRACTION MASS, mg (PERCENT RECOVERED)
LDD I	61.90	14.65	1.85	17.43	3.46	4.11	14.60	56.11 (91)
LDD II	142.03	0.20	10.11	69.49	15.12	11.38	27.21	133.51 (94)
HDD	63.24	1.49	9.48	8.23	5.61	12.07	23.40	60.28 (95)

   LDDI. For the LDDI engine, a total of 61.90 mg of total organic mass was
extracted from the particulate filter samples. The base, aliphatic, and high-
ly polar neutral fractions contained significant quantities of material, the
sum of which accounted for approximately 75 percent of the total extracted mass.
In general, the aliphatic and highly polar neutral fractions contained the
majority of mass in extract, however, the quantity of mass in the base fraction
was still unusually large. A possible reason for this large quantity may be
due to the presence of amines as a result of the high oil consumption of this
specific engine. The aromatic and moderately polar neutral fractions contained
about 6 and 7 percent of the extracted mass which are considered average.
Acids accounted for 3 percent of the total mass and is considered low. Typical
quantities in the acid fraction are 8 to 12 percent of the total mass. The
recovery of total mass through the fractionation procedure was determined to

be 91 percent and is considered satisfactory.

LDDII. The total organic mass extracted from the particulate filter samples was 142.03 mg. Again, the aliphatic and highly polar neutral fractions contained significant quantities of mass and accounted for 48 and 20 percent of the total extract. The base fraction contained less than one percent of the total mass, and the aromatic and moderately polar neutral fractions represent 10 and 7 percent of the total mass. Acids accounted for approximately 7 percent of the total mass. Recovery of the extracted mass through the fractionation procedure was 94 percent. In general, the distribution of mass within the fractions is typical for particulate extracts of light-duty diesel engines.

HDD. The total organic extract was 63.24 mg, which represented 3.1 percent of the total collected particulate mass. This low percentage of total organics as well as the distribution of mass within the six compound class fractions is typical for heavy-duty diesel engines operated at rated speed and rated load. The aliphatic fraction contained only 13 percent of the total extract while the polar and acid fractions account for the majority of the mass. Both the base and aromatic fractions contained average quantities of mass. Recovery of the extract through the fractionation procedure was 95 percent.

Ames Mutagenesis Bioassay Results. Table 3 summarizes the bioassay data using tester strain TA-98 without metabolic activation of the extracts and their individual fractions. The data is presented as the total number of revertants within each extract and fraction. These values were determined by multiplying the average mutagenic response at the 50 μg dose level by the total mass in the extract or fraction.

Both light-duty extracts contained greater than 300,000 total revertants whereas the HDD extract only contained about 50,000. A bioassay balance was calculated by comparing the sum of the revertants within the fractions to the total revertants found in the extract. The recovery of biological activity was as follows: LDDI, 72 percent; LDDII, 95 percent; and HDD, 97 percent.

Table 4 presents the bioassay data in terms of specific activity as revertants per milligram (rev/mg) of mass. The LDDI and LDDII extracts had specific activities of 5,049 and 2,290 rev/mg. Specific activity for the HDD was much lower at 819 rev/mg. The moderately polar neutral fraction of all three extracts is very active. For the two light duty diesel extracts, the specific activity of this fraction dominates the activity of the other fractions as well as that of the total extract. Activity of the moderately polar neutral fraction in the HDD extract is much less than that of the LDD

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fractions, however, it is still significant in relation to the other HDD fractions.

TABLE 3

AMES MUTAGENESIS BIOASSAY (TOTAL REVERTANTS) OF PARTICULATE EXTRACTS AND COMPOUND CLASS FRACTIONS USING TESTER STRAIN TA-98 (NONACTIVATED)*

ENGINE TYPE	TOTAL EXTRACT	BASE	ACID	ALIPHATIC	AROMATIC	MODERATELY POLAR NEUTRAL	HIGHLY POLAR NEUTRAL	TOTAL FRACTION ACTIVITY (PERCENT OF TOTAL)
LDD I	312.592	2,867	3,593	1,830	20,656	101,434	96,360	226,740 (72)
LDD II	326,180	132	39,865	—	22,227	220,020	25,894	308,125 (95)
HDD	51,824	1,036	14,424	—	777	15,547	18,555	50,340 (97)

*AVERAGE MUTAGENIC RESPONSE AT 50 μg DOSE LEVEL X MASS OF EXTRACT OR FRACTION

TABLE 4

AMES MUTAGENESIS BIOASSAY (REVERTANTS/mg) OF PARTICULATE EXTRACTS AND COMPOUND CLASS FRACTIONS USING TESTER STRAIN TA-98 (NONACTIVATED)

ENGINE TYPE	TOTAL EXTRACT	BASE	ACID	ALIPHATIC	AROMATIC	MODERATELY POLAR NEUTRAL	HIGHLY POLAR NEUTRAL
LDD I	5,049	1,549	5,019	104	5,969	24,679	6,600
LDD II	2,290	660	3,946	—	1,472	19,300	952
HDD	819	695	1,521	—	139	1,288	793

CONCLUSIONS

Fractionation into compound classes using the described procedure resulted in a satisfactory material balance for a variety of particulate extracts. Recovery of the total extracted material was 91 percent for the LDDI extract; 94 percent for the LDDII extract; and 95 percent for the HDD extract. A satisfactory biological activity balance was also demonstrated for these samples. Recovery of the total revertants was 72 percent for the LDDI extract; 95 percent for the LDDII extract; and 97 percent for the HDD extract.

Two important conclusions can be drawn from these data: (1) the mass and mutagenicity, using TA-98 (-S9), of the extract is approximately equivalent to the sum of the mass and the mutagenicity of the fractions, and (2) the fractionation procedure does not significantly influence the mutagenicity of the extract. The data presented in this paper demonstrates the utility of this fractionation procedure for the general chemical characterization of organics associated with diesel particulates. Furthermore, the ability to conduct a general biological screening for direct acting mutagenicity within the particulate extracts is also demonstrated. This procedure separates the particulate extracts into less complex fractions which can be analyzed by a variety of analytical techniques for identification of specific compounds.

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THE UTILITY OF BACTERIAL MUTAGENESIS TESTING IN THE CHARACTERIZATION

OF MOBILE SOURCE EMISSIONS: A REVIEW

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INTRODUCTION

In 1978 Huisingh et al.[1] reported that organic fractions chemically extracted from the exhaust particles of diesel vehicles were mutagenic in the Salmonella typhimurium plate incorporation (Ames) test. This report and an EPA cautionary notice[2] for laboratory workers exposed to diesel exhaust sparked expanded efforts in industry and government to understand whether or not these bacterial mutagens also presented any potential health risks. This question was especially important since the automotive industry was planning expanded light-duty dieselization for fuel economy. Although this original report was quite extensive — examining multiple vehicles, multiple fuels, chemical fractions of exhaust organics, etc. — some obvious questions had not been answered or even approached. Researchers in government and industry, striving to serve public interest, wanted to answer several critical questions as rapidly as possible:[3]

- Could these bacterial mutagens be artifacts of the testing process?
- Could these mutagens extracted from carbonaceous particles by the use of chemical solvents also be extracted by physiological fluids, enzyme systems, or cells within the human body?
- Would interaction with an ambient air situation alter the activity of these genotoxic materials?
- Could these bacterial mutagens cause hereditary or carcinogenic effects?
- What portion of the population would be exposed to these mutagens and to what degree would they be exposed?
- And ultimately, what hazard and risk would further dieselization present to man and to future generations?

Obviously, bacterial mutagenicity testing cannot answer some of these questions. Bacterial tests cannot define a substance as a human carcinogen and cannot be directly used for quantitative risk estimation. These microbial tests can be used, however, to aid in overcoming difficulties encountered while trying to understand the potential genotoxic effects of mobile source emissions. Table 1 lists some of the stages at which difficult research questions are

TABLE 1

DIFFICULTIES ENCOUNTERED IN THE INTERPRETATION OF MOBILE
SOURCE GENOTOXICITY STUDIES

Stage	Types of Difficulty
Generation	Characterization of the source; Repeatability of testing procedure; Vehicle-to-vehicle variation.
Collection	Artifact formation (NO_x, organic interaction, ozone); Control versus ambient conditions.
Extraction	Physiological versus organic; Selective loss and recovery of compounds; Artifact formation.
Fractionation	Recovery efficiencies; Complexity.
Bioassay	Relevance; Reproducibility; Endogenous activation; Bioassay versus human metabolism.
Statistical	Collection/recovery efficiencies; Data transformations; Data summary methods; Statistical/correlation methods.

generated and the problems to which these questions are related. Bacterial
testing has provided and will continue to provide many of the needed mechanisms
for recognizing, defining, and answering these difficult problems. Bacterial
tests have proven valuable because they are rapid, relatively inexpensive indi-
cators of genotoxic activity that can be used in a unified effort with other
research methods. For the cost of one life-time animal chronic-toxicity study
of one exhaust sample, more than 100 different vehicles can be compared in a
simple bacterial screen. When exhaust organics are fractionated by physical/
chemical means, bacterial testing can follow the distribution of genotoxic
activity before compound identification is complete. When road-way and smog
chamber samples are collected, microbial tests allow for comparison of genotoxic
activity with that of dilution tunnel samples. The purpose of this paper,
therefore, is to recognize and document the role that bacterial mutation tests

have played in characterizing mobile source emissions for genotoxic activity.
This presentation will be divided into four areas: 1) generalized observations,
2) assessing factors that modify the mutagenicity of mobile source emissions,
3) the comparison of various mobile source emissions, and 4) the effect of
physiological fluids and enzyme systems. This review will assume that the
reader has a general knowledge of mutagenicity testing and mobile source
research.

GENERALIZED OBSERVATIONS

Most of the bacterial mutagenesis research has been conducted in the same
manner as that originally reported by Huisingh et al.[1] They, and most other
investigators, have used the _Salmonella typhimurium_ plate incorporation assay
as described by Ames et al.[4] as the primary test protocol. Some investigators
have used all five strains as recommended by Ames. However, many investigators
have used only one or two strains (generally TA98 and/or TA100), primarily for
two reasons. First, the sample amounts available have been relatively limited,
and secondly, TA98 and TA100 have been the strains most responsive to organics
extracted from mobile source emission particles. TA1535, which responds by
base-pair substitution, has been negative or only marginally positive with total
extracts; however, since fractionation studies have not generally used this
strain, mutagens that cause base-pair substitution may be overlooked. Perhaps
even more interesting are the observations concerning strain TA1538. In
contrast to TA98, which exhibits the same or a decreased response with S9 addi-
tion, TA1538 exhibits an increased response upon the addition of an exogenous
activating system. This response means that TA1538 (although not providing as
many revertants per plate) is detecting indirect-acting mutagens that are not
readily recognized with strain TA98. Again, researchers may be failing to
identify another class of mutagens. Nitroaromatic activity in diesel exhaust
was first recognized by the use of another group of bacterial tester strains —
the nitroreductase-deficient strains.[5-7] Additional work by Rosenkranz et al.[8]
and Mermelstein et al.[9] has provided and characterized additional nitroreductase-
deficient strains since this initial work. Work with other bacterial strains and
systems has generally been neglected. While some investigators[6,10,11] have
demonstrated the usefulness of the 8-azaguanine forward mutation assay, most
others have not used this simple, more quantitative system.

Many investigators have also recognized that exogenous activation systems
reduce the mutagenic response of diesel exhaust organics, with the exception

of the TA1538 results. Few have emphasized, however, that these same exogenous activation systems generally enhance the mutagenic response of gasoline exhaust organics.[12] This simple observation clearly demonstrates the presence of different mutagenic compounds in exhaust organics from diesel and gasoline engines.

ASSESSING FACTORS THAT MODIFY THE MUTAGENICITY OF MOBILE SOURCE EMISSIONS

Fuels and oils

One of the first questions posed for diesel emissions work was: "Is the mutagenicity due to mutagens in the fuel or due to the combustion process?" The diesel fuel used by Huisingh et al.[1] was negative when tested directly in the Salmonella bioassay. Lebowitz[13] also reported diesel fuel as being negative. Wang[14,15] reported that a diesel fuel, JP-4, and two types of gasoline were negative when tested with TA98. Other investigators, however, have reported various crude oils and some of their distillates to be positive in the Ames test.[16-18] Calkins[18,19] reported that some natural, syncrude, and shale oil crudes, and some of their distillates are positive in the Ames test. In each case, however, the naphtha distillate was negative. Epler et al.[20] and Guerin et al.[21] demonstrated that coal-derived petroleum substitutes could exhibit ten times the bacterial mutagenic activity of a similar natural product and that the mutagenic response could occur over several orders of magnitude. They also reported that the activity of petroleum crudes was found mainly in the neutral fraction, while significant activity was found in both the neutral and basic fractions of derived fuels. Within the neutral fraction, Guerin et al.[21] found that aromatic amines were the predominant mutagenic constituent. When Henderson et al.[22] separated diesel fuel into an aromatic and an aliphatic fraction, they found both fractions mutagenic using strain TA100. They also noted that exposure to NO_2 dramatically increased the response of both fractions. This work was similar to the work of Pitts,[23] who exposed benzo(a)pyrene to NO_2 and generated a direct-acting derivative. The effect of fuel type upon the mutagenicity of the emission organics was shown by Huisingh et al.[1] They tested the effect of seven different fuels in two different vehicles and found a wide activity range in the mutagenicity for the emission organics. The results from McClellan[24] are similar, and both studies tend to demonstrate that fuels high in aromatic content produce a more notable mutagenic response. Crankcase oils have also been examined for mutagenic activity. Wang et al.,[14] Herman et al.,[25] and Löfroth et al.[7] each stated that unused crankcase oils are not mutagenic, but that used crankcase oils from gasoline engines give a positive response. In

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addition, Löfroth states that 1) metabolic activation increased any mutagenic response seen, 2) the response increased with vehicle mileage, and 3) this positive response was not seen with used oil recovered from a diesel engine. In no case has it been demonstrated that the mutagenic emissions of diesel or gasoline engines are due primarily to mutagens within the fuel.

Test cycles

Although most papers describe the test cycle(s) used when generating exhaust samples, only a few researchers have published any direct comparison of test cycles. When reporting data as revertants per microgram of organic material, Gabele et al.[26] found no great differences between six different test cycles. Gibbs et al.[27] was able to examine five different cycles with six different cars. They made the following observations:

- expressing the data as revertants per gram of particulate gave "widely divergent" results,
- expressing the data as revertants per mile, "cycle-to-cycle" trends were more pronounced and reproducible,
- when ranking cycles by revertants per mile, activity decreased in the order FTP > CFDS > HFET > 50C, and
- a general reduction in revertants per mile was found as the mileage of the vehicle increased (upon close examination of Gibbs et al.[27] data, it was noted that very low mileage cars {<4000 miles} demonstrated a very enhanced mutagenic response for all cycles except idle).

McClellan (July 1980)[24] examined four test cycles using a single automobile and noted that in his study "cycles with lower speeds and more stops and starts (NYCC and FTP) had higher mutagenic activity."

Collection methods and artifact generation

The greatest potential problem in collection methodology is the generation of artifacts, i.e., the generation of substances that do not exist in the natural situation or the elimination of substances that would normally exist. A number of investigators[5-7,28-33] have demonstrated by various means that mutagenic nitroaromatic compounds are contained in organic extracts of filter-collected particles. However, since diesel and gasoline also emit varying levels of NO_x gases that pass across the filters and collected particles, these nitroaromatic compounds may be artifacts. There are basically three places where these nitroaromatics may be produced: 1) in the combustion process, 2) in the exhaust process as organics interact and condense upon the particles, or 3) in the

collection process as an artifact. Pitts et al.[23] were the first to show that the passage of nitrogen oxides across a polynuclear aromatic hydrocarbon (PAH) compound upon a filter could generate a nitroaromatic (NO_2-PAH) that is direct-acting in the Ames bacterial assay. Henderson et al.[22] exposed 1-g samples of fuel aromatics and fuel aliphatics to excess NO_2 at 25°C and generated direct-acting mutagens for strain TA100. The aromatic NO_2 fraction was the most active and nitro-PAH compounds were identified in this fraction. In some preliminary experiments Bradow[34] and Claxton[35] passed artificial gas streams containing high levels of NO_2 across filters with diesel particles and observed increased mutagenic activity of the extracted organics. Gibson et al.[29,30] re-exposed filter-collected diesel particles to the gas-phase portion of similar diesel emissions and found increased levels of 1-nitropyrene, nitrobenzo(a)pyrene, and mutagenic activity. Although the extent and the relevance of this artifact problem has not been fully resolved, bacterial testing has paved the way in identifying and providing the methods for examination of the problem.

Extraction and chemical methods

The mutagenic response of different chemical fractions from the organic emissions of a diesel engine was initially done with an organic extract from emission particles of two heavy-duty engines.[1] In this study, the two most active fractions were eluted from a silica gel column and were designated as the transitional and oxygenate fractions. Choudhury and Doudney[36] fractionated organic emissions into three fractions and noted that 1) the acid fraction had the highest specific activity and showed direct-acting mutagenicity, 2) the basic fraction was enhanced by the addition of S9, and 3) the neutral fraction accounted for 94% of the mass and was predominantly direct-acting. Subsequently, Choudhury subfractionated the neutral fraction with adsorption chromatography methods into seven subfractions. Subfractions 3 to 7 were positive; however, the paraffinic subfraction was not active. Upon examining emissions from both a diesel and a gasoline vehicle, Löfroth[7] noted that the aromatic and an oxygenate fraction were the most mutagenic. McClellan's work,[37] using a Fiat with varying conditions, showed that upon Sephadex fractionation three of five fractions were mutagenic to bacteria. The classes of compounds said to contribute to the mutagenicity of these fractions were alkyl-substituted PAH compounds and oxygenated PAH. Ohnishi et al.[38] examined the fractionated emissions of two heavy-duty vehicles and one small diesel and found each fraction tested as being positive. Rappaport et al.[39] examined 16 liquid chromatography fractions of organic emissions from a Cummins turbodiesel engine and postulated that

pyrene-3,4-dicarboxylic acid anhydride and similar compounds accounted for a sizeable portion of the mutagenic activity. Siak et al.[40] summarized his fractionation study with emission organics from a GM 5.7 L diesel engine by stating that "more than 90% of the biological activity was accounted for in the neutral-nonpolar II, neutral polar, weak and strong acid fractions." Using normal phase and reverse phase thin layer chromatography, Pederson and Siak[31,32] showed that some major mutagenic constituents were the mono-substituted nitro-PAH compounds and other more polar compounds. The examination of diesel exhaust organics for active nitroarenes seems to have been spurred on by the earlier reports of Claxton[5] and Löfroth,[7] who demonstrated the presence of nitroaromatics by performing the bacterial bioassay with nitroreductase-deficient strains and anaerobic conditions. It is also interesting to note that very few of these investigators used the indicator strains TA1535 and TA1538; therefore, some mutagens that cause base-pair substitution and that need activation to be a frameshift mutagen will be missed in these bioassay-directed fractionations. Although the activity of these types of mutagens may have been missed in the total organic extract, bioassay-directed fractionation has been established as the primary means of identifying biologically active compounds in complex organic mixtures from combustion sources.

Ambient conditions

Ambient conditions can affect the condensation of organic compounds onto particles, influence the interaction between organics, alter the organic species emitted by a source, and provide for various other interactions. Most diesel and gasoline emission studies, however, have been done under non-ambient conditions, i.e., by the use of dilution tunnels, tail pipe filters, etc. The few studies that have been done have taken different approaches. Claxton and Barnes[41] studied ambient-like conditions in a controlled manner through the use of the Calspan smog chamber. In their studies, they found that the presence of ozone in the chamber tended to reduce the mutagenic response of the organic material collected; however, irradiation without other mitigating factors such as ozone did not alter the mutagenic response. A mutagenic sample also was collected when (without diesel exhaust) propylene, SO_2, NO, and NO_2 in an ambient atmosphere were irradiated, so as to produce ozone. Ohnishi et al.[38] examined road-side particles collected within a highway tunnel. When examined with TA100 and an activating system, they found 60 to 88 revertants/m^3 for particles collected during daytime hours. However, particles collected at night with a high density of diesel traffic exhibited 121 to 238 revertants/m^3. Alfheim and

Møller[42] collected ambient air particles at a road-side site, on a roof, and within a park. They found that the contribution of traffic to mutagenicity of air samples was significant and that the mutagenicity at street level varied with traffic frequency. In the Allegheny tunnel study,[43] it was shown that roadway-produced diesel aerosol is similar in activity to organics recovered in dilution tunnel studies. Secondly, it demonstrated that in revertants/km traveled, the mutagenicity of diesel vehicle exhaust is several times that of gasoline engine exhaust. The New York Port Authority bus terminal study[44] provided some real contrasts to other ambient air studies. Although the air particle concentration inside the bus terminal was three times the outside concentration, the mutagenicity outside based on revertants/m^3 was greater than that of inside air. (Jungers et al.[45] describes the technology available for ambient air studies.) These studies demonstrate that the production, chemical alteration, distribution, and concentration of mutagenic mobile source particles is dependent upon traffic patterns, amounts of reactive gases and vapors, level of ozone present, meteorological conditions, and the presence or absence of other ambient air particles.

COMPARISON OF VARIOUS MOBILE SOURCES USING BACTERIAL BIOASSAYS

A number of different diesel and gasoline engines and vehicles have been used for the generation of emission samples for mutation testing. Although a few authors have not given descriptions, most have described the engine and/or vehicles used in their studies; however, these descriptions are basically very limited. Generally, even less information is provided about the test cycle or run procedures, the fuel and lubricants, the description of the dilution and collection devices, and the precise methods of sample preparation. In addition, although most investigators described their bioassay as following the procedure of Ames et al.,[4] recent work by Toney and Claxton[46] shows that most "Ames testing laboratories" have made specific modifications to this somewhat standard protocol. The mutagenicity results are also analyzed and summarized in a variety of ways. Once these facts are understood, one is aware that the comparison of engines and vehicles between different studies (and even within the same study) must be done with caution and only in a qualitative manner.

In the first paper by Huisingh et al.[1] (Parts I and II), two heavy-duty engines and three light-duty vehicles were used. Although a direct comparison of these sources was not the primary purpose of this report, it provided the first mobile source comparison based on bacterial mutagenicity. In this study, particle exhaust organics from the heavy-duty engines were tested in five

tester strains (TA98, TA1535, TA100, TA1537, and TA1538) both with and without exogenous activation. Qualitatively, the two engines showed very similar results with the four positive strains having decreasing activity in the order TA100 > TA98 > TA1538 > TA1537. Without activation, TA1535 was negative with samples from both engines; however, with activation one engine (a Caterpillar 3208, 4-stroke V-8 engine) gave a marginally positive response. When examined in a more quantitative manner, the samples from these two engines demonstrated a difference greater than ten times in response for each of the tester strains; however, results were examined only as revertants/mg of particles. Due to sample amounts available, the exhaust organics from the three light-duty engines were tested using only strain TA1538. In this study, in which a comparison was made based upon the fuel used, results for even a single vehicle (using different fuels), could vary greater than 100 times. Other studies[5-7,47-49] report the results of multiple vehicle and/or engines in their reports. Qualitatively, the results are in agreement with the report of Huisingh et al.[1]

In order to demonstrate the effect of some sampling parameters, Claxton and Kohan[6] reported three specific types of comparison: 1) between different runs with the same diesel engine, 2) between gasoline vehicles of the same make, model, and configuration, and 3) between different makes of diesel vehicles. The results are summarized in Table 2. The coefficient of variation for the revertants/mi for the above three cases was respectively 0.11, 0.49, and 0.59. Assuming the coefficient of variation is a good estimation of the standard deviation in relation to the mean, and assuming a normal distribution for the test values, one can estimate the 99% confidence limits and percent of variation from the mean expected in all three cases. For the above three cases, a value could be within 99% confidence limit values and vary by 33, 147, and 177%, respectively. If multiple testing facilities, fuels, and bioassay laboratories are used, the variation between results would be expected to increase. Recognizing that the Ames assay is a semi-quantitative test for screening substances over a dynamic range of ~10^6 in dose/response slope, and recognizing that other parameters (such as percent extractable of the particles) show broad variation, then this seemingly large variance for a complex testing situation should not be considered excessive. Together, these studies indicate that semi-quantitative comparisons can be done within a single study and that cautious qualitative comparisons can be made using results from multiple studies.

TABLE 2

COMPARISON OF SUMMARY DATA DEMONSTRATING THE EFFECT OF DIFFERING SAMPLING
PARAMETERS[6]

	Slope[a] Rev/μg	% Ext.[b]	Rev x 10^5/ g Part[c]	P.E.R.[d] g/Mile	Rev x 10^5/ Mile[e]
Different Runs Within Same Automobile: (Diesel)					
Mean	3.68	11.8	4.35	0.524	2.27
SD	0.42	1.0	0.64	0.037	0.26
Coefficient Var.	0.11	0.09	0.15	0.07	0.11
Vehicles of Same Make, Model, and Configuration: (Gasoline)					
Mean	7.03	7.52	3.16	0.0102	0.032
SD	3.51	7.83	0.87	0.0048	0.016
Coefficient Var.	0.50	1.04	0.28	0.47	0.49
Different Diesel Vehicles:					
Mean	1.98	36.6	6.96	0.687	4.38
SD	0.80	18.0	4.06	0.256	2.59
Coefficient Var.	0.41	0.49	0.58	0.37	0.59

[a]Slope of linear regression line (revertants per plate per microgram organic material)
[b]Percent extractable
[c]Revertants x 10^5 per gram of particulate matter
[d]Particle emission rate
[e]Revertants x 10^5 per mile

EFFECT OF PHYSIOLOGICAL FLUIDS AND ENZYME SYSTEMS ON THE MUTAGENICITY OF
DIESEL EXHAUST

Since initial testing involved organic chemicals extracted from particles
with strong organic solvents, researchers questioned whether or not chemicals
bound to carbonaceous particles would be released into physiological fluids or
in the in vivo situation. McGrath et al.,[50] tested whole particles suspended in
dimethylsulfoxide (DMSO) and obtained results ranging from negative to moderately
positive in those tested using the Ames bioassay. However, DMSO is a moderately
effective solvent. In 1980, Siak et al.,[51] reported extracting particles with
four simulated biological fluids: fetal calf serum, 0.5% bovine serum albumin,
lung surfactant, and saline. The assay of each biological fluid in the Ames
test was negative except for a positive response with the fetal calf serum. The

fetal calf serum extract provided only about 6% of the response found with extraction by dichloromethane (DCM). Brooks et al.[52] found similar results with dog serum, lung lavage fluid, saline, dipalmitoyl lecithin, and albumin. However, they state that "the minimal mutagenic activity... may be due to a lack of removal of mutagens from the particles or an inactivation of removed mutagens by binding or some other process." When Clark and Vigil[53] tested a DCM diesel extract in the presence of Aroclor 1254 induced rat liver S9, an uninduced S9, an S9 without NADP, bovine serum albumin, and fetal calf serum, they showed a decreased mutagenic response in each case, suggesting that protein binding of mutagenic components was at least partially responsible for the lack of activity seen with incubated particles. By following the mutagenic activity of the DCM extracts in serum, lung cytosol, protease-treated serum and lung cytosol, and extracted particles, King et al.[54] demonstrated the release of mutagens from diesel particles and postulated that the lack of mutagenic response is due to either protein binding or metabolism. Siak and Strom[55] exposed rats to diesel particles, recovered the lung macrophages, and extracted the macrophages with DCM. They showed that although the particles continued to contain mutagens, "seven days after exposure, DCM extracts of alveolar macrophages had no detectable mutagenic activity, even though more diesel particles were recovered." These effects may be due to either protein binding or metabolism. Wang and Wei[56] and Wang et al.[57] gave evidence that the antimutagenic effect of S9 is not enzymatic by examining S9, heat-deactivated S9, S9 minus cofactors, and albumin effects. Somewhat in contrast, Pederson and Siak[32] used a nitroreductase-deficient bacterial strain to show that some mutagens in diesel particle extracts are activated by S9 and that 1-nitropyrene was also activated by NADPH-dependent S9 enzymes. Other studies presented within this volume will have an impact upon our understanding of this issue. In essence we now know the following: 1) mutagenic substances are released from diesel exhaust particles into certain physiological fluids and cells, 2) physiological fluids and S9 decrease the apparent mutagenic activity of diesel extracts and particles primarily because of protein binding, and 3) some mutagenic components (e.g., 1-nitropyrene) are activated by the microsomal fraction of S9, while other components are activated by the cytosol fraction.

SUMMARY

In summary, the work presented demonstrates that rapid, in vitro indicators
of genotoxicity have been and will continue to play a valuable role in under-
standing the toxicity of mobile source emissions. Bacterial assays have had
tremendous value in the characterization of mobile source emissions. Specifi-
cally they have had four major uses: 1) comparative screening, 2) analyzing
factors that alter the genotoxicants found in emission products, 3) directing
the chemical fractionation of emission organics for the identification of specific
genotoxicants, and 4) analyzing the interaction of complex emission products
with various mammalian systems.

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EMISSION FACTORS FROM DIESEL AND GASOLINE POWERED VEHICLES: CORRELATION WITH
THE AMES TEST

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INTRODUCTION

In 1978, initial findings on the mutagenic nature of diesel extracts
were reported.[1] Since that time, the Ames 'Salmonella typhimurium bioassay
has been used extensively in the investigation of mobile source emissions.
Both government and industry have carried out numerous studies on the many
variables effecting the mutagenicity of mobile source samples. These studies
for the most part fall into six general categories: 1) Sampling-exhaust
dilution ratios, gas phase vs particulate phase collection and examination of
filter types; 2) Bioassay sample preparation-extraction solvents, extraction
procedures, solvent exchange studies and sample storage; 3) Vehicle types-
light duty diesels, heavy duty trucks and gasoline powered cars; 4) Opera-
tional characteristics-driving cycles, fuel types, temperature, mileage and
engine malfunction conditions; 5) Artifacts; 6) Characterization studies-
qualitative and quantitative studies on the nature of mobile source mutagens
and their precursors.

The areas of sampling and bioassay sample preparation impact on all
resultant data. Some of the concerns in these areas will be briefly review-
ed. The question of artifacts, which is closely tied to sampling consider-
ations, is an area of on-going study. The status of these efforts is being
reported else-where in this symposium.[2] Diesel particulate extracts have
been characterized by many investigators. The majority have employed liquid
chromatography to resolve the extracts into non-polar (aliphatic and aromatic
hydrocarbons), moderately polar (transition) and highly polar (oxgenates)
fractions; the latter two fractions contain most of the Ames activity.
Various nitroaromatic compounds, e.g. 1-nitropyrene (1-NP) are potent muta-
gens which may be very important in the overall activity observed for diesel
extracts.[3,4] The mutagenic response of gasoline powered vehicle particulate
extracts relative to diesels is different suggesting the importance of other
compound classes (see text). Little characterization work has been reported
for gasoline particulate extracts, however.

The major emphasis of this report will be various operational parameters
and their effect on mutagenicity. Finally, the results from several recent

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studies on emission factors and Ames test mutagenicities of gasoline and diesel powered vehicles will be compared.

Sampling. The majority of mobile source testing consists of operating a vehicle or engine on a dynamometer and passing the exhaust into a dilution tunnel connected to a constant volume sampler (CVS). The total air flow remains constant even though the engine exhaust flow may vary due to transient operation (acceleration, deceleration). Sampling the diluted exhaust at a constant rate will give an integrated sample representative of a particular driving cycle. The average dilution of the exhaust is generally from 10:1 to 20:1 depending on the engine size and average operating speed. Actual dilution on the highway reaches 1000:1 shortly after the exhaust exits the tailpipe. Concerns were expressed that collecting diesel exhaust particulate samples under the relatively low dilution used in normal test procedures would not represent the roadway situation, especially with respect to the particulate bound organics. Several investigations have indicated that the composition of the particulate is fixed when it leaves the tailpipe and is not significantly influenced by further dilution.[5,6] No differences were observed in either the particulate emission rates or the molecular weight distribution of the soluble organic fraction (SOF). More recently, studies conducted at the Allegheny Mountain tunnel found that the diesel produced aerosol at Allegheny to be very similar to that encountered in dilution tube experiments with respect both mutagenicity in the Ames test and molecular-weight distribution.[7]

None of these above studies, however, address the question of what happens to the particulate organics on prelonged exposure to sunlight and/or to other chemical species present in the atmosphere such as ozone. The destruction of polynuclear aromatic hydrocarbons (PAH's) under atmospheric conditions is well known. For example, Falk, et al. found that benzo(a)-pyrene (BaP) absorbed on combustion soot was decomposed 10% on exposure to light and air for 48 hours and 18% on exposure to light and smog for only 1 hour.[8]

Preliminary results on the mutagenicity of diesel exhaust particulate exposed to light and ozone in a smog chamber found similar TA 98 activities for exhaust samples which had been irradiated (6 hours) or aged in the dark (4 hours). However, exposure to ozone at parts per billion levels (up to 650ppb) resulted in a significant decrease in TA 98 activity, the decrease being most pronounced without metabolic activation.[9]

Another major area of study concerning sampling has dealt with filter

types. Glass fiber filters had long been a standard medium for general particulate sampling. However, several studies indicated they were not the filter of choice for diesel application as they (Gelman GF/AE) were subject to adsorbing gas phase hydrocarbons.[5] This resulted in the recommendation of a teflon-coated glass fiber type filter, (e.g. Pallflex T60A20) for the determination of diesel particulate emission rates.

The effects the different filter media may have on the Ames activity of the SOF of diesels have also been studied. Diesel particulate was simultaneously collected on Teflon membrane (Zeflour), Teflon coated glass-fiber (Pallflex T60A20) and quartz fiber (Pallflex 25000A0) filters. No differences were observed in sample mutagenicities in strains TA 1538, TA 98 or TA 100.[10] Particulate loadings (0.3 to 0.7 $mg \times cm^{-1}$) were typical of those encountered in diesel studies. At these loadings the filter matrix is quickly covered with particles, and particles are then collected on particles; any interactions of the filter matrix with the particles is minimized.[11]

Bioassay Sample Preparation. Soxhlet extraction has been the method of choice for obtaining the SOF from mobile source particulate. In the case where gram quantities of the SOF are required for bioassay procedures such as skin painting, it is the only practical procedure. Investigations of solvents representing a wide range of polarities have generally found dichloro methane (DCM) to be the most efficient single solvent for extracting mutagens from diesel particulate.[12]

The more polar solvents also extract variable amounts of inorganics as shown in Table 1.[13] The % sulfate values are relative to the total amount of sulfate present in the particulate phase. On the other hand, a benzene-ethanol azeotrope was found superior for extraction of benzo(a)pyrene, a known carcinogen and mutagen.[6] DCM extracted only about 70% of the BaP obtained by benzene-ethanol.

Table 1

SOLVENT STUDY

Solvent	% Extractables	% Sulfate
Cyclohexane	12.65	0
Toluene	14.15	0
Methylene Chloride	14.09	0
Acetone	19.08	25 - 50
Acetonitrile	13.43	11 - 50
Benzene-Ethanol	21.32	16 - 25*

*Mass Balance Not Obtained.

Operational Characteristics. Malfunctions: The majority of emissions and Ames test data has been from vehicles operating according to manufacturers specifications. However, many vehicles in actual use on the highway may be operating under various malfunction conditions due to a lack of or improper maintenance, component failures, and engine wear. Some of these malfunctions may drastically effect emissions yet not noticably effect performance. [14]

Little data is currently available as to how these malfunctions effect mutagenicity, but indications are they may be considerable. Two studies have indicated that injector problems with diesels can cause significant increases in mutagenicity. A 1980 Volkswagon Rabbit diesel experienced a 42% decrease in Ames mutagenic activity (TA 98) by replacing a faulty injector.[15] Nissan Motor Company also demonstrated that problems with secondary fuel injection due to a lack of pressure pulse dampening in the fuel injection system can cause increased mutagenicity and BaP emissions.[16]

A recent investigation of in-use gasoline vehicles[17] included several with various malfunctions evidenced by their emission factors as shown in Tables 2 and 3. The specific malfunctions were not identified, except that the 1976 Fury obviously was an oil burner (82% SOF) and 1977 Dodge Aspen was suspected of having EGR problems. The presence of oil in the SOF of the Fury had a diluting effect on mutagenic activity expressed as revertants/μg, but mutagenic activity on a revertants per mile basis, particularly with activation was substantially higher than average for the unleaded gasoline vehicles. BaP emissions for the Fury were also the highest monitored for any of the catalyst vehicles tested (19.7 μg/mi vs fleet average of 2.1 μg/mi, excluding the Fury). The other catalyst vehicles which failed to meet certification values for regulated emissions generally had somewhat lower revertant per μg activities, but higher revertant per mile activites, with the exception of the 1981 Citation. While these vehicles with some apparent malfunction generally had increased revertant per mile activities, it should also be noted that a 1979 Chevette which met certification values for THC, CO, and NO_x, exhibited the highest revertant per mile activity (-S9 = 181.4 x 10^3; +S9 = 424.4 x 10^3) of any of the catalyst cars, including the Fury. BaP, Pyrene, and 1-nitro-pyrene emission rates as well as revertant per μg SOF activities were also much higher than the average.

Fuel effects. The composition of diesel fuel might be expected to in-fluence the mutagenicity of the SOF in two ways: 1) direct contribution of mutagens, e.g. BaP in Fuel, or 2) by percursor supply, e.g. Pyrene in the

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TABLE 2
EMISSION FACTORS FOR MALFUNCTION VEHICLES

| Vehicle | g/mi | | | mg/mi | |
	HC	CO	NO$_x$	Part.	SOF%
1976 Fury	24.5	32.2	3.0	192	81.7
1977 Aspen	2.30	28.0	6.1	30.0	45.0
1978 Dodge Truck	2.06	22.6	3.2	36.1	23.0
1981 Dodge Van	0.65	7.1	6.4	24.5	43.3
1981 Citation	0.79	10.1	3.3	15.3	11.8
1977-79 Cert.	1.5	15.0	2.0	(3.1 in 1976)	
1981 Cert.	0.41	3.4	1.0		

TABLE 3
PARTICULATE EMISSIONS FOR MALFUNCTION VEHICLES

| Vehicle | SOF mg/mi | BAP µg/mi | TA 98 Activity | | | |
| | | | Rev/µg | | Rev/mi * | |
			-S9	+S9	-S9	+S9
1976 Fury	156.9	19.7	0.4	1.3	65.9	204
1977 Aspen	13.5	3.8	3.6	7.5	48.1	101
1978 Dodge Truck	8.3	7.9	4.3	12.4	35.4	102
1981 Dodge Van	10.6	1.3	12.6	5.6	133	59.2
1981 Citation	1.8	0.1	3.8	6.8	6.9	12.3
Fleet Average	4.9	2.1	8.0	14.2	40.6	71.0

*x 10^3

fuel yielding increased levels of nitropyrene or more indirectly, certain components may aid or be more prone to combustion synthesis of particular mutagens. Table 4 lists several fuel parameters and their correlation with Ames activity in revertants per microgram of extract.

Highest correlations were seen with nitrogen content of the fuel, although the range is rather limited. In view of this correlation, experiments were conducted wherein fuel nitrogen levels were varied by doping a base fuel with isoquinoline. However, results thus far indicate no correlation between mutagenicity and fuel nitrogen levels when the nitrogen is introduced as isoquinoline.[14]

The aromatic content of the fuel tended to show slight correlation with activity, particularly without activation. Henderson, et al. have recently reported that treating diesel fuel with NO_2 greatly increased its mutagenicity. Furthermore, the activity of the NO_2 treated aromatic fraction from the fuel was 40 times greater than the NO_2 treated aliphatic fraction (strain TA 100). No 4 or 5 ring aromatics were detected in the work by

Henderson. However, the fuels used with the Caterpillar study in Table 4
were found to have pyrene levels from 185 to 14,000 µg/liter. (The correla-
tion of fuel aromaticity and fuel pyrene was 0.53). Given the high mutagen-
icity reported for the nitropyrenes,[4] the pyrene and other aromatics in the
fuel may be important nitroaromatic precursors. However, no correlation was
observed between specific aromatics in the fuel and the mutagenicity of the
extracts in the previous fuel studies.

TABLE 4

R-SQUARE CORRELATION COEFFICIENTS FOR FUEL PARAMETERS VS AMES ACTIVITY

| | | Cat. 3304 | DD-8V71 | |
Fuel Parameters	S9	TA 1538	TA 98	TA 100
Aromaticity (14-39%)	-	0.67	0.66	0.95
Aromaticity (14-39%)	+	0.82	0.37	0.59
Fluoranthene, µg/L	-	0.03	--	--
Pyrene, µg/L	-	0.09	--	--
BaP, µg/L	+	0.00	--	--
Nitrogen, wt.%	-	0.88	0.98	0.88
Nitrogen, wt.%	+	0.98	0.83	0.20

Cat. - 13 mode composite, 6 fuels, $\%N_2$ = 0.005 - 0.024
DD-1983 Transient Cycle, 5 fuels, $\%N_2$ = 0.006 - 0.61

The lack of correlation between specific PAH's in the fuel and mutagen-
icity is not totally unexpected, however, as a significant amount of muta-
genicity may apparently be derived from combustion synthesis of hydro-
carbons. Studies conducted at Pennsylvania State University with a single
cylinder diesel engine run on pure isooctane and tetradecane have yielded
SOF's with high mutagenicity.[18] Furthermore, the levels of 1-nitropyrene
found in the SOF were similar to those found in the SOF of a 1980 Oldsmobile
diesel operated on #2 Diesel fuel containing 21,000 µg/l pyrene.[19].

Temperature effects. The effects of temperature on the FTP emissions
of a 1980 Volkswagon diesel Rabbit and 1980 Oldsmobile diesel were recently
studied.[20] The vehicles were conditioned (soaked) overnight at a range of
ambient temperatures such that crankcase oil temperatures at the beginning
of each test ranged from 23°F to 82°F. The FTP's were conducted at ambient
temperatures (an average of 12°F above crankcase temperature) without heat-
ing of dilution air.

Decreasing FTP test temperatures resulted in slightly increased HC, CO,
and NO_x emissions (7-17%), increased particulates (17-30%), and SOF (40-63%)
and decreased fuel economy (-11 to -17%). The majority of the increased
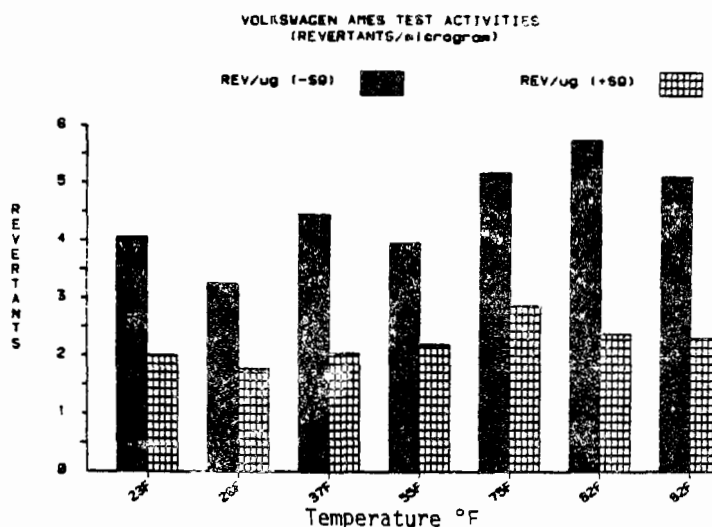
VOLKSWAGEN AMES TEST ACTIVITIES
(REVERTANTS/microgram)

REV/ug (-S9) ■   REV/ug (+S9) ▦

Fig. 1. Low Temperature Study - Volkswagen Ames test
activities (Revertants/microgram).

VOLKSWAGEN AMES TEST RESULTS
(REVERTANTS/mile)

REV/mi x 1000 (-S9) ■   REV/mi x 1000 (+S9) ▦
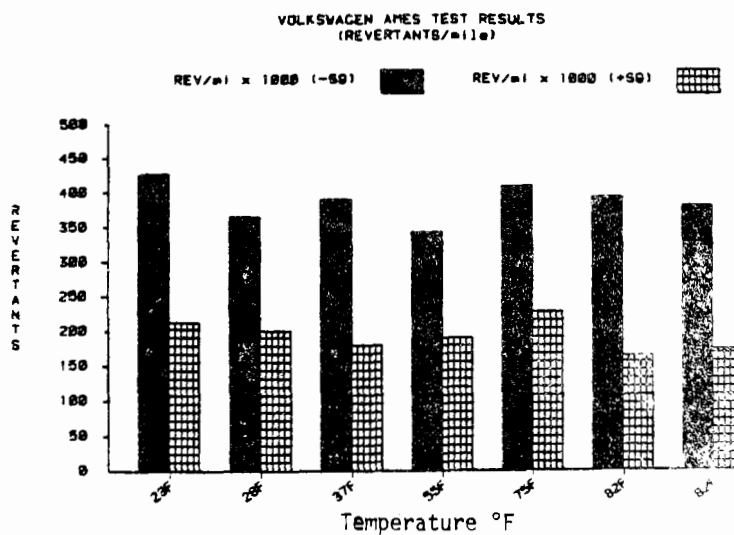
Fig. 2. Low Temperature Study Volkswagen Ames test
activities (Revertants/mi x10 ^{3}).

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particulate and SOF was found to be uncombusted diesel fuel. On a revertant per μg SOF basis, TA 98 activity without activation appeared to decrease with decreasing temperature (see Figure 1). A mild correlation of Ames activity with soak temperature was observed, r^2 = 0.73 for both vehicles. No correlation was found to exist between soak temperature and activity on a revertant per mile basis. (See Figure 2) This would be expected if the increased SOF emissions at low temperature were uncombusted fuel which is not Ames active and acts as a diluent.

Vehicle Types. As previously mentioned, the majority of mobile source emission characterization work has been carried out on test vehicles which are operating according to manufacturers specifications. However, the emissions from consumer operated vehicles may be appreciably different[21] and indeed, it is these emissions which impact air quality and public health. With this in mind the following data for comparison of various vehicle types was selected from representative in-use vehicle studies.

Tables 5 and 6 lists emission factors and Ames test data from recent studies of light duty in-use vehicles operated over the cold start Federal Test Procedure.[15,22] The diesel data is from 6 cars (4 General Motors, 1 Volkswagon, and 1 Mercedes Benz) while the gasoline data is from a 20 vehicle study (4 leaded gasoline (GM,Ford, Datsun, Honda) and 16 unleaded gasoline vehicles employing various catalytic control systems (8 GM, 4 Chrysler, and 4 Ford). Table 7 and 8 list some limited data for heavy duty trucks operated over the proposed 1983 transient cycle, a cold start, soak, hot start procedure similar to the light duty FTP sequence.[23] Several of the light duty gasoline vehicles had some apparent emission control malfunctions as previously discused (see tables 2,3).

The average as well as the minimum and maximum values observed for each emission factor are given in tables 5 and 6. In the case of the light-duty gasoline cars, the range was over two orders of magnitude. Vehicle to vehicle variation far exceeded those observed in repeditive testing of the same vehicle. (This implies it is important to test a large number of vehicles in establishing emission inventories which might be used in modeling studies). Excluding the oil burning Fury which was discussed under malfunctions, all the light-duty gasoline vehicle particulate associated organic emissions examined with the exception of 1-nitropyrene were higher for the leaded vehicles. Ames activity in revertants per μg of SOF were similar but the revertant per mile levels were considerably higher in the case of the leaded vehicles, mainly as a result of increased SOF emissions.

TABLE 5
FTP EMISSIONS FOR IN-USE LIGHT DUTY GASOLINE AND DIESEL VEHICLES

Emission Factors	Leaded Gas (4)[A]	Unleaded Gas (15)	Diesel (6)
THC, g/mi	2.74[B] (1.66-3.48)[C]	1.05 (0.22-2.94)	0.38 (0.21-0.60)
CO, g/mi	28.5 (15.0-61.0)	12.2 (1.6-28.0)	1.27 (1.03-1.86)
NO_x, g/mi	3.52 (2.5-5.2)	2.35 (0.2-6.1)	1.27 (0.8-1.9)
Particulates, mg/mi	102 (49.3-128)	21.0 (5.9-36.1)	607 (370-1070)
SOF, mg/mi	21.1 (6.8-33.5)	4.9 (0.7-13.5)	124 (45-290)

A Number of Vehicles
B Mean Value
C Minimum and Maximum Values Observed

TABLE 6
FTP PARTICULATE EMISSIONS FOR IN-USE LIGHT DUTY GASOLINE AND DIESEL VEHICLES

Emissions Factors	Leaded Gas (4)[A]	Unleaded Gas (15)	Diesel (6)
Bap, μg/mi	14.6 (1.1-35.5)	2.1 (0.1-12.4)	4.5[B] (0.9-7.7)[B]
1-Nitropyrene, μg/mi	0.20 (0.08-0.36)	0.19 (0.004-1.21)	7.8[B] (3.4-10.5)
TA 98 -S9, Rev/μg	7.3 (6.2-8.0)	8.0 (0.4-19.6)	4.1
TA 98 +S9, Rev/μg	12.5 (9.1-15.9)	14.2 (1.3-42.2)	---
TA 98 -S9, Rev/mi[C]	152 (51.1-256)	40.6 (2.3-181)	509 (260-670)
TA 98 +S9, Rev/mi[C]	258 (107-489)	71.0 (3.3-424)	---

A Number of Vehicles
B Typical Values, Not Reported In Ref. 20
C $x10^3$

Both vehicle groups exhibited higher cold start Ames activity with activation, suggesting the importance of PAH or other compounds requiring activation. BaP emissions of the catalyst vehicles (excluding the Fury) had a fair correlation with Ames activity in TA 98 with activation (r^2 = 0.81).

The Ames test data within the gasoline vehicle catagory may be quantitatively comparable as most all the vehicles were tested together in two experiments, one with activation (+S9) and one without (-S9). Comparisons of the data between the diesel and gasoline groups is more qualitative, however. Ames test results obtained by different laboratories or even the

TABLE 7
HDV TRUCK TRANSIENT EMISSION CHARACTERISTICS

	Gasoline		Diesel			
	Ford 370	HI 345	Cat. 320B	Mack 676	Cumm. 290	DD 8V71
GVW Lbs. (x10-3)	19.7	24.0	27.5	80.0	80.0	36.9
HC, g/mi	20.9	6.2	1.90	1.43	2.32	2.13
CO, g/mi	129	103	5.1	9.5	8.0	75.1
NO_x, g/mi	13.0	13.7	19.3	29.6	27.4	35.5
Particulate g/mi	0.58	0.89	0.90	1.95	1.61	3.33
SOF, mg/mi	40.6	14.5	475	193	336	537

TABLE 8

HDV TRUCK TRANSIENT EMISSION CHARACTERISTICS

	Gasoline		Diesel			
	Ford 370	HI 345	Cat. 320B	Mack 676	Cumm. 290	DD 8V71
BaP, µg/mi	61.0	17.1	1.61	0.92	5.30	1.33
TA98 -S9 Rev/µg	2.57	8.05	1.10	1.36	1.02	0.04
TA98 +S9 Rev/µg	14.9	17.4	1.08	1.22	0.95	0.09
TA98 -S9 Rev/mi[A]	104	117	523	262	343	21.5
TA98 +S9 Rev/mi[A]	604	253	515	232	319	48.3

[A] $x10^3$

same laboratory over a period of time may vary for various reasons related to assay protocol.[24-27] An additional source of variation may be related to the particular form of data reduction employed as several models and linear regressions procedures are available to calculate activities.[28,29] Table 9 compares the results obtained from the present study of four leaded gasoline vehicles (Group A) with those of four different leaded gasoline vehicles (Group B) obtained two years previously. Although the vehicle mixes are similar, major differences are observed in the Ames test results. If these earlier four vehicles were used for comparison with the recently tested unleaded vehicles, somewhat different conclusions would be drawn. Since the sample is so small, it is presently not known whether these diverse results simply indicate the range of valves possible or whether they also reflect variations in the Ames test itself.

TABLE 9
COMPARISON OF TA 98 AMES TEST RESULTS FROM TWO GROUPS OF LEADED GASOLINE
VEHICLE - HWFET DRIVING CYCLE

Test Group	HC	g/mi CO	NOx	mg/mi Part.	% SOF
A	1.35	8.15	6.0	276	10.9
B	2.21	32.1	3.5	222	7.27

Test Group	mg/mi SOF	Rev/µg -S9	+S9	Rev/mi x10 3 -S9	+S9
A	23.5	8.6	10.7	163	232
B	16.1	0.6	1.47	9.85	23.7

A 1970 Ford, 1973 Chevy, 1979 Datsun, 1979 Honda
B 1963 Chevy, 1971 Chevy, 1976 Honda, 1978 Datsun

In comparing the light duty diesel and gasoline vehicle groups, the average HC, CO, and NO_x emissions were lower for the diesels. These data suggest that the diesel's durability toward regulated emissions is superior to gasoline vehicles. Diesel total particulate FTP emission rates were 5.9 x the leaded gasoline vehicle rates and 19.1 x the unleaded gasoline. Diesel highway fuel economy test (HWFET) values (not shown) were 1.3 x the leaded and 13.5 x the unleaded gasoline. Diesel FTP SOF emission rates were 5.9 x the leaded and 8.6 x the unleaded gasoline .

Ames test results generally showed the gasoline vehicles to have higher activity with activation while the reverse was the case with diesels. This may also relate to the BaP and 1-nitropyrene (1-NP) emissions observed for each vehicle class. (Note: BaP & 1-NP levels for the diesels were not reported in reference 20 but should be representative values). The levels of cold start FTP BaP, an indirect acting mutagen, were higher than the diesels in the case of the leaded cars and similar for the unleaded. As previously mentioned, cold start FTP BaP emissions and activity in TA 98 with activation correlated fairly well (r^2 = 0.81) for the gasoline vehicles. On the other hand, the levels of 1-NP a direct acting mutagen, were 20 to 30 times greater for the diesels than the gasoline cars. Correlation of 1-NP emissions from the gasoline cars with activity in TA 98 without activation was very poor (r^2 = 0.25). The correlation of 1-NP emissions from diesels with activity in TA 98 without activation while not possible in this study was found to be significant in some unpublished work.[17] Driving cycle studies conducted on a 1980 Volkswagon Rabbit diesel resulted in an r^2

= 0.96 and artifact studies with a Cummins 290 heavy duty diesel had an r^2 = 0.97. On the other hand, the above Rabbit diesel had only an r^2 = 0.79 for the series of FTP runs made in the low temperature study previously discussed. The Ames activity therefore, is indicative of the types of compounds responsible but individual mutagenic species may or may not act as mutagenic markers. The highest levels of 1-NP observed in the studies with the Rabbit would have accounted for 5% of the activity, using 1.7 revertants/ng for the activity of 1-NP (4) and assuming their are no synergisms involved.

The transient heavy duty truck data in Tables 7 and 8 shows emission levels are much higher than the light duty vehicles, with the notable exception of BaP which is lower for the heavy duty diesels. Within the heavy duty (HDV) class, many features are similar to the light duty, i.e., HDV gasoline HC and CO emissions are higher and particulates and SOF are low compared to the HDV diesel. Because of the differences in gross vehicle weights (GVW), only the Caterpillar 3208 can be fairly compared to the two HDV gasoline vehicles. Even in this case, however, the gasoline vehicles were operated at wide open throttle during much of the transient testing which was not necessary with the more powerful caterpillar engine. The two stroke engine (Detroit Diesel, DD 8V-71) has noticably higher particulates and SOF, generally related to oil consumption in this type of engine. Ames activities in TA 98 in revertants per μg in general were much lower for the heavy duty diesels than the light duty diesels, but are similar on a revertant per mile basis due to the heavy duty's increased SOF emissions.

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ANALYSIS OF VOLATILE POLYCYCLIC AROMATIC HYDROCARBONS
IN HEAVY-DUTY DIESEL EXHAUST EMISSIONS

by

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The breakthrough and/or loss of polycyclic aromatic hydrocarbons (PAH) from particulate filters was investigated during the collection of heavy-duty diesel exhaust emissions. The concentration of PAH associated with heavy-duty diesel particulate has been monitored for several years. During these studies it was observed that appreciable quantities of organics including PAH pass through the particulate filter during the sample collection (1). The emissions from a heavy-duty diesel engine were sampled using 70-mm Pallflex TX40HI20WW filters. A portion of the gas passing through the particulate filter was sampled using 4.0-mm x 4.0-cm sorbent cartridges. Chromosorb 102 and Tenax GC were used to collect gas phase organics. The particulate filter was extracted in a micro-Soxhlet apparatus for 6 hours with methylene chloride. The sorbent cartridges were extracted by passing hexane/benzene (90/10, v/v) through the cartridge at a flow rate of 0.5 ml/min. Following concentration the PAH fraction was isolated using open column silica gel chromatography. The PAH in particulate and gas phase samples were analyzed using gas chromatography/mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC).

In the GC/MS analysis the samples were eluted on a 15.0-m $\times$ 0.31-mm i.d. SE-54 fused silica capillary column directly into the ion source of a Varian MAT 311A mass spectrometer operating in the repetitive scanning mode. The data were enhanced using a computer program by Dromey et al. (2) to locate peaks in the raw data and provide a set of clean mass spectra of the sample components free of contributions from background and overlapping peaks. Compounds were identified with a library matching search algorithm, and in the case of complex spectra the data was manually interpreted.

Twelve parent PAH were measured in the gas phase and particulate samples using reverse phase HPLC. The analysis was performed using two coupled 4.6-mm x 25.0-cm Zorbax ODS columns and an acetonitrile water gradient. The eluant were monitored using an ultraviolet absorbance detector at λ = 254 and 280 nm and a fluorescence detector, λ_{ex} = 280 nm and λ_{em} = 389 nm.

Over 40 compounds were tentatively identified in the gas phase after a particulate filter during the collection of heavy-duty diesel exhaust emissions. Thirty-five of these compounds were volatile PAH and included parent- and aldyl-substituted compounds ranging in size from two to five fused rings. A distribution quotient was defined as the ratio of the concentration of the PAH in the gas phase to their concentration in the particulate phase. It ranged from ~ 56 for fluorene to ~ 1 for benz[a]anthracene. Experiments to date show that at some operating conditions the concentrations of volatile PAH were significantly lower when measured on the particulate only. Since the role of these compounds in the formation of artifacts and mutagens is unresolved, the methods for measuring PAH in exhaust streams need to be modified to account for gas phase compounds.

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THE CHEMICAL CHARACTERIZATION OF DIESEL PARTICULATE MATTER

by

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INTRODUCTION

A great deal of research has been directed toward elucidating the potential health hazards of Diesel particulate matter. The potential health risks of the emmitted particles are due to a number of important factors. The mass median diameter of the agglomerated particles found in Diesel exhaust is less than 1 um (1-4), and their surface areas greater than 50 m^2/g (3,4). The large surface areas facilitate the adsorption of gas phase combustion products, while the small diameters allow appreciable residence times in the atmosphere. In addition, it is generally accepted that particles less than 1 um in diameter can be respired by humans, with a significant portion depositing in the pulmonary regions of the lung (5-7). An assessment of the potential health effects of Diesel particles must also consider the fact that many of the surface adsorbed species which have been isolated from the soluble organic fraction (SOF) of Diesel particulate matter are potentially carcinogenic (8-10).

A number of references have appeared in the literature in recent years regarding the identification of the individual constituents of the SOF (11-15). The commercial Diesel fuels and lubricating oils which were used in each of these studies contain such a wide variety of compounds that a study of the combustion mechanisms which lead to the particle-bound products becomes extremely difficult. An understanding of the mechanisms governing the formation of the adsorbed species is a requisite for complete comprehension of the potential health effects of Diesel particulate matter. The primary objective of this research was to simplify the combustion chemistry in order to better understand the overall mechanisms governing the formation of the particle-adsorbed species.

EXPERIMENTAL

Diesel particulate matter was generated from a single-cylinder engine, operated on a 1:1 by volume blend of n-tetradecane and 2,2,4-trimethylpentane. This prototype fuel was used in conjuntion with a synthetic lubricating oil, in order to simplify the exhaust chemistry. Air and argon/oxygen oxidant systems were employed. The Ar/O_2 was used in order to investigate the products of nitrogen-free combustion. Particle samples were collected on 142 mm Pallflex filters in an isokinetically drawn sample line, and gas phase emissions were monitored. Filters were Soxhlet extracted with dichloromethane, and the resulting extract blown to dryness under nitrogen and weighed. Samples were analyzed by the Ames Salmonella and Comptest (16) bacterial assays in order to assess their mutagenic and potential carcinogenic capaity.

The simplifications introduced in this study allow the separation and analysis of the entire SOF in a single pass. Particle extracts were analyzed by capillary gas chromatography, using flame ionization and thermionic specific detectors, and high performance liquid chromatography, using UV detection. Both positive and negative chemical ionization mass spectrometry were employed for direct analysis of the gas chromatographic effluents, and for analysis of collected HPLC fractions, using a Pt-filament, direct-insertion probe. Capillary gas chromatography/electron impact mass spectrometry was utilized for substantiating identifications. In addition, Diesel particles were heated under vacuum, and the evolved gases analyzed by chemical ionization mass spectrometry.

RESULTS

Figure 1 portrays a typical GC/PCIMS total ion profile for an air oxidant sample. Table 1 illustrates the compounds which have been identified in the air oxidant samples. Identifications were based upon gas chromatographic retention indices (17), molecular weights derived from CIMS, and EIMS library searchs. In general, the Ar/O_2 samples exhibited the same major components, with lower concentrations. GC/TSD results indicated a lower number of nitrogen containing compounds in the Ar/O_2 samples, however, specific identifications of the nitrogenous species were not possible. HPLC separations coupled with the mass spectra generated using the Pt-filament probe were especially useful for identifying higher molecular weight PAH, while GC/NCIMS data were particularly sensitive to the oxygenated species found in the SOF

Results indicate that unsubstituted, non-linear polynuclear aromatic compounds are the primary particle-bound combustion products from aliphatic hydrocarbon fuel components. The identified compounds are demonstrated to be fuel-independent products of the diffusion controlled combustion process which exists in the Diesel engine. This fact should be considered in any future health effects studies of Diesel particulate matter. The potent carcinogen, nitropyrene, was tentatively identified in the soluble organic fraction of the particles, and could possibly account for a large portion of the observed mutagenic properties of the extractable organics.

TICP FOR GC/PCIMS OF SAMPLE 1
TOTAL ION CURRENT: 3603972   BASE PEAK: 48657
MASS RANGE(S): 120-320
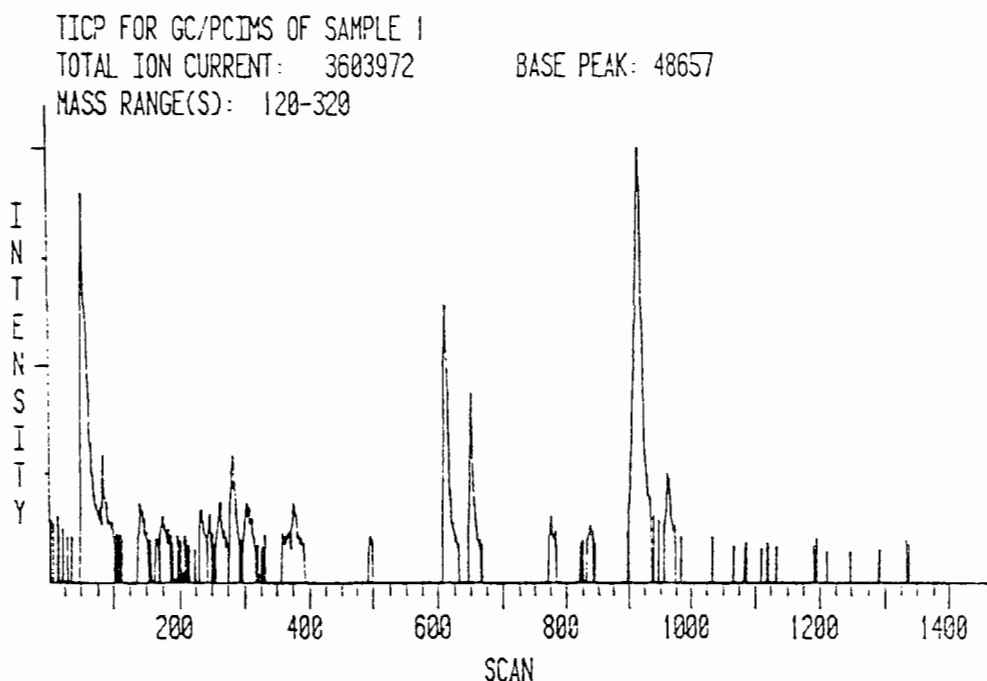
FIGURE 1. Total Ion Current Profile for GC/PCIMS of Air Oxidant Sample

Naphthalene	Phenanthrene
Benzofuran,7-methyl-	Methyl-9-Fluorenones
Inden-1-one,2,3-dihydro-	Benzo[c]cinnoline
Methylnaphthalenes	Fluorene Quinone
Phthalate-anhydride	Phenanthrene Quinone
Biphenyl	Cyclopenta-phenanthrene-5-one
n-Tetradecane	Naphtho[1,8-cd]pyran-1,3-dione-
1-Benzopyran-2-one	Fluoranthrene
Biphenylene, or Acenaphthylene	Pyrene
Acenaphthene	Methylpyrenes
Dibenzofuran	Benzo[ghi]fluoranthene
Fluorene	Cyclopenta[cd]pyrene
9-Fluorene	Chrysene or Triphenylene
Anthracene	Benzofluoranthrathenes or Benzopyrenes

TABLE 1. Compounds Identified in Air Oxidant SOF

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THE ANALYSIS OF NITRATED POLYNUCLEAR AROMATIC HYDROCARBONS IN DIESEL EXHAUST PARTICULATES BY MASS SPECTROMETRY/MASS SPECTROMETRY TECHNIQUES[1]

by

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INTRODUCTION

Recent investigations have indicated that organic solvent extracts of light-duty diesel exhaust particulates exhibit direct-acting mutagenicity when tested using the Ames assay. Preliminary estimates indicate that a significant portion of this direct-acting mutagenicity may be due to the presence of nitrated polynuclear aromatic hydrocarbons (nitro-PAH).[2] Mass analyzed ion kinetic energy spectrometry (MIKES) and triple stage quadrupole (TSQ) analytical techniques used to characterize these compounds in diesel exhaust are described.

EXPERIMENTAL

Light duty diesel exhaust particulate samples were collected on T60A20 Pallaflex filters using a dilution tube and a chassis dynamometer test facility. Filter samples were extracted with dichloromethane.

MIKES analyses were performed on a Vacuum Generators ZAB-2F mass spectrometer using both electron impact (EI) and negative ion methane chemical ionization (NICI) procedures. All experiments were conducted using a magnetic sector resolution of approximately 2000 and helium as a collision gas (1×10^{-7} torr).

A Finnigan TSQ mass spectrometer was used to perform collisionally activated dissociation (TSQ-CAD) and constant neutral loss studies. All experiments were conducted using positive ion methane chemical ionization (PICI) procedures. In the TSQ-CAD studies the first quadrupole was set to transmit a parent ion of interest into the second quadrupole which functioned as a collision cell (N_2, 5×10^{-3} torr). The third quadrupole was scanned repetitively to collect daughter ion spectra. In the constant neutral loss studies, the first and third quadrupoles were scanned in parallel with a 17 amu mass deficit. Under these conditions, only ions which experience the loss of a 17 amu neutral fragment when collisionally dissociated in the second quadruple are detected. This ion reaction was found to be characteristic of nitro-PAH compounds.

RESULTS AND DISCUSSION

The TSQ constant neutral loss analysis was found to be a very useful screening procedure for nitro-PAH compounds. Table I lists 20 different nitro-PAH derivatives which were tentatively identified in diesel particulate extract using this procedure. It must be emphasized that this technique only monitors a reaction characteristic of nitro-PAH compounds and does not confirm their presence. The specificity of the constant neutral loss analysis was assessed by examining the response of several compounds representative of the classes of PAH derivatives observed in diesel exhaust. The results of this study, as shown in Table II, indicate that the technique exhibits good selectivity for the nitro-PAH derivatives.

Table III illustrates the concentration of 1-NP in the exhaust particulate extract from four different diesel engines as determined by MIKES and TSQ-CAD analysis. These quantitation studies indicated that both MS/MS techniques lacked sufficient resolution on the first mass filter to eliminate positive interferences in the daughter ion spectra completely. The M-16 daughter ion fragment (M+1 - OH) was found to be specific for 1-NP and was used for quantitation by TSQ-CAD, but the electrostatic sector of the MIKES instrument did not resolve this daughter ion adequately. It was necessary to prefractionate the OP-1 and PG-1 samples by preparative scale high performance liquid chromatography and to use NICI techniques to accomplish an interference-free MIKES analysis of 1-NP.

Neither MS/MS technique distinguished between nitro-PAH isomers. This information was obtained by capillary GC-MS.

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Table I. Nitro-PAH Derivatives Tentatively Identified in Diesel Particulate
Extracts by TSQ Constant Neutral Loss Analysis

Nitroacenaphthylenes
Nitro(acenaphthlenes, biphenyls)
Nitronaphthaquinones
Nitrodihydroxynaphthalenes
Nitrofluorenes
Nitro(methylacenaphthalenes, methylbiphenyls)
Nitro(trimethylnaphthalenes)
Nitro(naphthalic acid)
Nitro(anthracenes and phenanthrenes)
Nitro(fluorenones and methylfluorenes)
Nitro(methylanthracenes and methylphenanthrenes)
Nitro(anthrones and phenanthrones)
Nitro(pyrenes and fluoranthenes)
Nitro(dimethylanthracenes and dimethylphenanthrenes)
Nitro(methylpyrenes and methylfluoranthenes)
Nitro(pyrones and fluoranthones)
Nitro(pyrene and fluoranthene)quinones
Nitro(dimethylphenanthrene and dimethylanthracene) carboxaldehydes
Nitro(methylbenzo(a)anthracenes, methylchrysenes and methyltriphenylenes)
Nitro(benzo(a)pyrenes, benzo(e)pyrenes and perylenes)

Table II. Selectivity of the TSQ Constant Neutral Loss Analysis for Nitro-PAH

Compound Class	Selectivity Ratio[a] (Nitro-PAH/Compound Class)
Amines	50/1
Aldehydes	
Quinones	
Carboxylic Acids	200/1
Acids	

[a] interference level <5%

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Table III. Quantitation of 1-NP in Diesel Exhaust Particulate Extract using MS/MS Techniques

Engine Sample	Instrument	Ionization	Concentration (ppm)
N1-1	TSQ	PICI	2285±230
	MIKES	EI	2080±220
OL-1	TSQ	PICI	204±30
OP-1	TSQ	PICI	77±15
	MIKES	EI	≤105
	MIKES	NICI	55±11
PG-1	MIKES	NICI	150±30

CONTRIBUTION OF 1-NITROPYRENE TO DIRECT ACTING AMES ASSAY MUTAGENICITIES OF DIESEL PARTICULATE EXTRACTS

by

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We have determined the percentage contribution, P, of 1-nitropyrene (1-NP) to the direct acting Ames assay mutagenicities of dichloromethane extracts of exhaust particles collected from three different diesel powered passenger vehicles. For strains TA98, 100, and 1538, respectively, P was (16, 1, and 7%) for vehicle 1; (24, 9, and 7%) for vehicle 2; and (13, 4, and 13%) for vehicle 3. We assumed:

$$P = C \times (M1/M2) \times 100\%$$

M1 and M2 are the slopes of the linear portion of the Ames assay dose-response functions for 1-NP and for diesel particulate extract respectively. C, the mass of 1-NP in the particulate extracts/mass of extract, was determined by collisionally activated dissociation mass analyzed ion kinetic energy spectrometry (CAD-MIKES). (CAD-MIKES is a double mass spectrometer technique with which compounds often can be analyzed in complex mixtures directly without chromatographic separation.) C for vehicles 1, 2, and 3, respectively, was 55 + 11, 2030 + 220, and 150 + 30 ppm. We used fused-silica capillary column GC-MS to show that the mass 247 (1-NP) CAD-MIKES spectrum was due to 1-NP and not to some other mass 247 isomer. Several isomers of nitrofluoranthene and nitropyrene were synthesized to assure adequate GC-MS separation of these isomers from 1-NP.

The slopes, M1 and M2, are approximately proportional to μN, where μ is the mutation rate per concentration of mutagen and N is the total number of histidine auxotrophs in the background lawn (1). N is proportional to the initial inoculum and the average number of auxotrophs per individual background colony. N cannot be measured in the Ames assay and it is not necessarily the same for M1 and M2. Consequently, whenever we carried out an Ames assay of diesel particulate extract we concurrently carried out an assay of 1-NP using equal amounts of the same broth culture, thereby ensuring that the initial inocula were equal. We then obtained photomicrographs of the

background lawn (100X) on each plate at the time of counting revertants and from these photomicrographs counted the background colonies and estimated their average size. If the background colony counts and their average sizes were the same for both 1-NP and diesel particulate extracts, we took this as evidence that N was the same and then used the corresponding slopes to calculate P.

Finally, we did experiments in which various known amounts of 1-NP were added to a constant amount of extract and obtained the Ames assay dose-response function of the 1-NP in the presence of the diesel extract. The added amounts of 1-NP were chosen to be within the same order of magnitude as that of the 1-NP already in the particulate extracts as measured by mass spectrometry. We found that the slope of the 1-NP dose-response function in the presence of particulate extract was the same as that for 1-NP alone in solution. This result supports an assumption, implicit in the above equation, that the mutagenicity of 1-NP is not altered by the other components in the mixtures.

These data show that 1-NP is an important contributor to the mutagenicity of these diesel particulate extracts. Even a 1% contribution is important, considering the thousands of compounds present in the particulate extracts. This observation is very encouraging to experimenters seeking to identify mutagens in complex mixtures because it suggests the possibility that a small number of compounds may account for the majority of the mutagenicity of these complex mixtures.

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DINITROPYRENES: THEIR PROBABLE PRESENCE IN DIESEL PARTICLE EXTRACTS AND CONSEQUENT EFFECT ON MUTAGENIC ACTIVATIONS BY NADPH-DEPENDENT S9 ENZYMES

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The direct-acting mutagenic activity of diesel exhaust particle extracts in the *Salmonella* mutation assay may be mostly due to nitroaromatic compounds activated by bacterial nitroreductase enzymes. Chromatographic separations have demonstrated that much of the extract's mutagenic activity is associated with components that are more polar than 1-nitropyrene or other monosubstituted nitro-PAH (Pederson and Siak, 1981, J. Appl. Tox., 1(2):54-60). The present studies, employing TLC and HPLC separation techniques and the recently developed dinitropyrene-resistant *Salmonella* strains (Rosenkranz et al, 1981, Mutation Res., 91:103-105), investigate the probable presence of dinitro-, trinitro-, or tetranitropyrenes in the polar mutagenic fractions of diesel particle extracts.

The direct-acting mutagenic activity of a diesel particle extract, reduced by 40% in the niridazole-resistant strain TA98NR, was decreased by 70% in the dinitropyrene-resistant strain TA98/1,8-DNP$_6$. After fractionation of the extract by silica gel TLC, all mutagenic fractions exhibited reduced activity in TA98/1,8-DNP$_6$, as shown in the figure. The most marked reduction occurred with the fractions which co-chromatographed with reference samples of the multisubstituted nitropyrenes. The mutagenicity of these fractions and multinitropyrenes was markedly reduced in TA98/1,8-DNP$_6$, but not in TA98NR. HPLC separations on a cyano phase-bonded silica column indicate that 1,8-dinitro-and 1,6-dinitropyrene are the predominant mutagenic components in the material recovered from TLC fractions 11 through 15. Some mutagenic activity was also attributed to 1,3-dinitro- and 1,3,6-trinitropyrene. The dinitropyrenes may account for 15-20% of the mutagenic activity in the particle extract, but they are very potent bacterial mutagens and would be present at concentrations of less than 1 ppm in the exhaust particulate.

The nitroreductase-deficient bacteria have also been used in the *Salmonella*/S9 mutation assay to examine the effect of mammalian enzyme activities on the mutagenicity of diesel particle extracts and the nitropyrenes. Under appropriate conditions, the activation of mutagens in diesel particle extract by rat liver S9 enzymes was evident as a difference between assays with and without NADPH (Pederson and Siak, 1981, J. Appl. Tox., 1(2):61-66). 1-Nitropyrene was similarly activated by S9 enzyme activity. The activation of 1-nitropyrene is located in the microsomal fraction of the S9 preparation, but activation of diesel particle extract was more evident with the cytosol fraction.

The mutagenicity of each dinitropyrene was greatly reduced by the NADPH-dependent activity of S9 enzymes. As shown in the table below, the NADPH-dependent inactivation is catalyzed by microsomal enzymes, but with the cytosol fraction, the mutagenic activity of the dinitropyrenes is increased. The NADPH-dependent increase in mutagenicity of the dinitropyrenes includes both a cytosol-independent reaction, which is probably a direct reduction of the compounds by reduced pyridine nucleotide, and an enzyme-catalyzed reaction. NADH is just as effective as NADPH in both the cytosol-dependent and independent activation reactions.

The NADPH-dependent increase in mutagenicity of diesel particle extract in the *Salmonella*/S9 assay involves both multiple extract components and multiple S9 enzymes. The dinitropyrenes presumably contribute to the cytosol-catalyzed activation. The much smaller effect of microsomal enzymes on the mutagenicity of the particle extract must reflect competing activation and inactivation reactions as evidenced by the difference between 1-nitropyrene and the dinitropyrenes.

Net TA98NR Revertants/Plate

		plus S9 microsomes	plus S9 cytosol
1,6-Dinitropyrene	-NADPH	282 +17	347 ± 16
10 ng/plate	+NADPH	19 ± 5	878 ± 98
	Change	-95%	+150%
1,8-Dinitropyrene	-NADPH	196 ±13	392 ± 21
2 ng/plate	+NADPH	7 ± 6	1129 ± 51
	Change	-95%	+190%
Diesel Particle	-NADPH	326 ± 6	344 ± 17
30 µg/plate	+NADPH	415 ± 8	605 ± 20
	Change	+25%	+75%
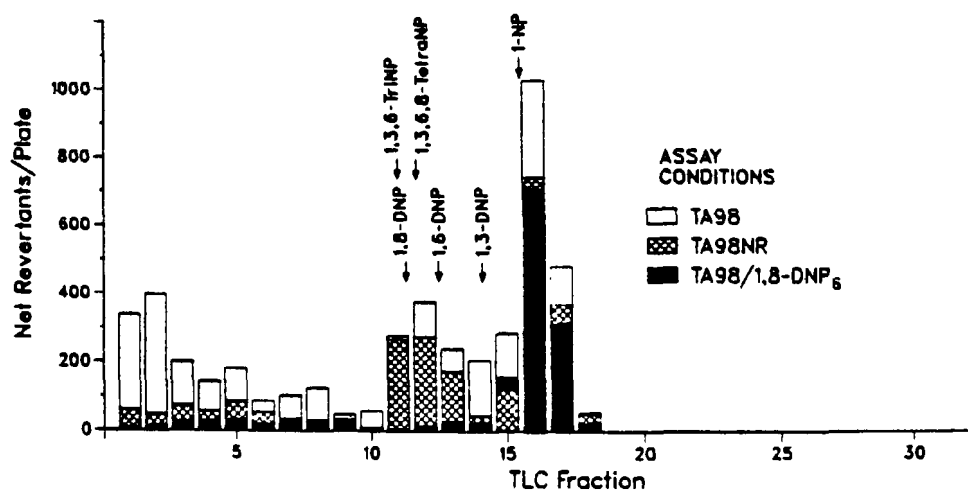
SECTION 3
PULMONARY FUNCTION

INHALATION TOXICOLOGY OF DIESEL EXHAUST PARTICLES

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INTRODUCTION

Diesel engines have found wide application in heavy duty vehicles and equip-
ment for many years and in recent years have been used to an increasing extent
in light-duty vehicles. This latter usage is projected to increase substan-
tially in the future, both in the United States and worldwide. It has been
estimated that 345,000 tons of diesel exhaust particles were emitted by diesel-
powered heavy duty trucks, off-road equipment and railroad engines in the United
States in 1977.[1] Further, it has been estimated that light-duty diesel vehicles,
if they comprise 20% of the U.S. automotive fleet in 1995, will release to the
atmosphere an additional 60,000 tons of diesel exhaust particles per year. This
estimate has been made assuming light duty vehicle emissions can be held to a
level of 0.12 gm/km, a level below that currently being attained by most vehicles
with existing exhaust emission control devices.

In recent years, increased concern has developed for the potential health
effects of diesel exhaust particles.[2] This concern stems from recognition that
(a) diesel exhaust particles are small in size, readily inhaled and deposited
throughout the respiratory tract with a substantial fraction in the deep lung,
(b) the relatively insoluble carbonaceous core of the particles results in their
tenacious retention in the lung, and (c) the cytotoxic and mutagenic properties
of organic solvent extracts of the diesel exhaust particles may result in func-
tional diseases or cancer, especially of the lung. Recent concern for the muta-
genic, and potentially carcinogenic, properties of the particles has no doubt
been fostered by the development, during the last decade, of relatively simple
techniques for assaying mutagenicity in bacterial and mammalian cells. However,
to some extent, the concern generated by recognition of the mutagenic properties
of diesel exhaust particle extracts has been countered by the failure to demon-
strate increased incidence of cancer in human populations occupationally exposed
to diesel engine exhaust.

The potential health effects of diesel exhaust coupled with the projected in-
creased use of diesel-powered vehicles has stimulated research to resolve health

risk uncertainties. The central issue being addressed by this international research effort is; does occupational or environmental exposure of people to diesel exhaust result in increased health risks? Because non-particulate emissions of diesel engines are qualitatively similar and quantitatively not markedly different than those of gasoline spark ignition engines, the research effort has focused on the effects of diesel exhaust particles. This paper will briefly review this research, summarize some of the most significant findings obtained to date and identify areas requiring further research.

EXPERIMENTAL APPROACH

The research program to develop an improved understanding of the health effects of diesel exhaust has proceeded along two inter-related avenues. The first is to develop as much information as possible from epidemiological studies of populations that have been occupationally exposed to diesel exhaust. Unfortunately, the information base obtained to date from the epidemiological studies has been limited because there are relatively few suitable populations available for study. The major research in this area was summarized at a 1979 Symposium.[3] The most extensive epidemiology study that has yielded significant, albeit negative to date, results is a study of London Transport Authority workers employed during the years 1950-1974. Although the findings of this study as regards lung cancer incidence are negative, Harris[4] has recently analyzed them to provide an upper boundary estimate of the lung cancer risk. (A 0.05 percent proportional increase in lung cancer incidence for an exposure of 1 microgram of particulate per cubic meter for 1 year.) Other epidemiological studies are currently being conducted. However, useful data from them is not likely to be available for at least several years. In the absence of adequate information obtained in man, it has been necessary to pursue a second avenue; the conduct of studies with laboratory animals and other biological systems. This approach is based on recognition of the many similarities between different species such that extrapolations can be made between species, including laboratory animals to man. Moreover, it is assumed studies of laboratory animals and simpler systems, i.e. organ, tissue, cell and subcellular entities can provide insight into the mechanisms by which the human body responds to foreign materials such as diesel exhaust.

The studies being conducted are directed to answering three questions: 1) Does exposure to high levels of diesel exhaust particles result in increased health risks? 2) What are the mechanisms by which the health effects are produced? and 3) Is there a basis for extrapolating these health effects to low level exposures

by considering the probable levels of human exposure, the kinds of health effects observed and the mechanisms by which the health effects are produced? A premise inherent in this approach is that studies can be conducted at exposure levels higher than those typically encountered in occupational or environmental settings and extrapolated to exposure levels likely to be of concern.

The overall approach being taken is shown schematically in Figure 1. As may be noted, the research program consists of several inter-related components directed to obtaining information that is required to provide the desired end product; an assessment of the potential health risks to man from exposure to diesel exhaust. Let us consider the results to date for each component.
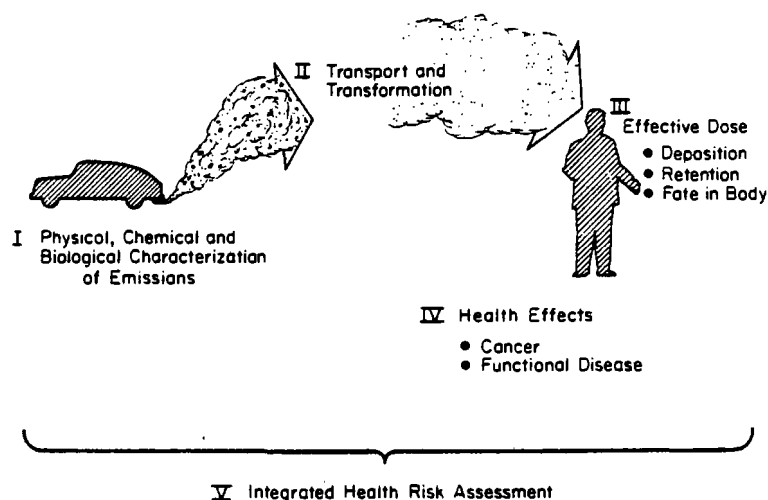
Fig. 1. Schematic representation of the research approach being taken to evaluate the health effects of diesel exhaust.

PHYSICAL, CHEMICAL AND BIOLOGICAL CHARACTERISTICS

Other papers in this symposium have provided an excellent review of the current status of our knowledge of the physical, chemical and biological characteristics of diesel exhaust emissions. Perhaps the most striking features of the data are the qualitative and, to a considerable extent, quantitative similarities between the exhaust emissions resulting from a wide range of vehicle, fuel and operating variables. This is important since it provides a basis for having confidence that the diesel exhaust exposure environments being utilized in the whole animal studies are representative of the varied environments likely to be encountered by man. Thus, the results of the animal studies are not likely to be unique to the specific exposure atmosphere being studied and its source.

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An additional point that is worthy of note is the extent to which studies with cellular and subcellular systems are starting to focus on consideration of the effects of individual chemical compounds and classes of compounds. They are providing more detailed dose-response orientation than earlier studies which were, of necessity, more of a screening nature. A more detailed level of knowledge should provide a better basis for understanding the mechanisms that may be involved in producing effects in the whole animal exposed to exhaust. Past research with extracts of particles has focused on their mutagenic properties. Looking to the future it would be appropriate for additional attention to be focused on the non-mutagenic properties of diesel exhaust particles and interactions between mutagenic and non-mutagenic effects in cellular and subcellular systems. This is especially important recognizing that to date no convincing evidence of carcinogenicity has been found in studies of animals exposed to whole diesel exhaust. Conversely, non-mutagenic changes, reflecting both tissue injury and repair, have predominated in the studies of animals exposed to whole diesel exhaust.

TRANSPORT AND TRANSFORMATION

In developing an assessment of the health risks of diesel exhaust, a key question is; what reaches the breathing zone of man? It is important that this question be kept in mind while considering the laboratory research findings since the majority of the research being conducted involves either animal exposures or sampling of diesel exhaust particles at relatively low dilutions and within seconds or minutes of their emission. It must be kept in mind that these conditions are not typical of all occupational or environmental exposure situations for man. In situations where people may be exposed to high levels of exhaust components (parking garage workers, for example), the exposure atmosphere will represent material that has had a residence time of at least several minutes in the atmosphere. Material having even longer residence times may predominate in other atmospheres such as the street canyons of large metropolitan areas. Further, the atmosphere is likely to contain not only freshly emitted exhaust, but also resuspended material that may have been exposed to sunlight or subjected to other environmental variables for extended periods of time.

The number of studies conducted on the transformation of diesel exhaust in the atmosphere has been limited; however, they do give rise to concern for significant changes in diesel exhaust particle constituents between the time they leave the tailpipe and reach the breathing zone of man.[5,6] The results of these studies emphasize the need for additional research in this area.

It is also important to consider the fate of the particles, and especially their organic constituents, after being released into the environment and whether they may affect man through other routes of entry. The release of several hundred thousand tons of particles from diesel vehicles to the atmosphere each year emphasizes the importance of this question. Obviously, we need to know the extent to which the organic constituents may be deposited on foodstuffs and be available for ingestion. A related need is to determine the fractional absorption of these materials from the gastrointestinal tract of man.

DEPOSITION AND RETENTION

Adequate assessment of the health risks of inhaled diesel exhaust particles requires knowledge of the deposition, retention and fate of these particles and their organic constituents in man. There is a substantial body of information available on the deposition of inhaled particles in man for a wide range of particle sizes. The kinds of information available may be found in recent papers by Lippmann;[7] Chan and Lippmann;[8] and Stahlhofen et al.[9] Unfortunately, most of the data have been obtained for particles larger in size than diesel exhaust particles. The most relevant human data were reported by Chan and Lippmann[8] who studied the deposition of 0.2 µm diameter particles in healthy non-smokers that breathed the test aerosol through a mouthpiece. The mean values obtained in their studies for tracheobronchial and pulmonary deposition are shown in Figure 2. For comparison purposes, data are also shown for dogs, rats and mice that had inhaled radiolabeled gallium oxide aerosols with a volume median
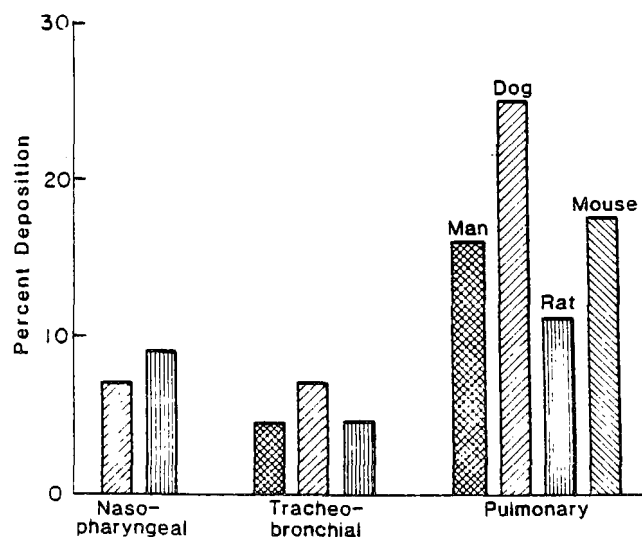
Fig. 2. Deposition of 0.1 or 0.2 µm particles in mouse, rat, dogs or man.

diameter of 0.1 μm (similar in size and shape to diesel particles). The labora-
tory animal data are useful in two ways; first, they provide a means for extend-
ing the more extensive observations on man to smaller particle sizes, and second,
they provide essential information for use in interpreting studies of inhaled
diesel exhaust particles in laboratory animals. With regard to the first con-
sideration, it is noteworthy that Cuddihy et al.[10] found good agreement between
data obtained in man and Beagle dogs for the fractional pulmonary deposition of
particles ranging from 0.4 to 5 μm in mass median aerodynamic diameter. Further,
these results (Fig. 3) are in general agreement with the predicted values based
on theory.[11,12] This lends confidence to using the fractional pulmonary deposi-
tion values obtained with 0.1 μm volume median diameter particles in the Beagle
dog[13] for estimating the fractional pulmonary deposition of diesel exhaust par-
ticles in people until such time as direct information can be obtained in man.
Looking to the future, plans are being developed at our Institute to conduct
studies of the deposition of surrogate diesel exhaust particles (radiolabeled
gallium oxide) in human subjects.
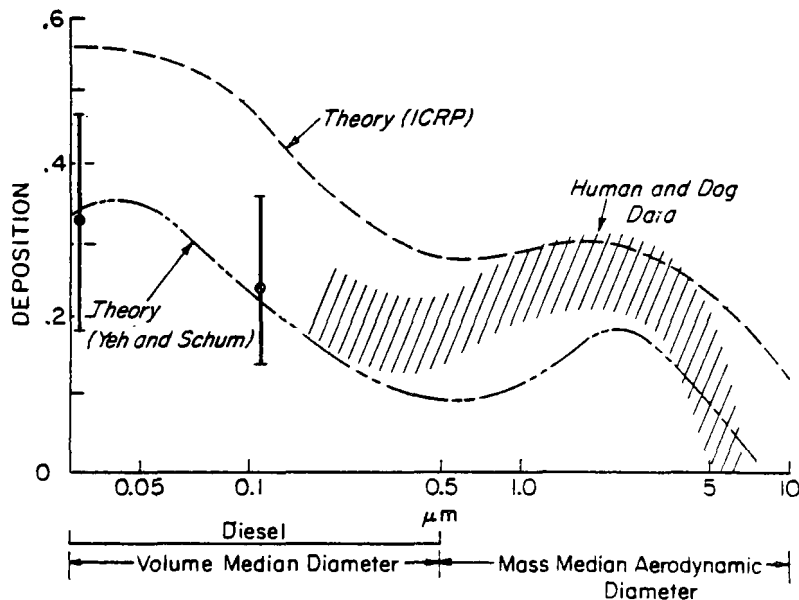
Fig. 3. Pulmonary deposition of inhaled particles in humans and dogs. The data
points in the diesel particle size range are from studies with Beagle dogs.[13]

In considering the data on deposition, it should be emphasized that the range
of uncertainty of the fractional deposition of diesel exhaust particles in the
respiratory tract of people is relatively low (approximately a factor of 2) and

as noted, this uncertainty can be further reduced by studies of people exposed to surrogate diesel exhaust particles. Unfortunately, information on retention of diesel exhaust particles in the respiratory tract of man is much more limited. Short-term observations of the retention of radiolabeled materials will provide insight into the rapid clearance phases in man for particles similar in size to diesel exhaust particles. It is unlikely that approaches can be developed that will give reliable information on the long-term retention of these small size particles in the respiratory tract of people. A possible exception is to study the retention of iron oxide particles in people using a magnetic detector system such as has been used for larger sized particles.[14] Until such time as information can be obtained directly in man, the best information available will be that obtained from laboratory animals such as shown in Figure 4. Chan et al.[15] have reported on the retention of carbon-14 labeled diesel exhaust particles in rats exposed for short periods of time to diesel exhaust. They have interpreted the retention curve as having two components with approximate half-times of 1 day and 62 days. These results are quite similar to those obtained in our institute with rats exposed to several different types of relatively insoluble aerosols when the observations have been restricted to a 100 days or so post-inhalation exposure. Typically, when the animals have been observed for longer periods of time, an additional component is observed with a longer half-time. Also shown in Figure 4, are data obtained by Griffis et al.[16] and Strom and Garg[17] who analyzed the lungs of rats for the carbonaceous particles at various times following extended exposure to diesel exhaust. These data suggest the presence of a
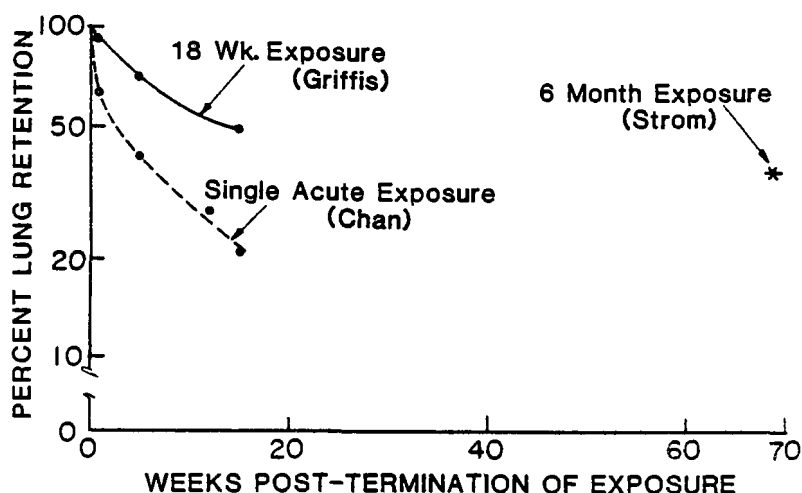
Fig. 4. Lung retention of inhaled diesel exhaust particles.

longer-term component in the pulmonary retention of diesel exhaust particles which is not apparent during short observation periods following acute exposures. There is also a suggestion that chronic exposure to high concentrations of particles may prolong the retention of particles. It should be recalled that pulmonary clearance of particles is generally more rapid, and thus retention is lower in rodents than in larger species such as dogs.[18] This suggests the need to evaluate diesel exhaust particle retention in dogs and perhaps subhuman primates as an aid in predicting particle retention in man. Retention data are essential for interpreting the health effects studies which will be discussed later. They permit comparisons to be made on the basis of dose-response relationships where dose is expressed as retained particle mass rather than the less supportable exposure-response relationships which do not consider species differences in deposition and retention.

The ultimate objective of the studies of the deposition and retention of diesel exhaust particles and their organic constituents is to develop an integrated model of the fate of diesel exhaust particles and their constituents as shown in Figure 5. The ideal model will provide a description of the temporal pattern of distribution of diesel exhaust particles and their constituents throughout the body with emphasis on the respiratory tract. It should extend to the cellular level providing a description of the time course of contact of
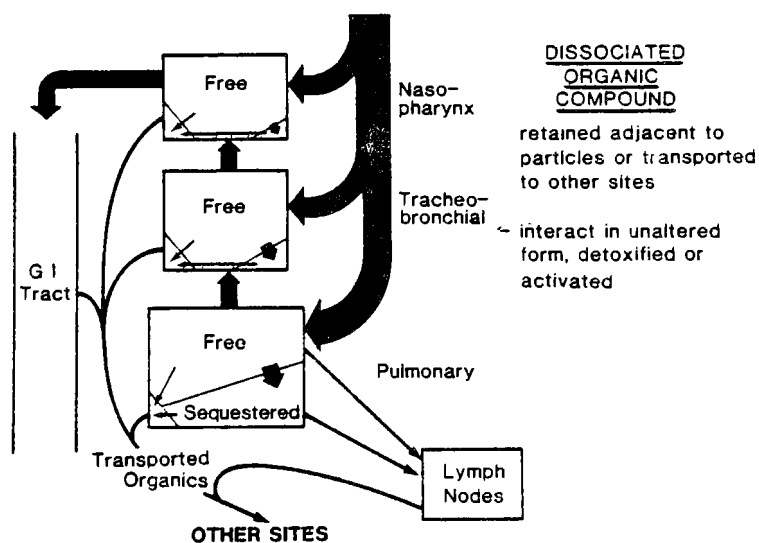
Fig. 5. Schematic model of the fate of inhaled diesel exhaust particles and associated organic compounds.

particles with the various cell types of the respiratory tract. Information of
this type has been obtained by serial sacrifice of exposed animals with subse-
quent morphological evaluation at both the light and electron microscopic level.
From such studies, it is clear that epithelial cells lining the conducting
airway receive only brief exposure due to the effective nature of mucociliary
clearance mechanism. Conversely the cells residing in or lining the alveoli are
in contact with the particles for a more protracted time due to the longer
residence in such structures.

Due to their enormous phagocytic capability, alveolar macrophages (AM)
receive the greatest potential exposure to inhaled diesel particles. This is
illustrated in a series of photomicrographs of lungs from rats exposed by inha-
lation for 19 weeks, 5 days a week, 7 hrs per day to a chamber concentration of
approximately 4300 μgm/M^3 diesel exhaust particles (DEP). Figure 6 shows an
increase both in number and in size of AM's with most distended by phagocytosed
DEP. Another characteristic feature is the aggregations of particle laden AM
in alveoli adjacent to terminal bronchioles (Fig. 7). Also shown in this figure
is the increase in number of Type 2 pneumocytes lining such occluded alveoli and
the presence of particles within alveolar and peribronchial interstitial tissues.
In most instances the particles reside within macrophages and the macrophages

Fig. 6. Section of rat lung exposed to 4300 μgm/M^3 for 19 weeks. Number of
Type 2 cells (arrow) increased in alveolus containing diesel particle laden
alveolar macrophages (arrowheads). H and E. X 320.
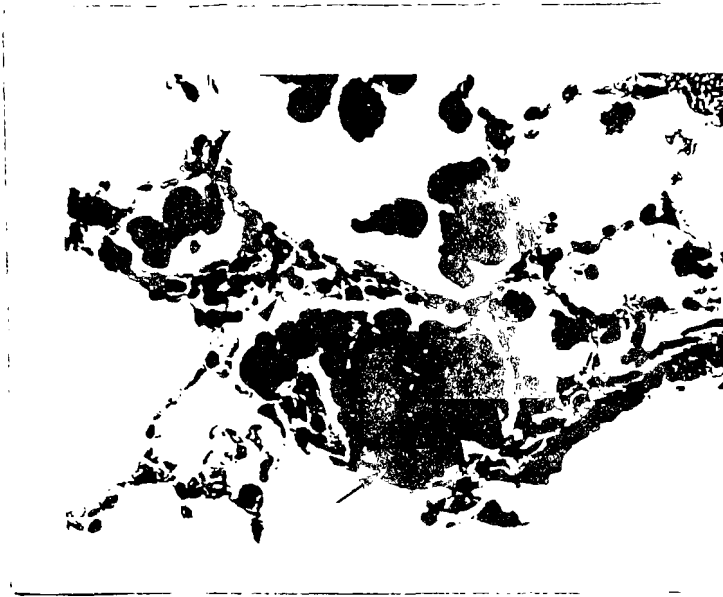
Fig. 7. Section of rat lung exposed to 4300 μgm/M^3 for 19 weeks. Clumped pigment laden alveolar macrophages (arrow) in occluded alveolus. Increase in Type 2 cells (arrowhead) lining alveolar septa. Interstitial pigment also present in wall of terminal bronchiole. H and E. X 320.

are in small lymphatics. Other studies have shown that Type 1 pneumocytes are also capable of phagocytosing particles and thus they represent another cell at potentially high risk.[19]

Although the precise mechanisms for subsequent transport to lung-associated lymph nodes is not known, it is clear that DEPs are concentrated in both bronchial associated lymphoid aggregates and in hilar lymph nodes (Fig. 8). Initially, they appear in histiocytes in the peripheral sinusoids but with time may be seen in both the medullary and cortical sinousoids. From these observations one may conclude that biological responses will most likely occur within cells comprising the terminal bronchiole, the respiratory bronchiole, alveolar structures, and in lung-associated lymph nodes.

The information already available emphasizes the need for the model to consider particles that are (a) either free or within relatively mobile macrophages, and (b) particles sequestered in other tissues of the respiratory tract. At this symposium, Soderholm[20] presented such a model and the associated analysis
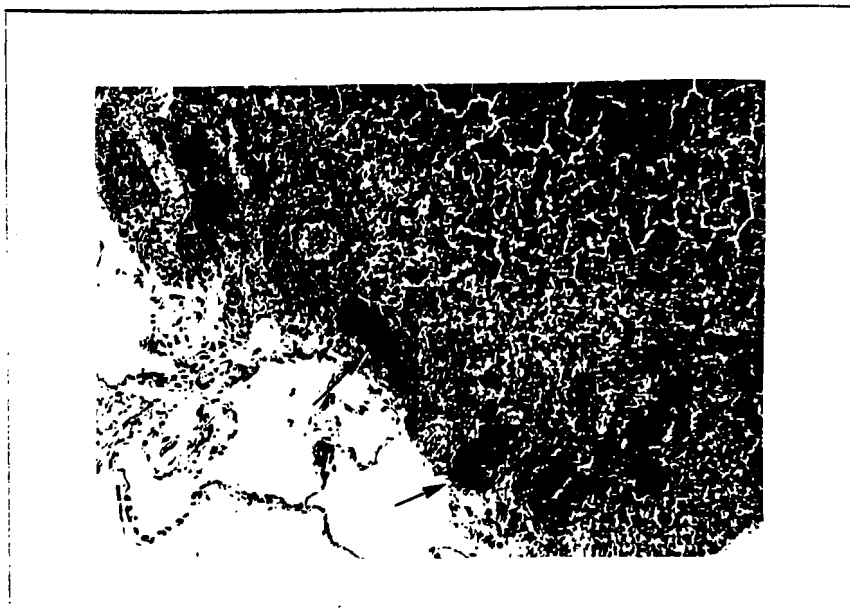
Fig. 8. Section of hilar lymph node from rat exposed to 4300 $\mu gm/M^3$ for 19 weeks. Particle laden macrophages (arrow) pack peripheral sinusoids. H and E. X 130.

of the particle kinetics. An additional key factor that must be considered in the model is the dissociation of organic compounds from the particles. These dissociated organic compounds may be retained adjacent to the particles or transported to other sites within the respiratory tract, the regional lymph nodes or to other tissues. Consideration must be given to the fate of these organic compounds, i.e. do they interact with cells and subcellular organelles in unaltered form, are they detoxified or activated? At the present time there are no direct observations of the dissociated organic compounds in vivo. This is not surprising recognizing that the compounds are probably released very slowly and at low levels. Thus, it is difficult to envision experimental approaches that will allow one to measure the disassociated compounds in vivo. An insight into the processes that may be involved can be obtained by studying simpler systems.

FATE OF ORGANICS ASSOCIATED WITH PARTICLES

Organic solvents such as dichloromethane readily remove the organic compounds from the diesel exhaust particles and the extracted material is mutagenic to bacterial and mammalian cells. However, dichloromethane is not typical of the

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solvents present within biological systems such as the respiratory tract. This raises a question as to the extent to which the organic compounds present in the diesel exhaust particles may be extracted by materials that are more typical of those found in the respiratory tract. Apparently, organic compounds can be removed by biological fluids, however, the removal process is not nearly as efficient as with dichloromethane. An example of the type of data that have been developed is shown in Figure 9. In this study a single cylinder diesel engine was operated with ^{14}C-labeled fuel and labeled exhaust particles collected. The ^{14}C-labeled material could be extracted from diesel exhaust particles by dichloromethane with a very short extraction time. Using serum, however, only about half of the ^{14}C-labeled material was extracted during a 72-hour period and a much smaller amount was extracted with saline. These data suggest that in the body, the organic compounds associated with particles are likely to be removed from the particles very slowly.
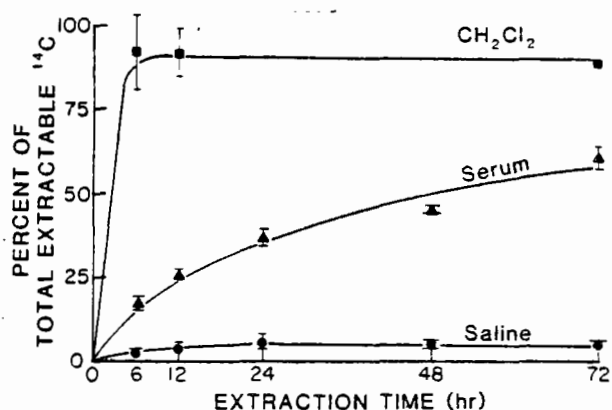
Fig. 9. Extraction of ^{14}C from ^{14}C-labeled diesel exhaust particles (Sun[21])

Having observed that biologically relevant materials such as serum can remove material from the diesel exhaust particles, it is important to determine the mutagenicity of material extracted by these more biologically relevant extracts. Brooks et al.[22] and King et al.[23] have developed information to address this question. Shown in Figure 10 are data obtained by Brooks et al.[22] In this particular study, Ames Salmonella strain TA-100 were exposed to extracts from 1 mg of diesel exhaust particles per bacterial culture plate. Substantial mutagenicity was observed with the dichloromethane extracted material while only a low level of mutagenicity was observed for serum and the levels of mutagenicity observed for material extracted with lavage fluid or saline were similar to the background levels. From the data presented in Figures 9 and 10, it is

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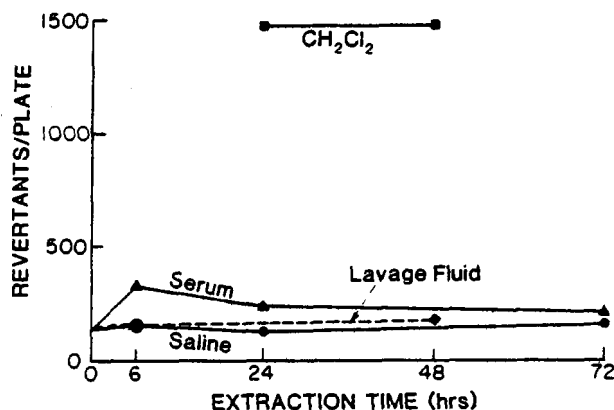
Fig. 10. Mutagenicity of diesel exhaust particle extracts prepared with different solvents.

reasonable to hypothesize that organic compounds were extracted by the serum and perhaps by the lavage fluid, however, these were rendered inactive to some extent in the test system. Support for this is apparent from the results of King et al.[23] as shown in Figure 11. In their studies, a high level of mutagenicity was apparent when the extract alone was evaluated in the Ames test system, but the level of mutagenicity was markedly reduced when extract plus serum were evaluated. When a protease was added, the activity level was intermediate between that of the extract alone and the extract plus serum. One explanation for these observations is that the compounds responsible for the mutagenic activity were inactivated by serum, but could be released by the protease and become available to the test system. Very similar results were obtained when lung cytosol was added instead of serum.

Li[24] studied the cytotoxicity of diesel particle extracts using Chinese hamster ovary cells. In his initial studies in which serum was included in the culture media at levels typical of those used in cell culture studies, he found little evidence for cytotoxicity. More detailed studies, however, with varying levels of serum present in the culture media demonstrated that the serum had a protective effect, i.e. the relative survival of Chinese hamster ovary cells was lowest when the lowest quantities of serum were present and survival was similar for extract treated and control cultures when more than 5 milligrams of serum protein were present per ml of culture media. Using this same system, he also studied the effect of addition of lung and liver S-9 fractions to the culture media and found that these afforded a protective effect (Fig. 12). This protective effect was further enhanced when co-factors NADP (nicotinamide-adenine

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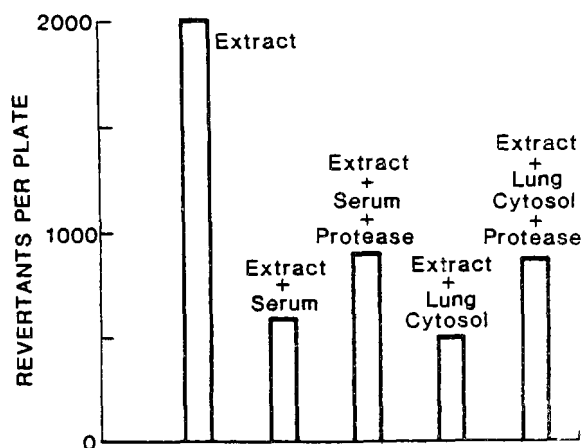
Fig. 11. Influence of serum and lung cytosol on mutagenicity of diesel exhaust particle extracts.
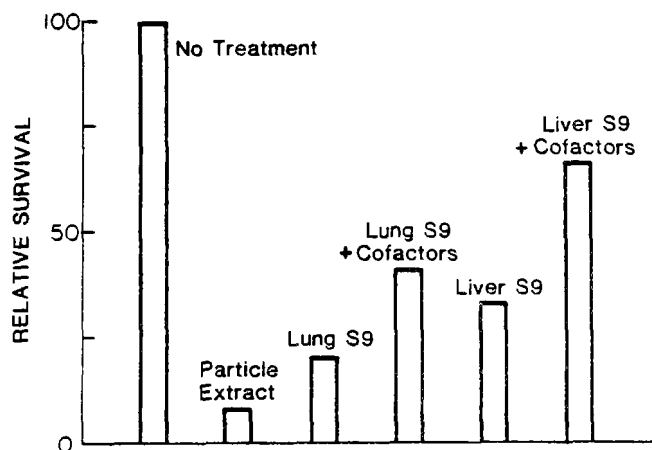
Fig. 12. Influence of liver or lung S-9 with and without co-factors on cytotoxicity of diesel exhaust particle extracts.

dinucleotide phosphate glucose-6-phosphate and magnesium) were added to the culture media.

Potential co-mutagenicity, which may lead to an altered carcinogenic response, is another factor that must be considered in evaluating the release of particle associated hydrocarbons and their effects. Li[25] has addressed this question by evaluating the mutagenic response of Chinese hamster ovary cells in in vitro cultures treated with benzo(a)pyrene alone, diesel exhaust particle extracts

alone or the two materials in combination. The response was more than additive suggesting an apparent synergistic response (Fig. 13). Additional studies of this type are needed recognizing that our ultimate objective is to assess the health risks for people that are exposed to a wide variety of materials in addition to diesel exhaust.
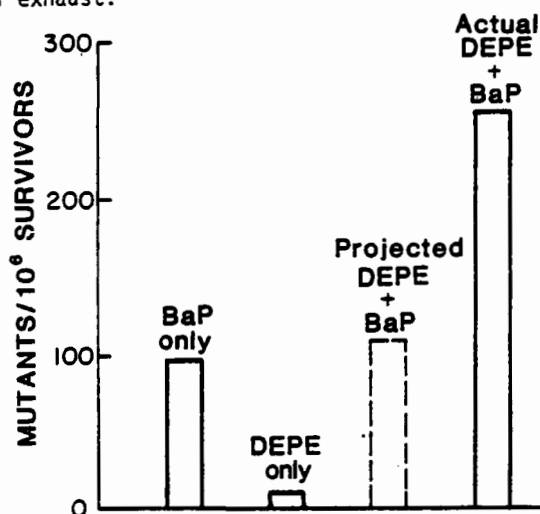
Fig. 13. Co-mutagenicity of diesel exhaust particle extracts and benzo(a)pyrene.

HEALTH EFFECTS OF INHALED DIESEL EXHAUST

In vitro studies with diesel exhaust particle extracts and in vivo assays such as skin painting have identified potential health risks of exposure to diesel exhaust. They have also provided insight into the mechanisms by which effects might be produced or be minimized, i.e. detoxification. However, studies with diesel particle extracts have typically involved delivery of organic compounds to cells over short times and at doses that are many orders of magnitude larger than those that could conceivably be encountered by cells in the human respiratory tract. The methods used frequently bypass normal protective mechanisms of the body and the endpoints measured provide only indirect information on the health effects of ultimate concern. Thus, to provide a more relevant basis for predicting the potential health risks in man, it is important that further studies be conducted in laboratory animals using the inhalation route of exposure to observe the risks for causing cancer and respiratory functional disease as they are the major concerns for man.

A number of studies in which animals have been exposed by inhalation to diesel exhaust have been completed, are in progress or planned. On first

review, one is struck by the number of species and the range of exposure levels studied. However, closer examination reveals that only a few of the studies involve observations for the total life span, or the majority of the life span, of the species being studied. These studies are listed in Table 1. In addition to those listed, it is understood that studies of the health effects of diesel exhaust in laboratory animals will be initiated during the next year in Japan by the Japan Automobile Research Institute, in West Germany by the Fraunhofer Institute and in Geneva by the Battelle Memorial Institute. Life span studies with larger numbers of animals are especially important since the effects being observed are subtle and are likely to occur in low incidence and late in life.

TABLE 1

MAJOR LONG-TERM STUDIES OF THE HEALTH EFFECTS OF DIESEL EXHAUST COMPLETED OR IN PROGRESS

Laboratory	Reference	Species	Particle Concentration $(\mu g/m^3)$
Environmental Protection Agency	26	Chinese Hamster, Mice, Rats, Cats	6000-12000
Fraunhofer Institute	27	Syrian Hamsters	4200[a]
General Motors	28	Rats, Guinea Pigs	250, 750, 1500
Lovelace Inhalation Toxicology Research Institute	29	Mice, Rats	350, 3500, 7000
Battelle-Northwest	30	Rats	8300
	31	Syrian Hamsters	7300
Southwest Research Institute	32	Syrian Hamsters, Rats, Mice	1:60, 1:120, 1:360 dilution[b]

[a]Also, exposures to gaseous emissions only without particles
[b]Particle concentrations not given

In studies completed to date, all of the observed health effects have been non-neoplastic in nature. Although there are some species-related differences, in general, the responses have been similar in all laboratory animals. After inhalation the biological sequence of events starts with the phagocytosis of particles by alveolar macrophages (AM). With time, there is an increase in both the number and size of AMs and an increasing concentration of DEP within their cytoplasm (Fig. 6). Type 2 pneumocytes also increase in number and size within alveoli containing pigment-laden macrophages. There is no evidence to suggest

that Type 2 cells participate in the clearance of DEP, but both neutrophils and eosinophils do appear to be recruited and to phagocytize particles under conditions of high pulmonary loading. [19,33] There is ultrastructural evidence that Type 1 pneumocytes also phagocytize DEP, particularly under conditions of high levels of particle exposure. [19] With time, particle laden AM form dense aggregates within alveoli, most notably adjacent to terminal bronchioles (Fig. 7). The surrounding tissue response to the macrophage clusters is highly variable. In some instances, there was a proliferation of interstitial cells and an increase in interstitial reticulin but in other cases, there was no elicited response. Particles are also translocated from alveoli to the interstitium where they are usually contained in interstitial macrophages. Finally, it has been shown that particles are transported to local and regional lung-associated lymphoid tissues (Fig. 8). Although at later times these tissues concentrate a significant mass of DEP within histiocytes, there is no evidence that other surrounding cells are affected by their presence. However, there is an indication that these nodes may have altered immunological competence. [34]

The responses in lung and lymph nodes observed to date represent the usual response of lung to inhaled relatively insoluble particles. Longer-term observations will be required to ascertain whether the lesions remain the same, or whether with time they become more functionally significant. Substantial effort has been directed to evaluating non-morphological responses, for example, biochemical and physiological alterations. The biochemical changes observed in tissues and airway fluids have in general been transient in nature suggesting injury followed by adaptation or repair. The physiological changes have been minimal to non-existent even at the highest exposure levels.

The lack of outstanding effects, and especially the lack of carcinogenicity, should be interpreted (and extrapolated to man) with caution for several reasons. First, as noted earlier, only a few of the studies have involved life span observations. Study of exposed animals for their life span provides the best opportunity for detecting late-occurring effects. Second, all of the longer-term observations have been made on rodent species. Ideally, one would like to study not only rodent species for their life span, but also longer-lived species. It is generally felt that rodents have more rapid clearance, and consequently, lower retention of particles than do other species including man. Thus, per unit of exposure, the actual dose to tissue may be less in the rodents than would be the case for man. The best basis for extrapolation between species will probably be particle or chemical dose-response relationships rather than exposure atmosphere — response relationships. This emphasizes the

need for periodic measurements of particle burdens in animals from the long-term exposure studies. Third, all of the laboratory animals have been in excellent health when placed on study and have been maintained under optimum conditions. It would be desirable to study some animals whose health status has been altered (for example, emphysematous animals) so they might be more representative of potentially sensitive individuals in human populations. Fourth, with the exception of the Fraunhofer study,[27] the treated populations have only been exposed to whole diesel exhaust. The Fraunhofer study is noteworthy in that it involved exposure of animals to diesel exhaust with and without particles and pre-treatment of animals with known carcinogens. The former should provide insight into the relative role of gaseous and particulate emissions in producing health effects. The pre-treatment of animals with known carcinogens may provide insight into those situations in which people are concurrently exposed to potentially toxic materials other than diesel exhaust. Finally, the majority of studies have been conducted at very high exposure levels. The effects at these levels may be dominated by alterations in protective mechanisms that may not be as significant at lower exposure levels.

SUMMARY

Our present knowledge of the health effects of diesel exhaust particles can be summarized as follows:

1. Diesel exhaust particles are very small in size and consist of a carbonaceous core with a myriad of adsorbed hydrocarbon compounds that are readily extracted with organic solvents.

2. The particle extracts are cytotoxic and mutagenic in in vitro bacterial and mammalian cell cultures.

3. The particle extracts are carcinogenic when painted on mouse skin along with a suitable promoter.

4. Inhaled particles readily deposit in the respiratory tract, a portion is rapidly cleared and a substantial portion is retained for long periods of time (over 100 days) in the lung.

5. Adsorbed hydrocarbon compounds slowly dissociate from the particles in biological media and presumably in the lung.

6. Detoxification mechanisms act on the hydrocarbon compounds released from the particles to minimize effects in in vitro systems and presumably in vivo.

7.   There is morphological and biochemical evidence of lung tissue injury and adaptation or repair after inhalation of very high levels of diesel exhaust.

8.   To date, the longest term studies have not been demonstrated carcinogenicity or major physiological changes.

RESEARCH NEEDS

From consideration of potential levels of diesel exhaust exposure for people[35,36] and the foregoing summary of our current knowledge of diesel exhaust-induced health effects, it appears highly unlikely that such exposures will produce substantial health effects. Indeed, there may be no health effects that will be attributable to diesel exhaust emissions. However, this should be viewed as a provisional assessment pending development of additional information. Some of this information will be obtained from research now in progress, while some will require the initiation of new studies. The following information needs are viewed as being of highest priority for obtaining an improved assessment of the health effects of diesel exhaust emissions in people.

1.   Additional information on the transport and transformation of particle associated hydrocarbons from the point of release to inhalation or ingestion by man. This should include studies of the environmental fate of the several hundred thousands of tons of diesel soot emitted per year into the atmosphere.

2.   Improved knowledge of the long-term retention of particles and their associated hydrocarbons in the respiratory tract of man or species with particle retention characteristics similar to those of man. Emphasis should be placed on evaluating the "microdosimetry" for tissue structures such as the bronchi and bronchioles that are known to be especially sensitive to cancer induction, and alveolar tissue, known to be sensitive to destruction, often resulting in major functional alterations.

3.   Information on the fate of major constituents of diesel exhaust particles in the body after inhalation. The results of such studies using exhaust particles with added concentrations of specific compounds should aid in defining exposure atmosphere — dose relationships. These may serve as a prelude to the conduct of exposure-dose-response studies with such particles. Such information should be obtained at several exposure levels to define the influence of exposure levels, and possible associated tissue alterations, thereby providing a better basis for extrapolating to lower exposure levels.

4.   Information on the response of model cell and tissue systems derived from respiratory tract epithelium. Studies with such model systems should aid

in linking *in vitro* results to those obtained in the whole animal. They should include the study of diesel exhaust particles and known mutagens and carcinogens.

5. Information on late-occurring tissue responses at various exposure levels to complement the information currently available for high levels of exposure and exposure times that have generally been one year or less.

6. Information on the response of individuals with altered health status that may render them more sensitive to diesel exhaust. This should include appropriate models of important respiratory diseases of man.

7. Information on the response of individuals following concurrent exposure to other materials known or suspected to be pulmonary toxicants. Studies have previously been conducted with animals exposed to diesel exhaust and coal or uranium ore dust. Similar studies should be conducted with oil shale dust in view of likely occupational exposures to shale dust and diesel exhaust. Recognizing the central role of cigarette smoking in human lung disease, the effects of smoking in combination with diesel exhaust exposure should be investigated.

8. Additional information is needed on tissue burdens of diesel soot particles and their organic constituents as a function of exposure level and duration of exposure in animals being studied in long-term experiments of the health effects of diesel exhaust. Such information will provide a basis for interpreting and extrapolating results between species on the basis of exposure atmosphere-tissue-dose-response relationships.

9. Additional epidemiological information on any human populations with sufficiently high exposure levels and population size to warrant study.

AN ANCILLARY MESSAGE

It is our opinion that the substantial amount of information obtained on the health effects (or lack of effects) of diesel exhaust particles and particle extracts conveys a message that extends well beyond the question originally asked — does exposure of people to diesel exhaust result in increased health risks? The message comes from consideration on the one hand of the cytotoxic, mutagenic and carcinogenic properties of diesel exhaust particle extracts. And on the other hand, recognition that inhalation and deposition of mg quantities of diesel exhaust particles in the respiratory tract of rodents has not elicited a carcinogenic response. These apparently contradictory results indicate the need for further research to evaluate the utility of using short-term *in vitro* studies for predicting late-occurring effects *in vivo* and thus to recognize the

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limitations of such extrapolations. The research conducted on diesel exhaust has indicated both the usefulness and the short-comings of short-term tests as predictors. It has also provided insights that help explain the apparent contradiction. Additional mechanism oriented research will not only aid in better understanding the likely human effects of exposure to diesel exhaust, but improve our ability to predict the health effects of other complex materials to which man may be exposed.

ACKNOWLEDGEMENTS

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EPA STUDIES ON THE TOXICOLOGICAL EFFECTS OF INHALED DIESEL ENGINE EMISSIONS

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INTRODUCTION

Rapidly rising fuel costs in recent years have resulted in attempts to increase fuel efficiency in passenger cars. Because the diesel engine uses less fuel and has proven to be practical for use in heavy trucks, buses, and farm machinery, the interest in utilizing the diesel engine as a power source for light duty vehicles has increased. The Environmental Protection Agency[1] has estimated that up to 25% of new U.S. passenger cars could be diesel powered by 1985. The Department of Transportation made a more modest estimate of 10%.[2] Even at the lower estimate, a larger number of light duty diesel equipped vehicles will be manufactured, and combined with heavy duty trucks and buses will make a significant contribution to environmental pollution.

The exhaust from diesel engines contains most of the pollutants common to the gasoline engine. These include carbon dioxide, carbon monoxide, nitric oxide, nitrogen dioxide, ozone, sulfur dioxide, alkanes, alkenes, aldehydes and many organic oxidants. The toxicological effects of most of these pollutants have been investigated in considerable detail. The particles emitted from diesel engines, however, differ greatly in both quantity and composition from those produced by gasoline engines. Even a well tuned engine produces 20 to 100 times more particulate matter than a catalyst equipped gasoline engine.[3,4] While the gasoline engine particles are primarily composed of sulfur compounds, diesel particles consist of a carbonaceous material with a large variety of high molecular weight organic compounds adsorbed onto the particle surface.[5] Over 80 such compounds have been identified in our own studies.

It is difficult, if not impossible, to predict accurately the carcinogenic and other toxicological effects of the diesel exhaust particulates. Quantitative measurements of all the components are not available. Some of the compounds present have not even been identified. Even if accurate, quantitative component data were available, effects would be influenced by bioavailability, efficiency of activation or detoxification mechanisms, transport and elimination as well as possible mutually synergistic and/or inhibitory actions of the many compounds present on the particle. Thus, in order to produce adequate data for risk assessment, a large scale animal exposure study of over

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two years duration utilizing a variety of animal species and testing a variety of endpoints was planned by the Environmental Protection Agency at Cincinnati.

The inhalation route was used as the primary means of exposure. During the second year of the study, however, intraperitoneal injections and intratracheal instillations of diesel exhaust particulate were used increasingly to produce greater exposures, and in some cases, to produce dose response curves. A single exposure level was set for the inhalation studies because of the limited chamber space available for the large number of planned experiments and because a single data point was considered adequate for cancer risk assessment using the linear no-threshold model.

Since cancer risk assessment was to be emphasized, a near maximum tolerated dose was selected to achieve the greatest chance of producing a positive response. In a preliminary 60 day study, exposure to a 1:14 dilution of exhaust for 20 hours/day resulted in decreased weight gains and food intake. It appeared that this exposure regime over a long time-period might result in mortality and shortened life span. As a result, the dilution ratio (DR) was adjusted to produce a particulate concentration of 6 mg/m^3 (DR $\cong$ 1:18) and exposure time was shortened to 8 hours/day. After the first year of exposure, it became apparent that the animals had adapted to the exposure conditions with little gross evidence of stress. In order to approach the maximum tolerated dose more closely the dilution ratio was decreased to produce a particulate concentration of 12 mg/m^3. This level of exposure was maintained until the completion of the study.

EXPOSURE CONDITIONS

Exposure facility

Exhaust was produced by one of two Nissan CN 6-33, 6 cylinder 198 cubic inch (3.24 liter) displacement diesel engines coupled to a Chrysler torque-flite model A727 automatic transmission. The engines were mounted on an Eaton Model 758-DG dynamometer and run in the federal Short Cycle Mode.[6] After mixing with filtered and conditioned air in a dilution tube, the exhaust was passed into a large mixing chamber, and from there into the exposure chambers. The 24 exposure chambers were constructed of stainless steel with plateglass windows and each had an interior volume of 100 cubic feet (2.8 cu m). Animals remained in the chambers continuously during the exposure period. They were housed in wire cages with the exception of cats which were allowed to roam free.

Aerometry

An on line data acquisition system was utilized for measurement of 6 gases: carbon dioxide (CO_2), carbon monoxide (CO), sulfur dioxide (SO_2), nitric oxide (NO), nitrogen dioxide (NO_2), and total hydrocarbons (THC). The atmospheres of each of 12 exhaust and 4 clean air chambers were sampled hourly to give 8 data points/day/chamber for each gas. Acrolein, formaldehyde and total aliphatic aldehydes were monitored daily from several (usually 4) exhaust chambers. Particulate mass concentrations were determined daily using samples collected on glass fiber filters from each of the exhaust containing chambers.

Concentrations of the automatically monitored pollutants, aldehydes, particulate mass, as well as dilution ratios are shown in Table 1. Actual particulate concentrations were slightly greater than the planned level of 6 mg/m^3 during the first 61 weeks and slightly less than 12 mg/m^3 during the second half of the study. The ratio of the two concentrations was thus nearer to 1.8 than 2.0. The ratios of the various pollutant gas concentrations for the 2 periods generally ranged from 1.6 to 1.8 if the background concentrations were first subtracted. The only major exception was SO_2 which was undoubtedly influenced by varying sulfur content in the fuel. For further details of the exposure system and aerometry see Hinners, et al.[6,7]

TABLE 1

CONCENTRATIONS OF HOURLY MONITORED GASES, ALDEHYDES, PARTICULATE MASS AND DILUTION RATIOS IN THE EXPOSURE CHAMBERS

Compound	Units	Clean Air Chambers Weeks 1-124	Exhaust Chambers Weeks 1-61	Weeks 62-124
CO_2	%	0.04 ± 0.002*	0.30 ± 0.04	0.52 ± 0.04
CO	ppm	2.20 ± 0.50	20.17 ± 3.01	33.30 ± 2.94
THC	ppm	2.82 ± 0.50	7.93 ± 1.42	11.02 ± 1.04
NO	ppm	0.05 ± 0.04	11.64 ± 2.34	19.39 ± 3.80
NO_2	ppm	0.03 ± 0.03	2.68 ± 0.80	4.37 ± 1.19
SO_2	ppm	0.03 ± 0.02	2.12 ± 0.58	5.03 ± 1.03
Acrolein	ppm	0.00	0.025 ± 0.003	0.034 ± 0.009
Formaldehyde	ppm	0.00	0.106 ± 0.029	0.251 ± 0.059
Total aliphatic aldehydes	ppm	0.00	0.177 ± 0.043	0.338 ± 0.057
Particulate Mass	mg/m^3	0.00	6.34 ± 0.81	11.70 ± 0.99
Dilution Ratio	---	---	18.16 ± 1.72	9.37 ± 1.13

*Standard deviation of weekly means.

Particle characterization

Particle size was estimated by collection of the particulates on a nucleopore membrane filter and by subsequent examination using a scanning electron microscope. Generally, the basic unit was found to be 0.1 micron or less in size. These units were intermixed with larger particles, consisting of agglomerates of the basic units, reaching almost a full micron across. Our observations were in general agreement with previous studies indicating that 90% of the particles (by mass) are less than 1 micron diameter and that 50% are 0.3 micron or less. Thus, they are almost all in the respirable range.

Chemical analysis of the diesel exhaust particulate was carried out by Pitts, et al.[9] Glass fiber filter samples were extracted with a solvent system containing benzene, methylene chloride and methanol 1:1:1 by volume. The extract was further separated into acids (27.7 mg), bases (2.0 mg) and neutrals (171.3 mg). The fractions were further divided into aliquots which were used for qualitative and quantitative GC-MS analysis.

Seventy compounds were detected qualitatively. These included aliphatic hydrocarbons, polynuclear aromatic hydrocarbons, alkylated polynuclear aromatic hydrocarbons, aliphatic acids, aromatic acids and a variety of other compounds not falling in the above groups. Quantitative analysis was carried out for 12 compounds deemed most important by concentration and activity. These are listed in Table 2.

TABLE 2

CONCENTRATION OF 12 SELECTED COMPONENTS OF DIESEL EXHAUST EXTRACT

Compound	Concentration ugm/gm
Phenanthrene	145.2
Fluoranthene	155.8
Pyrene	198.0
Benz(a)anthracene	53.8
Chrysene	71.6
Benzo(k+b)fluoranthenes	77.8
Benzo(e)pyrene	28.6
Benzo(a)pyrene	15.9
Perylene	3.5
Indeno(1,2,3-Cd)fluoranthene	10.9
Indeno(1,2,3-Cd)pyrene	14.8
Benzo(ghi)perylene	21.1

EXPOSURE ASSESSMENT

All exposures have been terminated. Most of the experiments are complete and have either been reported previously or will be at the present Diesel Symposium. Some important exceptions include a detailed biochemical and morphometric analysis of the lungs of cats exposed to diesel exhaust (DE) for 27 months. A multigeneration reproduction study in rats has been completed, but a compilation of results is still in progress. Also, positive control experiments for sperm quality assessment in cats exposed to DE is still in progress.

The types of experiments that were carried out generally fall into 3 categories emphasizing carcinogenic, mutagenic or toxicological endpoints. The experiments designed to assess cancer risk included lung tumor induction in Strain "A" and SENCAR mice and liver island induction in rats. Mutagenesis studies were designed to detect possible increases in heritable mutations in mice and fruit flies; sister chromatid exchange in Syrian Hamster lung cells and peripheral lymphocytes in mice and Chinese hamsters; micronuclei and cytogenetic changes in peripheral lymphocytes of mice and Chinese hamsters and finally; sperm abnormalities in mice and cats. Toxicological endpoints included behavioral and neurophysiological changes in rats, resistance to infection in mice, pulmonary function changes in Chinese hamsters and cats, reproductive effects in mice, teratological effects in rats and rabbits, and a variety of biochemical measurements in the lung. A discussion of genotoxicity and sperm quality will be covered in a separate chapter.

In the following studies exposure levels will be referred to in terms of either 6 or 12 mg/kg particulate matter. A few studies will also be reviewed that took place during the preliminary 60-day exposure period. Although the engines were run at a set dilution ratio of 1:14 in the preliminary study, the particulate mass averaged 6-7 mg/m^3. Thus, these exposures will also be listed as 6 mg/m^3 particulate, but can be differentiated from the main study by the fact that the engines were run 20 hr/day instead of 8.

Cancer risk assessment

Lung tumor induction in Strain "A" mice exposed via inhalation. The Strain "A" mouse was selected as a cancer risk assessment model because of it's sensitivity to induction of lung tumors, the relatively short exposure period required, and the large volume of background data available.[10] The mice were exposed from about 6 weeks to 9 months of age unless stated otherwise. They were then sacrificed and lung tumors counted using Standard

Methods.[10,11,12] The first experiments of this series were carried out by Orthoefer, et al.[12] at a particulate concentration of 6 mg/m^3. No significant changes in lung tumor incidence could be detected in 400 males exposed to DE, although in 100 of these animals that were sacrificed at 11 months of age instead of 9 there was a tendency for a lower tumor incidence as compared to controls. In the second experiment 170 females per group were exposed to either DE or clean air. One half the mice in each group received an initiating dose of 1 mg urethane prior to the start of exposure. The incidence of lung tumors in both initiated and non-initiated DE exposed mice was slightly but significantly greater than those of clean air controls. The tumor incidence in controls, however, was very low compared with historic controls rendering the results inconclusive.

Since the previous report a series of 3 experiments were carried out at a particulate concentration of 12 mg/m^3. In the first experiment half the exposed and half the clean air controls received an initiating dose of 5 mg urethane. In the second experiment none of the mice received urethane but were exposed until 12 months of age instead of 9. In the final experiment the light cycle was altered so the chambers would be dark during engine operation with the animals presumably awake, active and respiring at a higher level.

The results are shown in Table 3. In every case the average number of tumors per mouse was less in DE exposed mice compared with their respective clean air controls, and differences were significant with only one exception. The lower incidence values in exposed mice was particularly noticeable in the ones pretreated with urethane.

The reason for the decreased tumor incidence in DE exposed mice is uncertain. Possibly, diesel exhaust inhalation results in an inhibition of the induction of enzymes responsible for converting procarcinogens to their active forms. It is also possible that the immunocompetence of the animals was altered by the inflammatory reaction to deposited materials.

Lung tumor induction by IP injection in Strain "A" mice. This study is one of several being conducted by the EPA to compare the relative carcinogenicity of diesel exhaust with that of other environmental pollutants known to be carcinogenic in humans. Comparisons were made between exhaust samples from the Nissan diesel, an Oldsmobile Diesel run at a steady 40 mph, cigarette smoke condensate, coke oven mains and roofing tar condensate.

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TABLE 3

EFFECTS OF DIESEL EXHAUST INHALATION ON LUNG TUMOR INDUCTION IN STRAIN "A" MICE

Treatment	Age (Mo)	Illumi- nation	Sex	Survivors/ Initial	Mice with Tumors	P	Tumors/Mouse	P
Clean air	9	Light	M	44/45	10		.227 ± .071	
			F	43/45	11		.349 ± .080	
			M&F	87/90	21		.287 ± .054	
Exhaust	9	Light	M	37/45	5	NS	.189 ± .077	.130
			F	43/45	4	.05	.093 ± .080	.05
			M&F	80/90	10	.05	.138 ± .056	.055
Clean air + 5 mg urethane	9	Light	M	38/45	32		2.368 ± .263	
			F	37/45	34		3.243 ± .314	
			M&F	75/90	66		2.800 ± .260	
Exhaust + 5 mg Urethane	9	Light	M	39/45	26	.10	1.025 ± .260	.001
			F	36/45	16	.0001	0.861 ± .318	.0001
			M&F	75/90	42	.0001	0.947 ± .206	.0001
Clean air	12	Light	M	38/	22		0.684 ± .090	
Exhaust	12	Light	M	44/	11	.01	0.250 ± .083	.001
Clean air	9	Dark	M	97/108	28		3.24 ± .047	
			F	140/142	31		.234 ± .034.	
			M&F	237/250	59		.271 ± .028	
Exhaust	9	Dark	M	111/115	13	.01	.135 ± .047	.01
			F	139/143	9	.001	.065 ± .034	.001
			M&F	250/258	22	.0001	.096 ± .027	.0001

The particulate from our own study was collected from the large mixing chamber on Pallflex T60 A20 (teflon coated) filters during the course of the inhalation study. The Oldsmobile sample was provided by the Environmental Monitoring and Support Laboratory of EPA. Both samples were soxhlet extracted with dichloromethane. Cigarette smoke condensate was produced from the Kentucky reference 2 RI cigarettes. Coke oven mains and roofing tar were collected using procedures described by Buisingh, et al.[13] Strain A/Jax mice approximately 8 weeks of age were injected 3x weekly for 8 weeks with the test substances. They were sacrificed at 9 months of age and examined for presence of pulmonary adenomas.

Two separate experiments were carried out. Results are shown in Table 4. The increase in tumor rates in the mice injected with urethane was comparable

to that reported in earlier studies[10] and showed that the mice were re-
sponding normally. The lack of a consistent increase in tumors in mice in-
jected with the environmental pollutants indicated that either the carcinogens
present were very weak or that the concentration of carcinogens reaching the
lungs was below detectable limits.

TABLE 4

EFFECTS OF INJECTED POLLUTANTS ON THE INDUCTION OF LUNG TUMORS IN STRAIN "A"
MICE

| Group | | Experiment I | | | Experiment II | |
	Sex	Tumors/mouse	Dose	Sex	Tumors/mouse	Dose
Uninjected	M	0.6 ± 0.2	–	M	0.2 ± 0.1	–
controls	F	0.6 ± 0.2	–	F	0.4 ± 0.2	–
Vehicle	M	0.9 ± 0.5	0.05 ml/inj	M	0.5 ± 0.2	0.05 ml/inj
controls	F	0.7 ± 0.2		F	0.3 ± 0.1	
Urethane	M	22.5 ± 1.9*	20 mg/mouse	M	7.3 ± 0.7*	10 mg/mouse
	F	21.8 ± 1.5*		F	11.3 ± 0.9*	
Nissan par-	M	0.4 ± 0.1	4 mg/inj	M	0.3 ± 0.1	2 mg/inj
ticulate	F	0.5 ± 0.1		F	0.3 ± 0.1	
Nissan	M	1.4 ± 0.3**	1 mg/inj	M	0.4 ± 0.1	1 mg/inj
extract	F	1.0 ± 0.3		F	0.7 ± 0.1	
Olds	M	0.4 ± 0.1	1 mg/inj	M	0.4 ± 0.1	1 mg/inj
extract	F	---		F	---	
Cigarette	M	0.8 ± 0.3	.20 mg/inj	M	0.4 ± 0.1	.20 mg/inj
smoke	F	1.1 ± 0.2		F	0.2 ± 0.1	
Coke oven	M	1.2 ± 0.3	.02 mg/inj	M	0.4 ± 0.1	.02 mg/inj
	F	0.5 ± 0.2		F	0.9 ± 0.2**	
Roofing tar	M	0.7 ± 0.2	.02 mg/inj	M	0.7 ± 0.2	.02 mg/inj
	F	0.7 ± 0.3		F	0.3 ± 0.1	

*Significantly different from both vehicle controls and uninjected
 controls (P < .05).
**Significantly different from uninjected controls (P < .05).

Tumor induction in Sencar Mice. This study was designed with several pur-
poses in mind; (a) to evaluate the effects of chronic DE exposure for a near
lifetime upon both pulmonary and nonpulmonary tumorigenesis, (b) to separate
the tumor promoting from the tumor initiating effects of DE, and (c) to pro-

vide further information on the nononcogenic pathological effects of diesel engine emissions, especially in the lungs.

Groups of approximatly 780 mice, half males and half females were exposed from conception until sacrifice to either DE or clean air. Each group was separated into 3 subgroups, initially numbering 260 animals each, receiving either a single injection of the tumor initiator urethane, multiple injections of the tumor promoter butylated hydroxytoluene, or no injections. The mice were sacrificed between 15 and 16 months of age. The particulate concentrations in the chambers were maintained at 6 mg/m^3 during the first 3 months of exposure and 12 mg/m^3 thereafter.

Preliminary inspection of histopathological results indicate no dramatic differences in lung tumor incidence between the exhaust and clean air groups. Results are very preliminary, however, and no final conclusions can be reached at this time. Further details will be presented by K. I. Campbell et al. at the 1981 Diesel Emissions Symposium.

Liver Island Assay. The liver island test was developed as a relatively short term in vivo carcinogenesis bioassay. It is a 2 stage initiation/promotion test using either a choline deficient diet or phenobarbital for promotion and partial hepatectomy to enhance initiation.[14] The endpoint is the focal appearance of hepatocytes staining positive for gamma glutamyl transpeptidase (GGT). These studies were carried out by Pereira et al. and have been partially published.[15]

In the first study young adult male Sprague Dawley rats were exposed to DE at a particulate concentration of 6 mg/m^3 for 3 or 6 months following partial hepatectomy and/or after placement on a choline deficient diet. No islands could be detected in any of the groups after 3 months exposure. Results of 6 months exposure are shown in Table 5. Fewer foci were detected in the diesel exposed rats, a difference that was significant in the most sensitive group, those partially hepatectomized and fed a choline deficient diet. The results generally agree with those found in Strain "A" mice exposed via inhalation. It is premature, however, to conclude that exposure to DE inhibits island development since results were quite variable and there was no indication of a toxic response in the liver, suggesting that little of the test chemicals may actually reach the liver.

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TABLE 5

EFFECTS OF DIESEL EXHAUST ON THE DEVELOPMENT OF LIVER FOCI

	Number of GGT (+) Foci/cm^2	
Treatment	Clean Air	Diesel Exhaust
Partial hepatectomy, choline deficient	5.68* $\pm$ 3.07	0.20 $\pm$ 0.20
Partial hepatectomy, choline sufficient	0.29 $\pm$ 0.29	0.00
Sham operated, choline deficient	2.82 $\pm$ 1.91	2.28 $\pm$ 2.28

In order to increase the concentration of exhaust components reaching the liver, an additional group of rats was injected with 667 mg/kg body wt of DE extract following partial hepatectomy and using phenobarbital as a promoter. Under these conditions DE had no effect with treated animals averaging 0.37 islands/cm^2 compared with 0.33 for controls.

Mutagenesis

Heritable effect on drosophila. The drosophila sex-linked recessive bio-assay was chosen as one of our tests because it is considered an excellent screen for genetic hazards[16] and also because of its relative economy and small exposure space requirements. For details see Schuler and Niemeier.[17] Male flies were exposed 8 days to whole exhaust filtered to remove particles larger than 0.3 microns diameter, resulting in a mean particulate concentration of 2.2 mg/m^3. The males were mated one and 8 days post exposure, thus utilizing sperm exposed while mature and while in the spermatocyte stage, respectively. The F_1 generation females were mated with their brothers. The F_2's were scored for a sex linked lethal event. A portion of the F_2's were mated to produce an F_3 generation which was also scored.

Among F_2 generation flies sex linked lethal events were detected in 4 of 1350 vials (0.30%) for exposed versus 5 of 1354 (0.37%) for control flies. Among F_3's no sex linked lethal events were detected in the diesel group versus one of 680 among controls. With the numbers used the test is capable of detecting mutagens exhibiting 3 to 5 times the background rate of 0.1 to 0.6%. It appears that either no mutagen was present or the test was not sufficiently sensitive to detect the weak activity present.

Heritable effect in mice. The effectiveness of inhaled whole diesel exhaust on the induction of heritable effects in mammals was studied by a battery of tests utilizing mice. The assays chosen were designed to detect a

number of endpoints, namely chromosome breakage, chromosome interchange and point mutations. Both sexes were examined for effects. These experiments were designed and carried out by L. B. Russell and co-workers at Oak Ridge National Laboratories.

The mice were exposed 8 hr/day, 7 days/week to a particulate concentration of 6 mg/m^3. In the first experiment the incidence of heritable point mutations were assessed by exposing recessive T stock males, then mating them with females homozygous for 7 easily detected phenotypic traits. No definite mutations were detected among 42,512 offspring ruling out with 97.5% confidence that, at the exposure level encountered by man, the induced mutation rate could exceed 0.01 times the spontaneous rate.

To test for induction of dominant lethals, male T-Stock mice were bred to 4 different stocks of females following exposure to DE, and the pregnant animals examined for dead implants. Again, no effects of diesel exposure could be detected following examination of about 280 animals/group.

To test for heritable translocations, 160 T stock mice were bred to (SEC X C57 BL) F_1 females after 4.5 weeks of DE exposure. The male progeny were weaned and subsequently tested for sterility. One partially sterile translocation was found among 1466 control male progeny compared with none in 350 male progeny of diesel exposed mice.

Effects of DE on oocyte killing in females was studied by measuring reproductive performance of 60 (SEC X C57 BL) F_1 females/group after 8 weeks exposure to DE. The average litter size of 11 for both exposed and unexposed groups indicated a lack of detectable chromosomal or cytotoxic effects in the oocytes.

The possible induction of dominant lethals in females was evaluated following exposure of 54 (101 x C3H) F_1 females for 7 weeks to DE followed by mating to the same strain of males. While there was no evidence for the induction of dominant lethal effects, fewer corpora lutea were found and a longer interval between caging and copulation occurred in exposed females.

In the final experiment of this series, the effects of DE on spermatogonial survival was tested in JH and H strain mice after 5 or 10 weeks of exposure to DE. No effects of exposure could be detected among the 8 spermatogonial classes tested.

In summary, results of all the heritable tests using flies or mice were negative. The only significant change was a decrease in corpora lutea in mice, slightly depressing reproductive performance. The absence of genetic effects indicated that either no active metabolites reached the germ cells or

the germ cells were refractory to the induction of mutational events. In any case it appears that diesel exhaust does not pose a major hazard.

Toxicological effects

Behavioral effects.

Certain behavioral endpoints, such as activity levels, have been shown to be quite sensitive to the inhalation of engine exhaust and other environmental pollutants.[18] An experiment was therefore designed to assess the effects of DE exposure in both adult and neonatal rats using spontaneous locomoter activity, forced activity and an operant conditioning task as endpoints. For further details see Laurie et al.[19]

Spontaneous locomotor activity (SLA) was measured using Sprague Dawley rats housed in Wahman LC-34 running wheels. All exposures were conducted at a particulate concentration of 6 mg/m^3. SLA in adults exposed 20 hr/day was decreased to less than 10% of controls after 6 weeks in diesel exhaust. Rats exposed 8 hours/day showed similar but less dramatic SLA declines in exposures lasting 16 weeks. A separate series of experiments was also carried out using rats exposed to DE, 20 hr/day on days 1-17 of age, and 8 hr/day on days 1-21, 1-28, or 1-42 post parturition. Testing, however, was conducted in clean air starting after the rats reached 6-7 weeks of age. The post-exposed rats were less active than controls with the largest decrements in SLA, again, noted in the 20 hr/day exposure group.

Forced activity was measured during the final week of a 42 day exposure in young adult males. Maximum tolerance was determined as the time to refusal to run on a treadmill at a speed of 19 meters/second. The animals were removed from the chamber and breathed clean air during the test. The criterion averaged 40.9 minutes for the exposed rats compared with 107.5 minutes for controls, a difference that was highly significant (P .01).

Another test involved learning a bar pressing task to obtain food pellets. The rats were exposed 20 hrs/day and remained in clean air thereafter. The training period started at 15 months of age and was continued for 42 days. The controls showed a short rise in the learning curve starting at day 5 of shaping and all learned to press the bar shortly thereafter. After 25 days only one rat previously exposed to exhaust learned to press the bar.

It was concluded that exposure to diesel exhaust resulted in alterations in voluntary activity, forced activity and learning ability. Moreover, at least some of the changes were permanent since exposure as early as the first week of life could affect adult learning.

Neurophysiological effects. Investigations in this area were designed to provide information explaining the behavioral changes observed during exposure to diesel exhaust. The endpoints assessed were somatosensory and visual evoked potentials (SEPs and VEPs, respectively) collected from rat pups at varying ages. For further details see Laurie and Boyes.[20]

Sprague Dawley pups were exposed 8 hr/day, 7 days/week from birth to 7, 14, 21, or 28 days of age. The exhaust was diluted to produce a particulate concentration of 6 mg/m^3. Measurements were made immediately after completion of exposure. SEP was elicited by a 1 m amp electrical pulse to the tibial nerve of the left hind limb. VEP was elicited by a flash of light. Evoked potentials were recorded from silver ball electrodes located over the appropriate projection area on the skull. The responses consisted of a series of positive and negative peaks.

A discussion of the details of the responses is beyond the scope of this review. However, the primary change detected was an increase in pulse latencies in the diesel exposed groups. This was thought to be due to differences in the rate of nervous system development, which is most rapid in 2 week old rats. While the particular process is uncertain, it was hypothesized that the increased latencies were related to a lesser degree of myelinization in exhaust exposed rats.

Susceptibility to infection in mice. Enhanced susceptibility to infection by inhaled Streptococcus and Klebsiella pathogens has been shown following exposure to both gasoline[21] and diesel engine exhaust.[22] The present studies were designed to further evaluate diesel exhaust with respect to enhancing susceptibility to infection under conditions of longer-term exposure and the use of pathogens in addition to Streptococcus.

Mice were exposed for acute, subacute or chronic (up to several months) periods, 8 hr/day, 7 days/week, to DE diluted to a particulate concentration of 6 mg/m^3. Immediately after test exposure the mice were removed and exposed very briefly to an infectious challenge aerosol of Salmonella typhimurium, Streptococcus pyogenes or A/PR8-3 influenza virus.

Test exposures to DE significantly and consistently, as was the case for gasoline engine exhaust, enhanced susceptibility to lethal infection by the streptococcal pathogen. Susceptibility to the viral or Salmonella pathogens, on the other hand, was not significantly affected by previous DE exposure. At least part of the DE effect was considered possibly caused by the NO_2 and short-chain aldehydes present in exhaust. Further details are available in Campbell, et al.[23]

Teratological effects

Although many of the organic compounds present in diesel engine emissions may be potentially teratogenic, no previous studies have been carried out to specifically address this issue. An experiment was therefore designed to evaluate the potential for diesel emissions to produce malformations in rat and rabbit fetuses.

Twenty Sprague Dawley rats and 20 New Zealand white rabbits were exposed to DE at a particulate concentration of 6 mg/m^3 during the critical period of gestation for development of abnormalities (days 5-16 for rats and days 6-18 for rabbits). The animals were sacrificed one day prior to the predicted birth date. A portion of the fetuses were fixed in Bouins for determination of soft tissue abnormalities. The remainder were fixed by the Alizarin Red S procedure and examined for skeletal anomalies.

No effects of diesel exposure were noted for either fetal visceral or skeletal abnormalities in either rats or rabbits.

Lung function and pathology in Chinese hamsters. This study was designed to elucidate the effects of intermediate term exposure to diesel engine emissions in a small laboratory animal species. For further details see Vinegar, et al.[24,25]

Groups of young adult male Chinese hamsters were exposed 8 hr/day, 7 days/week for 6 months to DE diluted to produce particulate concentrations of 6 or 12 mg/m^3. After completion of exposure, lung volumes, diffusing capacity, and pressure volume curves were measured. The lungs were then fixed for pathological evaluation.

Microscopic examination of the lungs of exposed animals revealed the presence of numerous black alveolar macrophages, almost filling the alveolar spaces. The lining of the alveoli was thickened due to hyperplasia of the type II alveolar cells. Adding to this thickening was the presence of edema along with a possible increase in lung collagen. Functional parameters are listed in Table 6. Dose dependent increases in lung weights, along with decreases in vital capacity and diffusing capacity were detected. Plots of vital capacity versus transpulmonary pressure (not shown) also indicated a loss of recoil pressure. Despite the large decrement in lung function, the animals apparently adapted to the exposure conditions since body weights were normal.

TABLE 6

EXPOSURE TO DIESEL EXHAUST UPON LUNG VARIABLES IN CHINESE HAMSTERS

	Clean Air	DE (6 mg/m^3)	Clean Air	DE (12 mg/m^3)
Body wt. (gms)	36.0 ± 4.1	35.2 ± 3.4	31.5 ± 3.4	34.3* ± 1.7
Lung wt. (gms)	0.23 ± 0.02	0.37* ± 0.00	0.20 ± 0.02	0.48* ± 0.06
VC (ml)	0.96 ± 0.10	0.75* ± 0.12	0.98 ± 0.10	0.61 ± 0.10
DC (ul/ml/mmHg)	2.9 ± 1.1	1.5** ± 0.9	8.0 ± 1.6	2.2* ± 1.6

*Significantly different from control (P < .01).
**Significantly different from control (P < .05).
VC - Vital Capacity
DC - Diffusing Capacity

Pulmonary function in cats. This study was designed to evaluate the effects of chronic exposure to DE upon lung function and pathology in a large animal species having a lung complexity more similar to that of humans than is found in rodents. Functional measurements have been completed. Pathological evaluation along with quantitative morphometric measurements of the lung, however, are still in progress and results are not yet available. For further details of methodology and results of testing after one year exposure see Pepelko et al.[26]

Twenty-five adult male disease-free cats of uniform age and genetic background were exposed to DE for approximately 2 years and 3 months. During the first year of exposure the exhaust dilution ratio was adjusted to produce a particulate concentration of 6 mg/m^3. During the second year the concentration was increased to 12 mg/m^3 and remained there until completion of exposures. The cats were removed from the chambers and anesthetized with Ketaset Plus (Ketamine 100 mg., Promazine 7.5 mg/ml) at a dose of 42 mg/kg during testing.

Results (Table 7) were generally negative after one year of exposure. By contrast a clearly defined response was noted after the second year. The decrease in vital capacity and total lung capacity compared with normal values for most functional measurements indicated a lesion which restricted breathing but did not cause airway obstruction or loss of elasticity. The restrictive disease found was compatible with a diagnosis of pulmonary fibrosis, along with chronic inflammation, interstitial edema, or vascular engorgement. In agreement with this diagnosis was the decrease in diffusing capacity. The comparable decreased diffusing capacity, decreased lung volumes, and patho-

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logical alterations in the lungs of DE exposed hamsters provide additional support for this diagnosis.

TABLE 7

PULMONARY FUNCTION AND LUNG VOLUMES IN CATS EXPOSED TO DIESEL EXHAUST

		One Year Exposure		Two Years Exposure	
	Units	Clean Air	Exhaust	Clean Air	Exhaust
RV	ml	104 ± 37.7	86.1 ± 37.0	80.3 ± 28.2	66.9 ± 14.3
VC	ml	369 ± 42	348 ± 43	410 ± 58	369* ± 42
TLC	ml	450 ± 75	415 ± 56	484 ± 68	428* ± 56
MEF	ml/sec	1041 ± 174	1016 ± 185	952 ± 111	887* ± 98
MEF 50%/VC	ml/sec	761 ± 160	728 ± 196	864 ± 122	801 ± 125
MEF 25%/VC	ml/sec	491 ± 199	490 ± 187	574 ± 153	518 ± 154
MEF 10%/VC	ml/sec	222 ± 157	197 ± 107	234 ± 102	223 ± 110
Compliance	ml/cmH$_2$0	23.7 ± 9.3	23.5 ± 7.2	26.2 ± 7.1	27.4 ± 4.9
Resistance	cmH$_2$O/ L/sec	10.3 ± 4.4	10.7 ± 4.6	5.7 ± 2.3	5.6 ± 3.2
Diffusion	ml/min/ mmHg	1.21 ± 0.40	1.18 ± 0.43	1.01 ± 0.14	0.90* ± 0.27
CV	ml	36.0 ± 16.0	25.6*± 13.4	25.2 ± 19.3	27.2 ± 17.6
N$_2$ washout	%N$_2$	29.0 ± 30.2	32.0 ± 20.6	21.0 ± 18.2	39.0* ± 26.0

*Significantly different from controls (P < .05).
RV - Residual Volume   MEF - Maximum Expiratory Flow
VC - Vital Capacity   CV - Closing Volume
TLC - Total Lung Capacity

Deposition and clearance. The large concentration of respirable particulate in DE can enhance the inherent toxicity of adsorbed organics by carrying them deep into the respiratory tract where they can be slowly released. Slow clearance of the particles will result in an increased exposure to the released toxic substances.

In order to investigate the deposition and clearance of inhaled DE, Charles River suckling rats were exposed 20 hours/day for 54 days; 20 hours/day for 16 days; 8 hours/day for 5 days, and 8 hours for one day to exhaust diluted to a particulate concentration of 6 mg/m^3. Following completion of exposures a small number of animals from each group were sacrificed and histologically evaluated for particle deposition. The remaining rats were sacrificed at varying times post exposure. For further details see Moore, et al.[27]

The rats exposed for 8 hours and then sacrificed showed only an occasional black particle in a few alveolar macrophages (AM). Twenty-eight days after exposure, no particles could be seen in the AM. The rats exposed for 5 days all contained a moderate number of granules in the cytoplasm. After 28 days

post exposure a few of the AM contained no particles with varying amounts pre-sent in the remainder. The AM in the rats exposed 20 hr/day for 16 or 54 days were found to be loaded with phagocytized particles at the end of exposure with no detectable decrease in particle numbers even after 90 days post exposure.

Moore et al.[27] concluded that animals exposed for a single 8-hour period received a low enough dose to effectively clear the lungs within a 28 day period, whereas the longer exposures overwhelmed the clearance mechanisms.

Biochemical alterations

Enzyme induction. The primary function of xenobiotic metabolizing enzymes is to detoxify and/or to transform harmful environmental chemicals into more readily excretable forms. Unfortunately, some of the metabolites formed during those reactions are active mutagens and/or carcinogens. Therefore, enzyme induction could lead to increased risk. This study was designed to evaluate the potential for induction of these enzymes in mice exposed to either inhaled DE or to intraperitoneal injections of DE extract. For further details see Peirano.[28]

Male strain A/J mice were exposed for either 6 or 8 months to DE at a particulate concentration of 6 mg/m^3. Separate groups of male and female strain A mice were injected with DE extract prepared by soxhlet extraction with methylene chloride and dissolved in DMSO. Each mouse received 250 mg/kg body wt/day on 2 consecutive days. The liver cytochrome $P_{448-450}$ enzymes present in microsomal preparations were measured using a modified version of the method of Omura and Sato,[29] while the enzyme aryl hydrocarbon hydroxylase (AHH) in liver and lung was determined by modified methods of Van Canfort et al.[30] The sensitivity of the cytochrome $P_{448-450}$ assay was tested by injection of 20 mg/kg body wt of 3-methylcholanthrene (3-MC) or 80 mg/kg body wt phenobarbital (PB) into separate groups of mice.

PB induced 75% and 131% increases in liver cytochrome P_{450} type enzymes in males and females, respectively, while 3-MC induced 39% and 37% increases in liver cytochrome P_{448} type enzymes. Liver cytochrome $P_{448-450}$ levels and liver AHH activities were not influenced by the inhalation exposure. Lung AHH activities calculated on a per mg microsomal protein basis were decreased 31% and 21% in mice exposed for 6 or 8 months to DE, respectively. When calculated on a per lung weight basis, however, 6 and 8 month exposed males showed 23% and 47% increases, respectively. Injection of DE extract resulted in a 23% increase in liver cytochrome $P_{448-450}$ levels in males (P < .02), but

only a nonsignificant 7% increase in females. AHH activity was not determined in DE extract exposed mice.

The changes in lung AHH were considered artifactual. This was based on the likelihood that the non-microsomal protein of the DE exposed animals was increased due in part to inflammation, increased collagen synthesis, etc. The limited inducibility by IP injection, despite the very high dose, suggested either that DE contains only a limited amount of inducing chemicals, induction inhibitors are present, or the bioavailability of chemicals present on the diesel particle is very low.

Benzo(a)pyrene metabolism in mice. One means of estimating the carcinogenic potential of inhaled DE is to measure the effects of DE exposure on the absorption, distribution, metabolism and excretion of a polycyclic aromatic hydrocarbon (PAH) known to be present in the exhaust. Benzo(a)pyrene (BP) was selected as a characteristic PAH that is found adsorbed to DE carbon particles. The following study was performed by Tyrer et al.[31] and Cantrell et al.[32]

Male strain A/J mice were exposed for 9 months to DE diluted to produce a particulate concentration of 6 mg/m^3. Following completion of exposure the mice were intratracheally instilled with ^{14}C-BP and then sacrificed 2, 24 and 168 hours later. Immediately after sacrifice the mice were frozen in liquid nitrogen, sectioned and autoradiographs made. The metabolism and excretion of intratracheally instilled ^{3}H-BP was determined in separate groups of mice. The mice were instilled and sacrificed at 2, 24, and 168 hours as before. The liver, lungs and testes were removed, frozen and later extracted and analyzed via HPLC. Urine and feces were also collected and analyzed.

The results indicated there were no differences in clearance of soluble metabolites between clean air or DE exposed mice. The DE exposed mice appeared to have less free unmetabolized BP in their tissues, which at the time was speculated as possibly due to enzyme induction. The only meaningful differences found between DE and clean air exposed mice was the inability of the DE exposed mice to clear small amounts of BP one week after instillation. It was suggested that this was due to adsorption of BP onto deposited DE particulates.

Biochemical assessment of exposure induced lung damage. Since the lung is the prime target organ for inhaled pollutants, a series of experiments were conducted to evaluate lung damage using a variety of biochemical parameters. Some of the studies have been published by Lee, et al.[22] The remainder are unpublished.

All the experiments were conducted using rats exposed either 8 or 20 hours/day, 7 days/week for periods ranging from one to 63 days. Particulate concentrations were maintained at 6 mg/m^3.

Assays were carried out on lung lavage fluid, pulmonary macrophages and lung tissue homogenates. Lung lavage was analyzed for ^{131}I-albumin leakage into the alveolar spaces as an estimate of lung integrity. Lysozyme and total protein concentrations in the lavage were monitored to assess the degree of cell lysing and subsequent protein release. The number and viability of pulmonary macrophages were determined as an estimate of the lung's defensive response to an irritant pollutant insult. Analyses were carried out on lung tissue homogenates for the following: total lung proteins to assess for possible lung fibrosis and general tissue injury; lung lipid peroxidation as an indicator of the presence of free-radicals; superoxide dismutase assessing the ability of the lung to destroy harmful superoxide freeradicals that may result from DE exposure; glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase to assess any diesel induced effects on the pentose phosphate metabolic pathway.

The only significant changes were substantial increases in lung homogenate protein concentration. It was concluded that there were no significant early detectable biochemical alterations that would indicate diesel induced lung tissue injury.

Influence of DE on lung proteins. Diesel engine emissions contain many potentially harmful components capable of injuring lung tissue. Such injury may lead to increased synthesis of lung proteins, especially collagen, and subsequently to an accumulation of lung connective tissue matrix. This can lead to morphological alterations, such as fibrosis, or to lung scarring and loss of structural integrity as in emphysema.[22,33]

To assess the potential of DE to induce these lung alterations, several studies were conducted using rats exposed 20 hours/day, 7 days/week, or mice exposed 8 hours/day, 7 days/week to exhaust diluted to produce a particulate concentration of 6 mg/m^3. The animals were assessed for total lung protein and the ability of the lungs to synthesize and accumulate collagen and non-collagen proteins using radiolabelled proline and leucine incorporation assays and, in some cases, lung prolyl hydroxylase activity.[22,33]

The total lung proteins in rats exposed for 56 days were increased 47% over that of controls. The in vivo leucine incorporation, however, was decreased 38% suggesting a decrease in overall protein synthesis in exposed animals. The increase in protein concentration, despite an apparent decrease in overall

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protein synthesis, was considered as possibly due to either an increased accumulation of circulating proteins and migrating cells in the alveolar interstitium or other compartments, or to connective tissue proliferation and increased deposition of connective tissue matrix.

In _vivo_ proline incorporation, an estimate of collagen synthesis, was not affected by 59 days exposure to DE. However, prolyl-hydroxylase activity, an in _vitro_ assay, was increased in rats exposed 33 days to DE and in rats exposed to DE in _utero_. The results of both of these assays suggest relative increases in the synthesis of collagen as compared with the synthesis of non-collagen proteins in DE exposed rats.

In order to extend these studies to compare effects upon another species, an experiment was designed utilizing mice exposed for 3.5, 6 or 9 months to clean air or DE. Large increases in lung protein content were found. Collagen synthesis also increased in the exposed mice, reaching a value 1.5 times that of controls after 9 months exposure, while overall protein synthesis decreased.

The relative increase in collagen synthesis in both mice and rats suggested the occurrence of lung injury, leading to proliferation of connective tissue and possible fibrosis. The data from the mice suggests further that continued exposure to diesel emissions may exacerbate lung injury.

SUMMARY

There was little evidence that inhalation of DE resulted in the induction of tumors. In fact, some of the results suggested a possible inhibitory effect of DE on tumorigenesis. Injection of DE particulate or particulate extract into Strain "A" mice or rats likewise failed to produce significant increases in the incidence of either lung tumors or liver islands. Essentially negative effects were also noted in attempts to induce increases in heritable mutations in mice or fruit flies; teratological effects in rats or rabbits; or enzyme induction in mice (with the exception of a small increase in males, but not females, injected with a very large dose). Results of most genotoxic studies, which have been discussed by Pereira in another chapter, were also negative using inhalation or injection of particulates as the means of exposure. Only after injection of large doses of particulate extract, or measuring effects in the lungs were positive results obtained. The most likely reason for the relative lack of effects is a low degree of bioavailability, which could stem from slow leaching of the particulate coupled with rapid inactivation and excretion of potential carcinogens and mutagens.

By contrast, a wide range of non—oncogenic toxicological effects were found. Exposure to DE resulted in a decreased level of both voluntary activity and exercise tolerance. Exposure early in life had a detrimental effect on learning in adult rats. In agreement with behavioral changes was evidence for delayed neuronal maturation. A variety of changes were detected in the lungs. Functional decrements suggested the development of restrictive lung disease. Biochemical changes indicative of increased collagen deposition, along with alterations in histology supported this conclusion. Resistance to infection decreased markedly after DE inhalation, and clearance mechanisms were overwhelmed. The marked effects found in the non—oncogenic studies are likely due to the presence of vapor phase components such as nitrogen oxides, aliphatic aldehydes, etc., which would be expected to show a much greater degree of bioavailibility than the potential carcinogens.

In the areas where dramatic effects were noted, such as behavior, lung morphology, infectivity, etc., further studies are necessary to delineate threshold limit values and to isolate and identify components of exhaust responsible for producing these changes.

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DEPOSITION AND CLEARANCE OF DIESEL PARTICLES FROM THE LUNG

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INTRODUCTION

A projected increased use of light-duty diesel engines on U.S. roads has generated considerable interest in determining the potential health impact of inhaled diesel emissions. Of primary concern is the potential effect of the submicron carbonaceous particulate fraction of the exhaust, which is present in concentrations of 30-100 times those of a spark-ignited, catalyst-equipped, gasoline-fueled engine.[1] Since these particles have a mass median aerodynamic diameter of approximately 0.2 μm,[2] they have a settling velocity which is almost nil, and will persist in the atmosphere for a considerable time after emission, allowing time for airborne transport and possible chemical transformation in the atmosphere. Studies have shown the particle to consist of smaller, elemental, carbonaceous particles approximately 300 Å in diameter, which are fused together into agglomerates containing up to several hundred elemental units.[3] Closely associated with this core is a variable fraction of benzene-soluble organic material,[2] known to contain literally thousands of compounds, including polycyclic aromatic hydrocarbons and other compounds known to be biologically active in various assays.[4,5,6,7] Since airborne particulate material in this size range is not effectively removed in the upper respiratory system, it is anticipated that a certain fraction of the particulate material inhaled with each breath will be deposited in various regions of the respiratory system, including the deep lung. The exact amount of diesel particulate material, and where in the respiratory system it will be deposited, are determined by the physics of the particle interaction with the inspired current of air and the adjacent walls of the airways. The total mass deposited determines the dose to a given organ, and together with other factors such as the bioavailability of materials on the particles for interaction with the surrounding tissues and the rate of clearance, causes the response which may occur in these tissues. It is this issue, the determination of dose to the lung, which this chapter will address through experimental measurements of deposition and clearance of inhaled diesel particles.

Deposition efficiency in this context will refer to the mass fraction of particulate which deposits on the surfaces of any of the respiratory airways, divided by the total mass of particulate material entering during respiration. This percentage of deposition has been studied extensively in man, and has been expressed as a function of the diameter of the

inhaled particle by the well known International Commission Radiological Protection (ICRP) model.[8] On a smaller scale, similar work has been done for some species of laboratory animals; however, the experimental data available to date is much more limited.[9] The form of the deposition relationship is derived from consideration of the deposition mechanisms of inertial impaction, sedimentation, and Brownian diffusion applied to a particle during its transit of the nasopharyngeal, tracheobronchial, and deep pulmonary regions of the lung. The model has been well verified for particles > 0.5 μm mass median aerodynamic diameter in human exposure tests to innocuous monodisperse aerosols through measurements of the inspired and expired mass concentrations and the air volumes respired. Recent data obtained for submicron aerosol inhalation studies in humans has shown that the amount deposited may be over-estimated by the ICRP model,[10] and that experimental measurements may still be necessary to accurately predict the deposition of airborne materials in this size range.

The second process closely associated with particle deposition is the clearance of deposited material from the respiratory system via the mechanisms of physical transport or dissolution. Since this process may account for the removal of a significant amount of deposited material from the airways, it is an important consideration in determining the amount of material retained in the lung, and thereby the long-term dose to this organ. To this end, the following series of experiments was performed using diesel particles, and a mathematical model of particulate transport in the respiratory system was developed to evaluate and interpret the experimental findings.

METHODS

Test animals were exposed to diluted diesel exhaust at controlled particle concentrations in a large-volume exposure facility[11] by methods described in an earlier publication.[12] Tracing the deposition, clearance, and subsequent retention of the diesel particles was accomplished using a second exposure apparatus in which radioactively-labelled diesel particles were inhaled by the animals, and their subsequent fate determined by tracing the radioactivity. The radioactive tagging was achieved by introducing the compound (1-^{14}C)-n-hexadecane (ICN Pharmaceuticals) into the diesel fuel (AMOCO Type 2D), based on its representative boiling point, molecular weight, and chemical structure. A ^{14}C tag of an aliphatic hydrocarbon in the fuel has several advantages over other markers which were considered for the study. These include the fact that the tagged carbon atom is incorporated into the diesel particle during the combustion process in a manner indistinguishable from atoms of other fuel compounds, and the fact that the tagged atom cannot be leached from the particle by body fluids. The latter characteristic is particularly important for clearance and retention studies as discussed in detail by Chan et al.[13]

In order to utilize the radioactive compound most effectively, a single-cylinder direct-

injection diesel engine (Farymann K-54) with a 242 cm^3 displacement was used. The engine was operated at a constant speed of 3600 rev/min, and particles were sampled at various engine loads on microquartz glass fiber filters to determine the distribution of ^{14}C radioactivity between the insoluble particle core and the extractable fraction. Generally, the extractable fraction decreased with increasing engine load both for the mass distribution determined gravimetrically and for ^{14}C activity distribution determined radiometrically. In view of the fact that the diesel fuel contained a large number of aliphatic and aromatic hydrocarbons and the ^{14}C tag was only present in a single compound (^{14}C-n-hexadecane), the activity distribution did not exactly follow the mass distribution. Under conditions of high engine load, 99% of the ^{14}C was preferentially incorporated in the carbonaceous particle core. The particle size distribution was determined at different engine loads using a multi-jet Mercer cascade impactor, and it was determined that when the engine was operated at loads higher than 30% of full load, the size distribution was essentially identical to that observed in the GM 5.7 L diesel engine operated at 20 mph under road load conditions.

Groups of 24 male Fischer 344 rats (*Rattus norvegicus*) weighing approximately 325 g or 24 Hartley guinea pigs (*Cavia porcellus*) weighing approximately 450 g were exposed in a nose-only inhalation chamber to ^{14}C-tagged diesel particles generated from the Farymann engine operated at full load. The exposure chamber was constructed of 6 mm thick stainless steel with exposure ports on each of three tiers. Radioactive diesel particles entered through the bottom section of the chamber, and rapid mixing with dilution air was achieved in a 15 cm high mixing section inside the exposure chamber below an annular perforated plate. Precalibrated orifice meters in the airflow measurement section allowed precise adjustment of the flow of dilution air and diesel exhaust to the chamber. The total flowrate through the chamber was 200 L/min, and a negative pressure of 1.5 cm of water maintained within the chamber prevented any possible radioactive contamination leaks. Immediately after the exposure, i.e., generally within 30 minutes from the conclusion of the exposure, groups of animals were sacrificed by an intraperitoneal injection of Na-pentobarbital. A blood sample was obtained by cardiac puncture, and the lungs, heart, spleen, liver, trachea with hilar lymph nodes, and thymus with mediastinal lymph nodes were removed. The tissues were processed in a biological material oxidizer (Harvey). The resultant ^{14}CO$_2$ was absorbed in a trapping solution (three parts of ethanolamine and seven parts of 2-methoxyethanol) for radiometric assay of ^{14}C activity using a liquid scintillation counter (Searle Mark III). Expired air, feces, and urine were also collected and analyzed for ^{14}C activity during the first ninety-six hours post-exposure. The measurement of the ^{14}C activity in blood, urine, and expired air indicated that the elimination of ^{14}C activity from the blood through the urine and expired air was surprisingly fast, and more than 95% of the initially absorbed activity was eliminated from the circulating blood within the first

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six hours.

Although a diffusion scrubber greatly reduced $^{14}CO_2$ concentration in the diluted exhaust, a relatively large amount of remaining radioactive CO_2 could still be absorbed in the circulating blood and significantly influence the measurements of particle retention by ^{14}C activity assay. To correct for the potential artifact, the amount of blood remaining in the excised organs was determined by the analysis of iron using atomic absorption spectrophotometry, and the ^{14}C activities in the tissues were corrected for the absorbed ^{14}C bicarbonate contamination in the circulating blood. Based upon these observations, a numerical correction was necessary only for the initial deposition determination. After 24 hours, no correction was necessary, since 99% of the activity in the blood had already been eliminated. Typically, the numerical correction for the blood contamination in the initial deposition was 10% of the total activity.

In each of the ^{14}C studies, the deposition efficiency was calculated by dividing the corrected lung particle activity determined immediately after the termination of exposure by the inhaled dose. The inhaled dose was calculated as the product of the specific activity of the exhaust particles in the inhaled air, estimated lung ventilation, and exposure duration. The specific activity of particles in the exhaust was determined from the radioactivity assay of filter samples, the exposure duration was experimentally known, and literature data were used to estimate the lung ventilation of test animals.[14,15,16] Since the lung ventilation correlates well with the 3/4 power of the body weight, an empirical relationship was also used to estimate the lung ventilation based on variations in body weight of the test animals.[14]

EXPERIMENTAL RESULTS

Three separate projects investigated the kinetics of deposition and clearance using the ^{14}C-tagged diesel particles:

Long-term Clearance

Chan et al[13] and Lee et al[17] measured the long-term lung clearance of inhaled diesel particles in animals acutely exposed to two different exhaust concentrations. At the lower diesel particulate concentration (2000 $\mu g/m^3$) and extended exposure duration (140 min), the deposition efficiency of diesel particulates in the lung was estimated to be 20 ± 5% of the inhaled dose for the exposed Fischer 344 rats. This agreed well with the calculated deposition efficiency of 17 ± 2% of the inhaled dose for the same strain exposed to a higher concentration of diesel particulates (7000 $\mu g/m^3$) for a shorter exposure duration (45 min), and it indicated that the deposition efficiency was not significantly influenced by the inhaled concentrations of particles within the range of our observations. It was encouraging to note that the value of 15-20% deposition efficiency for inhaled diesel particles in the Fischer 344 strain was in good agreement with experimental data reporting 15-20%

deposition efficiency for particles of comparable size (0.2 μm) in humans.[10,18] It also agreed well with the Schum and Yeh theoretical model,[19] which suggests that the deposition efficiency of inhaled submicron particles in rats was more similar to humans than any other mammalian species used in laboratory testing.

The activity of inhaled ^{14}C-tagged diesel particles retained in the lungs at various time periods after the exposure was determined by liquid scintillation counting of the retained ^{14}C activity in the lung. Since the initial lung burden could not be determined for every test animal, a normalized value to account for the variability in the initial lung dose has been estimated. No significant difference in particle clearance has been observed between the two experimental groups exposed to two different particulate concentrations with comparable inhaled dose (7000 μg/m^3 for 45 minutes and 2000 μg/m^3 for 140 minutes) for an observation period up to 28 days after exposure as shown in Figure 1. This indicates that at the two particulate concentrations studied, there were no major alterations in the lung clearance process.

At the inhaled particulate concentration of 7000 μg/m^3, the retention of particles was determined up to 6 months after the exposure. The experimental data, shown in Figure 1, were analyzed by a curve-stripping procedure into three components with approximate half-times of 1 day, 8 days, and 80 days, respectively[17]. The biological meaning of these
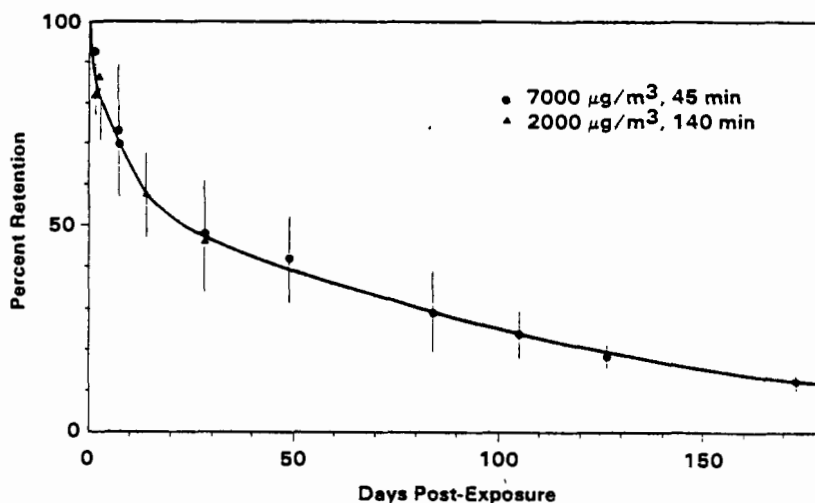
Figure 1. Long-term clearance of retained ^{14}C-tagged diesel particles from the lungs of Fischer 344 rats exposed to two concentrations of diluted diesel exhaust. Vertical bars indicate standard deviations of the measurements. From Lee, et al.[17]

components can be understood in terms of clearance mechanisms. The first mechanism deals preferentially with particles deposited in the tracheobronchial tree, and represents their transport by the mucociliary escalator. The particles are finally cleared through the gastrointestinal tract, and their elimination is clearly documented by the presence of ^{14}C activity in the feces. The second mechanism can be interpreted as the transport of material deposited in the proximal respiratory bronchioles, where only a short distance is required for transferring the particulates to the mucociliary escalator. The third mechanism involves the removal of particulate matter from the alveolar region where the clearance may involve endocytosis, passive and active absorption, and dissolution or metabolism of the deposited particles.

Species Differences

Chan et al[20] analyzed the species differences in the deposition and clearance of inhaled diesel particles using rats and guinea pigs. Lung clearance of inhaled diesel exhaust particles in the rat was divided into two distinct phases of clearance[13] up to 105 days post-exposure. An exponential clearance half-time of 1 day for particles removed from the tracheobronchial region by ciliary action represents the 'rapid' clearance phase. A slower clearance phase, mediated by the alveolar macrophages through phagocytosis and transport of the particles out of the respiratory airways, has a half-time of 62 days. In the guinea pig, the clearance rates for diesel particles removed in the first few days by the same mechanisms showed a similar clearance half-time of 1 day. The percent of initial lung particle deposition cleared during the rapid phase by the guinea pig was 17%, compared to 34% in the rat.

The long-term clearance phase, however, was surprisingly very different. As shown in Figure 2, almost no clearance was observed in the lungs of the guinea pig between day 14 and day 105 post-exposure. This would indicate a greater potential for the guinea pig to accumulate diesel particles in the pulmonary regions, compared to the Fischer rat. This seeming lack of 'normal' alveolar clearance in the guinea pig would account for the relatively large amount of diesel particles recovered from lungs of guinea pigs exposed to diesel exhaust in a chronic inhalation study.[21] In an earlier study,[13] the clearance of diesel particles to the lymphatic system was observed in the Fischer rats, where a few percent of the initial deposition was measured in the mediastinal lymph nodes as early as a few days post-exposure. These measurements have followed the accumulation of inhaled diesel particles in the lymph nodes of the guinea pig and the rat up to 105 days post-exposure. Clearance of the diesel particles from the pulmonary regions to the lymph nodes occurred in both species during the first few days with 2% of the initial lung dose retained after 105 days in both the rat and guinea pig. Although this observation is not uncommon for insoluble particles that have been known to reside in the lymphatic system for long periods of time, the transport of diesel particles from the lungs to the lymph nodes was faster than
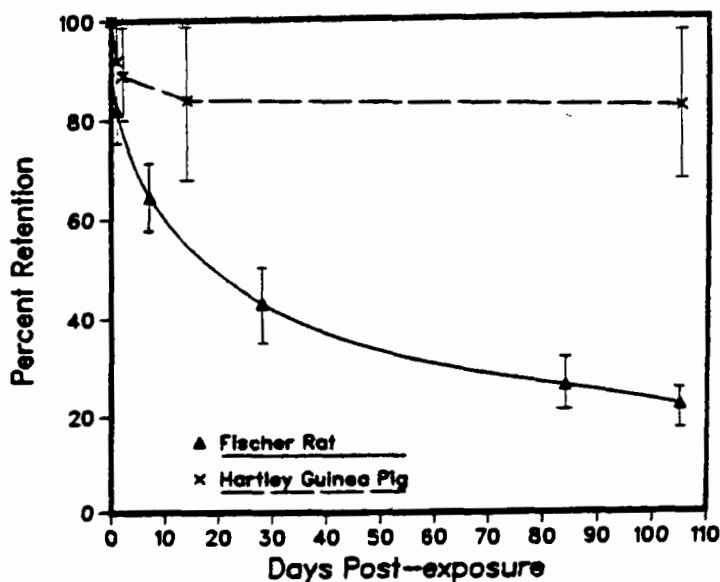
Figure 2. Differences in the clearance of ^{14}C-tagged diesel particles from the lungs of rats and guinea pigs after a single acute exposure. At each measurement, N = 4. From Chan and Lee[20]

that of fused-clay particles reported by Thomas.[22] Diesel particles of 0.15 μm diameter could conceivably evoke faster lymphatic drainage, as compared to the larger 1.5 μm clay particles. In this study, only the largest thoracic lymph nodes (hilar and mediastinal) were analyzed, so that the absolute amount of diesel particles in the entire lymphatic system might be slightly higher than the values computed. Furthermore, the alveolar clearance of diesel particles in the guinea pig was extremely slow, with more than 80% of the initial dose retained after 105 days. The pulmonary clearance half-time for inhaled diesel particles in the guinea pig is estimated to exceed 300 days which strongly contrasts with 60-80 days in rats (determined by fitting experimental data collected so far to two- or three-phase clearance models.) The differences observed in this study demonstrate a greater long-term retention of inhaled diesel particles in the guinea pig possibly caused by slower clearance processes in the deep lung of this species. The actual biological dose to the respiratory epithelium would also be different in both species. This clearly indicates the difficulty in comparing studies on potential health effects of inhaled diesel particles among different species and in extrapolating experimental animal data to man.

Accumulated Dose Effect

Chan et al[23] also measured the effect of accumulated particle mass in the lung on the kinetics of clearance of acutely inhaled diesel particles. Preliminarly results obtained on

groups of Fischer 344 rats pre-exposed to substantial doses of diesel particulate in the large-volume chambers (6000 μg/m^3 for seven and 112 days, and 250 μg/m^3 for 112 days) indicate that the clearance rate of ^{14}C-tagged diesel particles inhaled for 45 minutes is substantially altered by the condition of the animals. Preliminary results were obtained from pooled measurements on 4 animals. In the group exposed to 6000 μg/m^3 for 112 days and having an accumulated lung burden of 10 mg particles per gram of lung at the time of exposure, a retardation of clearance was noted which persisted over the first 49 days with only approximately 15% of the lung-deposited material being cleared in this interval. Lung clearance in animals exposed to the lower pre-loading doses (approximately 0.5-1.0 mg particles per gram of lung) was also reduced, but to a lesser extent as depicted in Figure 3, and compared to age-matched control animals, which had only clean air exposure prior to inhaling the ^{14}C-tagged diesel particles. At this time in the analysis, it appears that an increase in the retention of the tracer particles has occurred as a result of the massive accumulation of diesel particulate material in the lung, and that the effect is dose-dependent.
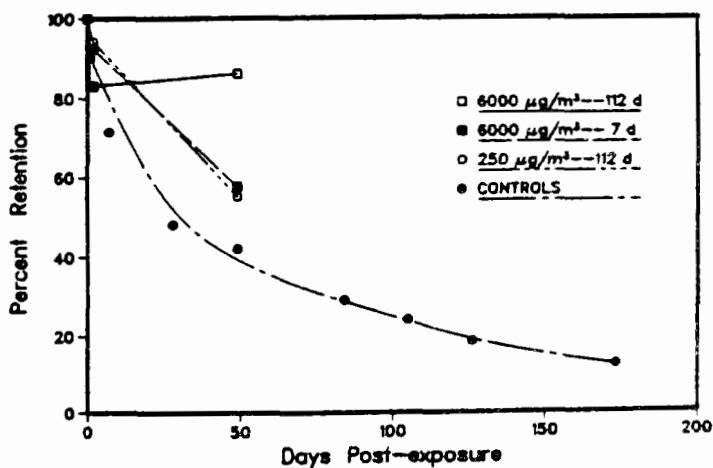
Figure 3. The effect of pre-exposure to various doses of diesel particulate on the lung clearance rate of Fischer 344 rats after acute exposure to ^{14}C-tagged diesel particles. From Chan et al.[23]

PARTICLE TRANSPORT MODEL

Summary of experimental data

Briefly, the experimental results of the deposition and clearance studies and additional information available in the literature on the clearance of submicron particles may be summarized as follows:

About 8% of the inhaled particulate deposits in the head of rats and rapidly enters the GI tract;[9] another 6-8% deposits in the tracheobronchial region of the lung (ciliated airways) and is cleared to the GI tract with a clearance half-time of about 1 day,[9,13] and about 12% deposits in the pulmonary (alveolar) region of the lung.[9,13]

The particulate which deposits in the pulmonary region is fairly quickly engulfed by pulmonary macrophages and possibly other cell types. One estimate of the half-time of phagocytosis in mice is 1 day.[24]

Some of the scavenger cells containing particulate travel to the ciliated airways and are cleared to the GI tract. Others travel through the lymphatic system to the lymph nodes.[25] The overall clearance half-time from the pulmonary region was estimated to be 60-80 days in rats.[13,17] In another experiment, the fraction of lavageable macrophages which contained diesel particulate decreased after the exposure ended with a half-time of 40 days in rats and 37 weeks in guinea pigs.

Particularly after excessive exposures, some particle-laden macrophages do not carry particulate out of the lung, but aggregate in pulmonary connective tissue,[24] in lymphoid foci in the lung parenchyma,[25] and in complexes near terminal bronchioles.[26,27]

Model development

Soderholm[28] reviewed the data and developed a particle transport model with compartmental divisions illustrated in Figure 4. Each compartment represents a significant "reservoir" in which particles can be found. Four lung compartments are included: particulate in the tracheobronchial region (T), free particulate lying on deep lung surfaces (F), particulate in macrophages or other mobile scavenger cells (M), and sequestered particulate (S). Two compartments are external to the lung: gastrointestinal tract (G) and lung-draining lymph nodes (L).

The model consists of a set of differential equations specifying the rate of transport of particulate mass among compartments. The kinetics are assumed to be first order since it is the simplest assumption which can be made and since it leads to exponential clearance curves, the type found experimentally. Because the kinetics are assumed to be first order, the clearance rate for each pathway is proportional to the amount of material in the compartment being cleared. Each proportionality constant is written in terms of the half-time of clearance. The half-times are designated by four-letter symbols (HTxy) where the third letter is the compartment which the particulate is leaving, and the fourth letter is the compartment which the particulate is entering. The other parameters in the model are the deposition rate into the tracheobronchial compartment (RT) and the deposition rate into the free particulate compartment (RF). Each of these deposition rates can be calculated from the deposition efficiency of the compartment, the animal's respiratory minute volume, and the airborne concentration of particulate. The product of minute

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volume and the deposition efficiency of a compartment is called the specific deposition rate of that compartment.
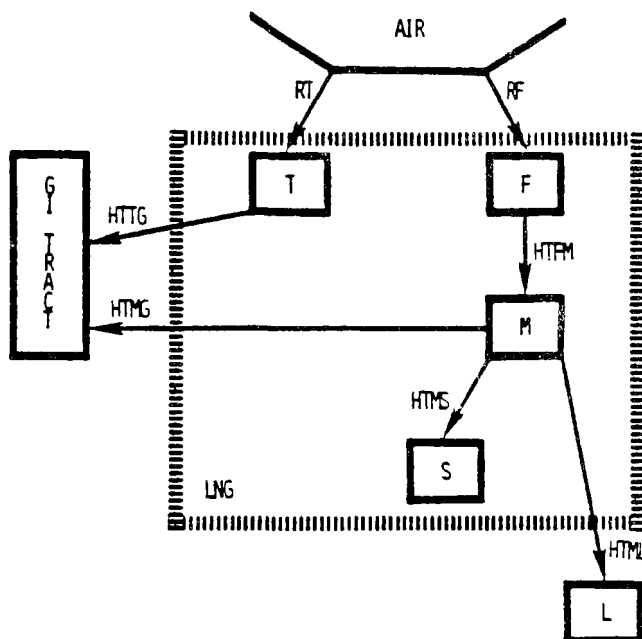
Figure 4. Compartments and parameters in the lung particulate transport model: T = tracheobronchial, F = free particulate on the deep lung surface, M = macrophages, S = sequestered particulate, L = lymph nodes, G = gastrointestinal tract, LNG = total lung, RT = tracheal deposition rate, RF = deep lung surface deposition rate, HTxy half-time for clearance from compartment x to y.

In a "short exposure" experiment, the animals are exposed to high concentrations for only a short period, and their changing particulate lung burdens are measured starting at the end of the exposure. In a "continuous exposure" experiment, the animals are exposed to high concentrations continuously or nearly continuously over a long period and particulate lung burdens are periodically measured. This model is a first approximation in that the clearance rates of the individual compartments are assumed to be independent of the amount of accumulated particulate and constant over the period of observation, even for long-term continuous exposure experiments.

Before analyzing the experimental results, it will be illustrative to consider the general transport kinetics derived from the model for the two illustrative cases of "short exposure" vs. "continuous exposure." Considering first the change in the total lung burden with time after a short exposure, we note that at the end of the exposure all of the particulate

resides in the "tracheobronchial" and "free particulate" compartments. The material in the "tracheobronchial" compartment quickly clears with an assumed half-time of 1 day. Since in rats a significant fraction of the initial total lung burden was assumed to have deposited in the "tracheobronchial" compartment, its fast clearance out of the lung is evident in the decrease in the total lung burden over the first day after the exposure. Meanwhile, the material in the "free particulate" compartment is picked up by scavenger cells with an assumed half-time of 2 days, causing movement of particulate mass from the"free particulate" compartment to the "macrophage" compartment. After about 10 days, the "free particulate" compartment is empty and all the particulate is in the "macrophage" compartment. It clears with an overall half-time consisting of contributions from clearance into the GI tract and clearance into the lymph nodes. The effect of a sequestering region would be to cause the total lung burden to decrease to some level above zero, rather than continuing to decrease to zero, as with no sequestering.

For the "continuous exposure" case, particulate continuously deposits directly into the "tracheobronchial" and "free particulate" compartments. The "tracheobronchial" compartment burden reaches a plateau after a few days at a relatively low level equal to 144% of the deposition rate times the clearance half-time. Similarly, the "free particulate" compartment quickly plateaus, reaching a level of 144% of its deposition rate times its clearance half-time. After several clearance half-times, the "macrophage" compartment content also plateaus, causing no further increase in the total lung burden with continuing exposure without sequestering. The effect of a "sequestered particulate" compartment would be to cause the total lung burden to increase linearly with time after the particulate burdens in the other compartments have all plateaued. It may be observed that after the first few days of exposure, the lung burden of chronically exposed animals is insensitive to the parameters of the faster clearing compartments.

Chan et al [13] fit the post-exposure clearance data with a two-phase exponential curve whose coefficients and half-times were interpreted as indicating that 34% of the initial lung burden was deposited in the tracheobronchial region and cleared with a half-time of 1 day, while 66% of the initial lung burden deposited in the pulmonary region and cleared with a half-time of 62 days. These results can be translated into values for the model parameters. When the calculated parameters were plotted in Figure 5 against the original data, using clearance half-time of the free particulate compartment abitrarily set to 2 days, the analysis showed that the variation of the half-time between 1 or 4 days did not significantly change the curve for the total lung burden. The satisfactory fit to the long-term clearance data by the model when the experimentally determined deposition and clearance half-times are used shows that the model is compatible with the approach originally used to interpret the data.

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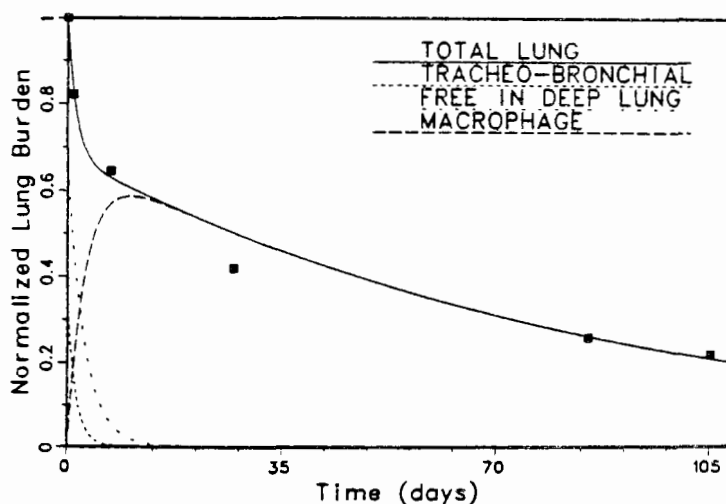
Figure 5. Comparison of acute clearance data of diesel particles in the rat (Chan, et al.[13]) with the lung clearance curve computed from the transport model using the experimentally determined half-times. From Soderholm (1981)[29]

It is interesting to note, however, that if the parameters derived from this acute exposure to diesel exhaust are used in the model and the analysis is cross-checked with data obtained after chronic exposure of animals to non-radioactive diesel exhaust[12,21] a significant mismatch occurs. The parameters derived from the acute exposure of the long-term clearance data predict that the lung burden of animals chronically exposed would eventually plateau as shown in Figure 6. However, the data from the chronic exposure experiment showed that the lung burden continually increased over the period of observation. The continual increase was originally interpreted as indicating that at least some of the deposited particulate was cleared very slowly, if at all.[21]

A revised set of model parameters was derived which provided a better description of the chronic exposure data. Figure 7 shows that the model calculations, using the revised parameters, do not agree with the acute exposure experimental data, but predict higher lung burdens at longer times than were observed. Further consideration of the model solutions and the experimental data reveals that no single set of model parameters can satisfy all the available data. This is a significant result, since it indicates that there is an actual unaccounted for difference among the experiments in the respiratory system's reaction to particulate. It is conceivable, if not likely, that the respiratory system responds differently during a short nose-only exposure than during a long-term whole-body exposure.

The revised set of parameters used to calculate the curve in Figure 7 are not the only set which would fit the chronic exposure data. The parameters were obtained as follows:

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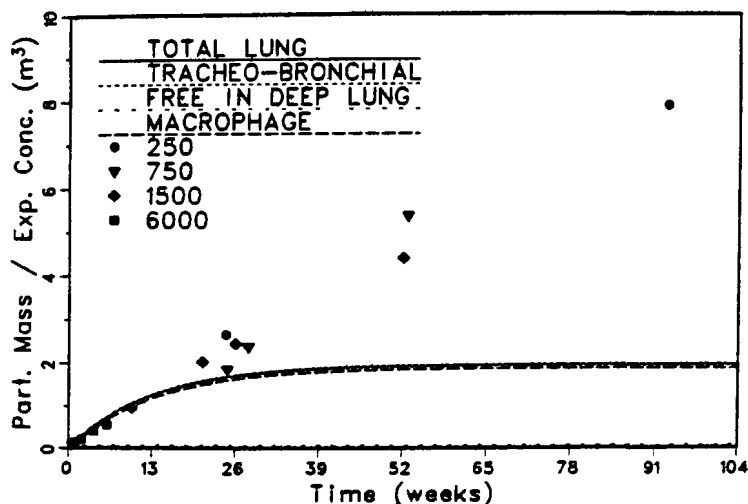
Figure 6. Comparison of chronic exposure lung burden data normalized by the exposure concentration with model calculations using the actual intermittent exposure schedule. The model parameters were taken from the original analysis by Chan et al[13] of their data which describe clearance after a short exposure. Despite the fact that the model adequately describes the clearance kinetics for low doses (Figure 5), a significant mismatch occurs when the parameters are used to model clearance for higher doses of particulate. From Soderholm (1981)[29]
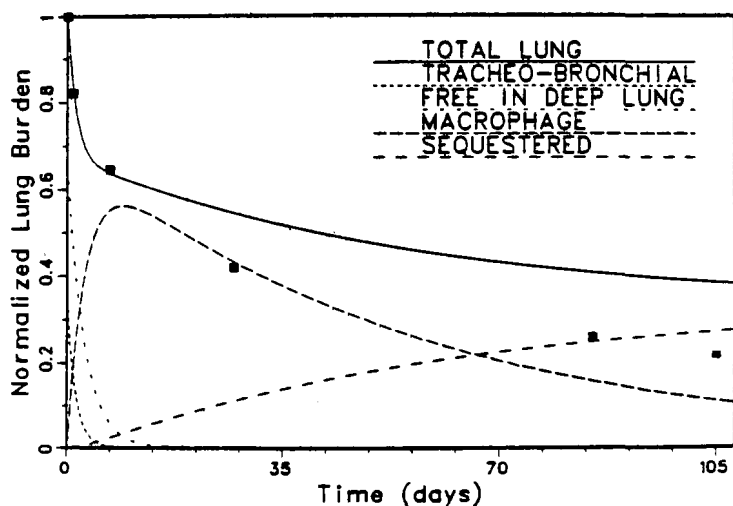
Figure 7. Comparison of Chan et al.[13] data with model calculations of particulate transport after acute exposure using the set of parameters modified to fit the long-term clearance data. In each case lung burdens were normalized by the total lung burden following acute exposure. The comparison illustrates that a significant mismatch and over-estimation of lung retention occurs when parameters derived from high lung dose experiments are used to predict clearance at low doses. From Soderholm (1981)[29]

the deposition efficiencies in the "tracheobronchial" and "free particulate" compartments and the clearance half-time of the "tracheobronchial" compartment were taken from the acute exposure and clearance study using ^{14}C, the minute volume was assumed to be 200 mL for rats weighing approximately 400 g, the overall clearance half-time of the "macrophage" compartment was taken from experimental data, and the half-time of clearance from the "macrophage" compartment to the "lymph nodes" was set to 10^7 days. This effectively lumps all clearance out of the deep lung into one overall clearance half-time since there is little data available now on the separate rates of clearance to the lymph nodes and GI tract. With these assumed values, the overall clearance half-time out of the deep lung and the clearance half-time into the "sequestered particulate" compartment would each have to be 80 days in order to fit the slope of the chronic data. There may be little significance in the values of these two half-times because of the large number of assumptions which went into their derivation. The principle result is that no set of parameters fits all the data.

The major conceptual difference between this model of particulate kinetics in the lung and previous ones[8,22] is that the deep lung was assigned three compartments based on microscopic observations of the fate of diesel particulate in the respiratory system. This approach allows quantitative measurements of the particulate burden in a single compartment, for example, measurements of lavageable macrophages,[26] to be treated within the model. The ICRP clearance model[8] includes a nasopharyngeal compartment and emphasizes particle dissolution. These features could be added to the present model, although they were deemed irrelevant to the diesel particulate body burden data available. Inclusion of the "free particulate" compartment does not significantly change the fit to experimental data, but serves as a reminder that particulate resides on the lung surface for a short time.

The acute exposure and long-term clearance data give the deposition rate into the tracheobronchial and deep lung regions and the clearance half-time of the tracheobronchial region. The contribution of the model to the analysis of that data is to point out that the long-term clearance phase may be interpreted in two ways. It may be clearance of particulate out of the deep lung with a half-time of 62 days as originally interpreted,[13] or it may be a combination of deep lung clearance and sequestering. Only data taken at longer times showing whether the lung burden plateaus above zero can resolve which interpretation is correct.

The buildup of particulate in the lungs of chronically exposed animals was interpreted as indicating that after excessive exposures some portion of the deposited particulate was retained for long times and indicated the presence of a compartment with a prolonged residence of diesel particles. Since morphological studies[27] reported the formation of alveolar macrophage aggregates, it may be that the sequestering compartment actually corresponds to this physical mode of isolating the diesel particles from the functional cells

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of the deep pulmonary region. The contribution of this work to the analysis of that data is to relate the rate of buildup to the fundamental transport parameters of the system. To date, there is insufficient data to precisely set the values of all the parameters. However, the calculations have shown that no single set of model parameters fit the data available from experiments and more data must be collected to identify the source of the apparent discrepancy.

DISCUSSION AND CONCLUSIONS

Data from a variety of inhalation exposure studies has been presented and compared in an effort to better understand the deposition, clearance, and long-term retention of diesel particles in the lung. In response to observations of the apparent linear increase of the lung burden after inhalation of high concentrations of diesel exhaust particulate (250, 750, 1500, and 6000 $\mu g/m^3$) for a long period of time, a model was derived which included a "sequestering" compartment.[29] Material in this compartment clears very slowly, if at all, and is thought to be associated with the aggregations of macrophages which have been described by White and Garg.[30] Analysis of the chronic exposure data revealed that for the rat, 7% of the inhaled particulate (assuming a respiratory minute volume of 200 mL) appears to be held in the sequestering compartment which has a clearance half-time in excess of one year. Using this model, the long-term retention parameters which had fit the chronic exposure data were used to predict the long-term results of the post-exposure clearance experiments of Chan et al.,[13] who used radioactively-tagged diesel particles. The results were seen in Figure 7, and show the predicted lung burdens to be significantly higher than the actual ones for long times. It appears likely that the discrepancy would have been even greater if the post-exposure clearance had been followed for longer times. Further model calculations show that no single set of model parameters (minute volumes, deposition efficiencies, clearance half-times) could be found which would predict both sets of experimental data.

Thus, it is clear that under conditions of high-level exposures, the model does not accurately reflect all the major lung clearance mechanisms, and the changes in their contributions with exposure conditions. The model calculations have quantitatively shown that long-term retention does not occur to nearly as great an extent after a single exposure in which less than 10 μg of radioactive particulates is deposited in the pulmonary region as during chronic exposure, when hundreds of micrograms up to several milligrams have deposited. This is not too surprising, since the long-term retention during chronic exposure to high concentrations is assumed to be associated with aggregates of macrophages, a feature not seen in unexposed lungs, or lungs containing very small particulate burdens. Thus, it appears that there is a threshold effect in the amount of long-term retention of diesel particulate in rats. The threshold might correlate with mass loading, exposure

concentration, or exposure duration. Only further studies using lower concentrations of particulate in chronic exposure experiments, and different exposure conditions in the short exposure, tagged-particulate clearance experiments can help to refine our understanding of the threshold effect in long-term clearance.

One paper on this subject by Lewis and Coughlin[31] measured lung burdens of acid insoluble material (soot) in man at autopsy. They reported an apparent linear increase in lung burden with age and claimed that a fraction of the inhaled soot was retained for long times. On the other hand, studies of carbonaceous particle content in the lungs of coal mine workers by Stober et al[32] suggests that a very active clearance of particles exists in the human lung with a half-time of approximately 5 years. Consequently, the relevance of the available inhalation data to real-life conditions must be carefully weighed, especially when extrapolating long-term retention data for health effects evaluations.

In conclusion, this work has shown that low doses of diesel particulate material are rapidly cleared by the lung defense system but that species differences do exist in clearance and retention mechanics. Therefore, test animal models should be carefully chosen when studying the long-term retention of diesel particles to assure that the kinetics will appropriately relate, if possible, to those of the human lung. Furthermore, the data available to date indicates that clearance and retention rates determined with high particle concentrations and high associated lung burdens, are not equivalent to those measured at lower levels of exposure and particle burdens. It is clearly evident from this work that information obtained at high levels of exposure cannot be scaled down to predict effects at expected ambient levels, since above certain, as yet undetermined thresholds, the lung's defense system deals with the deposited particles in different ways than it does at lower levels. In particular, the data indicate that the particle retention rates that have been found after excessive inhalation exposures were much higher than those occurring at minimal exposures. Consequently, the dose of inhaled particles and their resulting biological activity which could be responsible for potential adverse health effects are disproportionately larger when the inhaled concentrations are high than when the exposures are minimal. Based on the projected ambient levels of diesel particles, and the expected minute real-life lung particle accumulations, which may range several orders of magnitude below those of this study, it is concluded that the particle dose administered to the respiratory system by the increased penetration of diesel engines into the light-duty vehicle fleet will be well within the coping capacity of the human lung clearance mechanisms, and that the pulmonary defense system may be expected to be highly effective in protecting the lung against the inhaled diesel particles.

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A SUBCHRONIC STUDY OF THE EFFECTS OF EXPOSURE
OF THREE SPECIES OF RODENTS TO DIESEL EXHAUST

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INTRODUCTION

The projected increase in the use of diesel-powered automobiles for fuel economy has led to considerable concern over potentially adverse health effects from exposure to the emissions of these engines. The principal concern is with the particulate matter produced by diesel engines. Diesel exhaust contains 30 to 100 times more particulate than exhaust from a catalyst-equipped gasoline engine of comparable performance[1]. These particulates are small, readily respirable and are composed of a carbonaceous core to which a variety of toxic, mutagenic and carcinogenic chemicals are adsorbed. These chemicals, when extracted and concentrated, have been shown to be mutagenic and carcinogenic by a variety of in vitro and in vivo assays[2-5]. However, carcinogenic effects have not been demonstrated in chronic inhalation studies with experimental animal models, although the number of such studies has been limited until recently.

Early last year, a large inhalation exposure facility was constructed at the Southwest Foundation for Research and Education in order to investigate the potential health effects of exposure to diesel exhaust emissions. A chronic 15-month inhalation exposure study involving three dose levels of diesel exhaust particulate and three species of rodents was initiated in June, 1980. In preparation for this study, a subchronic pilot study was conducted at the high dose level of diesel exhaust particulate. Primary emphasis of this study was on potential carcinogenic effects, alterations in pulmonary ultrastructure and morphometry and proliferative changes within lung epithelium as a result of exposure to diesel exhaust. This paper reviews some of the results of this study.

MATERIALS AND METHODS

Diesel Exhaust Generation, Monitoring and Control

Diesel exhaust was generated by a 5.7 liter Oldsmobile engine operated continuously at 40 mph 20 hours per day, 7 days per week. Hydrocarbons, CO, CO_2, NO_x and particulates were monitored on a periodic basis and dilution was ad-

justed, as necessary, to maintain a 1500 $\mu g/m^3$ particulate level. The details of the generation, monitoring and control system, as well as results of analyses of particulates and gaseous components, are described by Springer[6].

Exposure Chambers

Two 8-ft cubical inhalation exposure chambers, constructed of stainless steel and glass, were used in this study (Figure 1). Each chamber, including its pyramidal top and bottom, had a volume of 14.5 m^3 and was configured to hold four stainless steel racks. Each rack (Figure 2) was designed to hold 14 compartmented stainless steel cages at 7 levels. The cages were equipped with removable feeders and the chambers, racks and cages were equipped with the components for an internal automatic watering system. Rat cages (Figure 3) were compartmented with removable dividers into 12 units and hamster and mouse cages were divided into 24 units. In this study, one rack each was assigned to rats and hamsters and two racks were used to hold mouse cages. With this arrangement, each chamber had the capacity for 168 rats, 336 hamsters and 672 mice.

Figure 1. View of four 8-ft cubical inhalation chambers with diluted diesel exhaust/air inlet systems on pyramidal tops of chambers. Pyramidal bottoms of chambers project into basement area. Each chamber has a volume of 14.5 m^3 and accomodates four cage racks. Access to chamber is provided by double doors on both sides of chamber.

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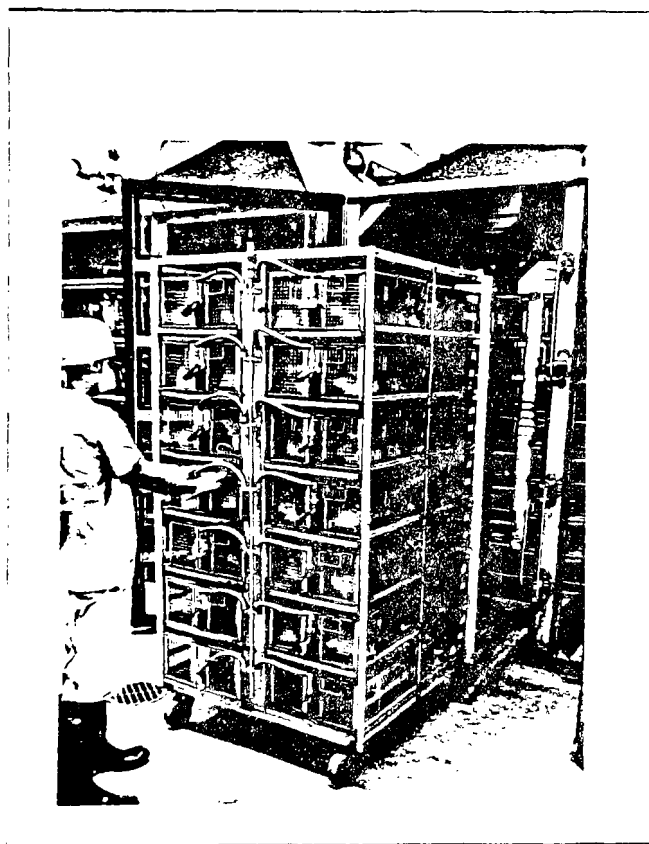
Figure 2.   Stainless steel rack holding 14 compartmented rat cages at 7 levels.
Each rack is equipped with an automatic watering system manifold and
trays for collection of urine and feces.

Figure 3. Stainless steel cage, compartmented with removable dividers to pro-
vide capability for 12 individually-housed rats. Each cage is
equipped with a central removable feeder and 12 lixits for automatic
water supply.

Animals

The animals used in this study were male Fischer 344 rats, Syrian golden
hamsters and Strain A/J mice. The rats and hamsters were obtained from Charles
River Breeding Laboratories and the mice were obtained from the Jackson Labora-
tory. All animals, at arrival, were approximately six weeks of age. Upon
receipt, the animals were quarantined for a minimum of two weeks prior to their
introduction into the exposure chambers. Standard Purina rodent laboratory chow
and water were available ad libitum during all phases of the study.

The Fischer 344 rats were supplied in one shipment and were randomly and
equally divided into experimental and control animals at the end of the quaran-
tine period. Hamsters and mice were received in two and four shipments, respec-
tively, at approximately weekly intervals. Therefore, hamsters were identified
in the study as Group 1 or 2 hamsters and mice as Group 1, 2, 3 or 4 mice. Each

group was randomly and equally divided, at the end of its quarantine period, into control and experimental animals which were simultaneously introduced into the two exposure chambers. Although exposure of each group of hamsters and mice began at different times, all groups of each species were exposed for the same length of time prior to removal from the chambers.

Experimental

In one of the two exposure chambers, the Fischer 344 rats, Syrian hamsters and Strain A/J mice were exposed to diluted diesel exhaust containing 1500 $\mu g/m^3$ of particulate 20 hours per day, 7 days per week. In the second chamber, control groups of the three animal species were exposed under the same regimen to the filtered air used to dilute the raw diesel exhaust. Temperature was maintained at 22 ± 2°C and relative humidity at 50 ± 10% within the chambers. The animals were exposed to the diluted diesel exhaust or air for a total of 90 days, with the exception of the hamsters which were removed from the chambers at the end of 86 days of exposure.

During the exposure period, rats and hamsters were caged individually. The mice, however, did not adjust satisfactorily to the automatic watering system when they were separated during the second week of quarantine. Therefore, dividers were removed from the compartmented cages and the mice were caged in groups of three. Difficulties in obtaining water were not subsequently observed.

Engine operation and exposure of animals continued on a 12:30 PM to 8:30 AM schedule during the exposure phase. During the four-hour daily engine shutdown, a number of data recording and animal care activities were accomplished. Upon inactivation of the engine, animal racks were removed to permit washing of chamber interiors with pressurized hot water and detergent. Feeders were emptied and fresh feed was added daily. Antibiotic impregnated cardboard for collection of feces and urine was replaced on alternate days to reduce ammonia formation within the chambers. Cages were rotated within each rack and racks were rotated within each chamber at regular intervals to equalize the exposure of animals. Animals were rotated to clean cages and racks on a weekly schedule.

Animals were observed daily and body weights were obtained biweekly. Sickly animals were identified for further evaluation and dead animals were removed and necropsied, unless tissue autolysis was too advanced. The disposition of all animals, including spontaneous or accidental deaths and scheduled removals, was recorded.

At the end of 86 days of exposure, the hamsters were removed from the two chambers. The mice and rats were removed after 90 days of exposure. From these

animals, thirty control and thirty experimental animals of each species were randomly selected, sacrificed by an intraperitoneal injection of pentobarbital sodium and exsanguinated by severing the femoral arteries. Each animal received a complete necropsy prior to removal of organs. Lungs were inflated in situ by tracheal instillation of a measured quantity of 10 percent buffered formalin prior to removal. Other organs collected for histopathological examination included the heart, liver, kidneys, spleen, squamous and glandular stomach, ileum, colon, duodenum, pancreas, urinary bladder, testicle, adrenal glands, lymph nodes, larynx, tongue, salivary glands and external ear. Heads, denuded of skin and muscle, were decalcified and three coronal sections were made of the nasal cavity and paranasal sinuses. A coronal section was also collected that included brain and middle ear, as well as bone, bone marrow, skeletal muscle and, often, Zymbol's glands and pituitary. Tissues were processed into paraffin by standard methods, cut at 6 microns and stained with hematoxylin and eosin.

Additional control and experimental animals of each species were randomly selected for study of pulmonary ultrastructure and morphometry and proliferative changes in lung epithelium. Lungs from these animals were inflated with Karnovsky's fixative for examination by light and electron microscopy.

The remaining animals were placed in a separate holding facility for post-exposure recovery studies. During the recovery period, animals were observed daily and weighed at monthly intervals.

When the Strain A/J mice reached nine months of age, 500 control and 500 experimental animals were randomly selected for study of the pulmonary adenoma response. This animal model was first applied as a quantitative bioassay of chemicals for carcinogenic activity in 1940 by Shimkin[7] and, since then, has been used to detect the carcinogenicity of a number of chemicals[8,9]. The mice were sacrificed by an intraperitoneal injection of pentobarbital sodium and the lungs were immediately removed, rinsed and placed in Tellyesniczky's fluid for fixation. Prior to counting of adenomas, the lobes of the lungs were severed from the primary bronchus to facilitate their examination. The adenomas were readily detected as pearly-white, discrete nodules, on the surface of the lungs, visible to the naked eye or under the dissecting microscope (Figure 4). Light microscopy was used to confirm a sampling of the nodules as adenomas. A positive control group of Strain A/J mice was used to verify the pulmonary adenoma response to chemical carcinogens. These animals were injected intraperitoneally with urethane at a dose of 1 mg/g of body weight at two months of age and sacrificed for measurement of tumor response four months later.

At the end of six months post-exposure, thirty control and thirty experi-
mental animals of each of the three species were sacrificed for histopatho-
logical examination. The protocol was the same as that followed in the histo-
pathology study at the termination of the 90-day exposure.

Figure 4. The lungs of a control (left) and an experimental (right) Strain
A/J mouse showing an adenoma on each lung. The experimental
animal was exposed to diesel exhaust containing 1500 μg/m^3 particu-
late for 90 days.

RESULTS

The results of the studies of pulmonary architecture and morphometry and of
proliferative changes within lung exithelium are not included in this paper.
These results will be published with the results of the chronic study at a later
date.

Mortality

Spontaneous deaths of animals during the three-month exposure and six-month
recovery periods consisted of 3 control and one exposed rats, 35 control and
31 exposed hamsters and 10 control and 7 exposed mice. These mortality data
indicate that exposure to diesel exhaust containing 1500 μg/m^3 of particulate

did not result in increased mortality rates in any of the 3 species.

Growth Patterns

The mean body weights of control and exposed animals of the 3 species are shown in Figures 5 through 11. Separate growth curves are shown for each of the two groups of hamsters and each of the four groups of mice since these groups differed slightly in age and weight when introduced into the chambers. No significant differences were found at the 0.05 significance level in any of the species when the average changes in body weight between successive weighing dates of exposed animals were compared with those of control animals, using a two sample t test[10].

COMPARISON OF RAT GROWTH CURVES

3-Month Exposure and Recovery
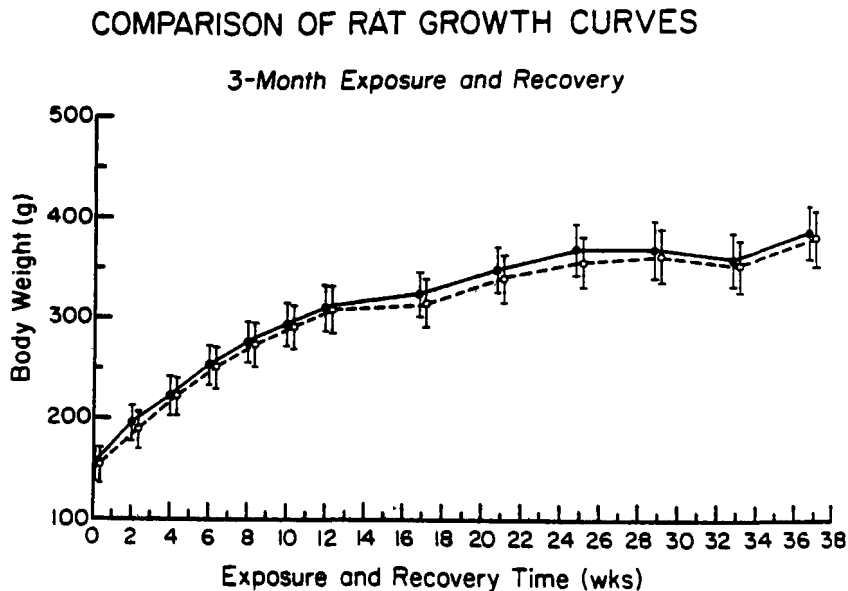
Figure 5.   Mean and S.D. of body weights of Fischer 344 rats exposed (•———•) to diesel exhaust containing 1500 µg/m³ particulate and controls (•--------•) during a three-month exposure and a six-month recovery period.

COMPARISON OF HAMSTER GROWTH CURVES
Group-1
3-Month Exposure and Recovery
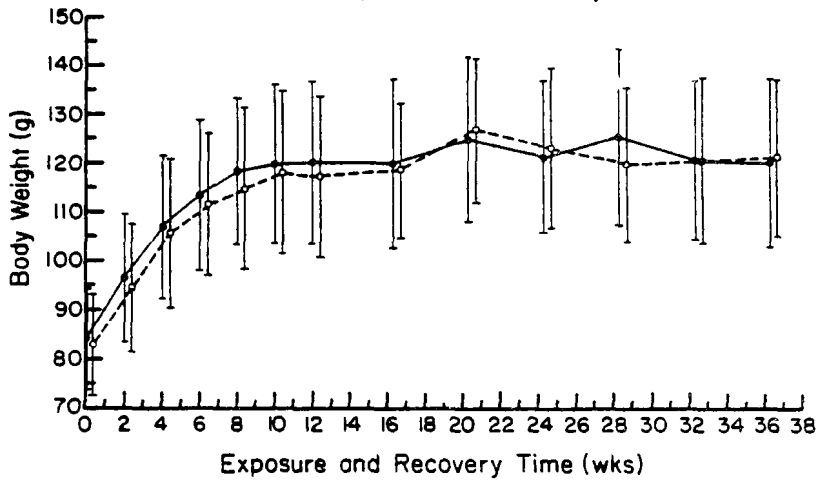
Figure 6. Mean and S.D of body weights of Syrian hamsters exposed (·————·)
to diesel exhaust containing 1500 µg/m³ particulate and controls
(·--------·) during a three-month exposure and a six-month recovery
period.

COMPARISON OF HAMSTER GROWTH CURVES
Group-2
3-Month Exposure and Recovery
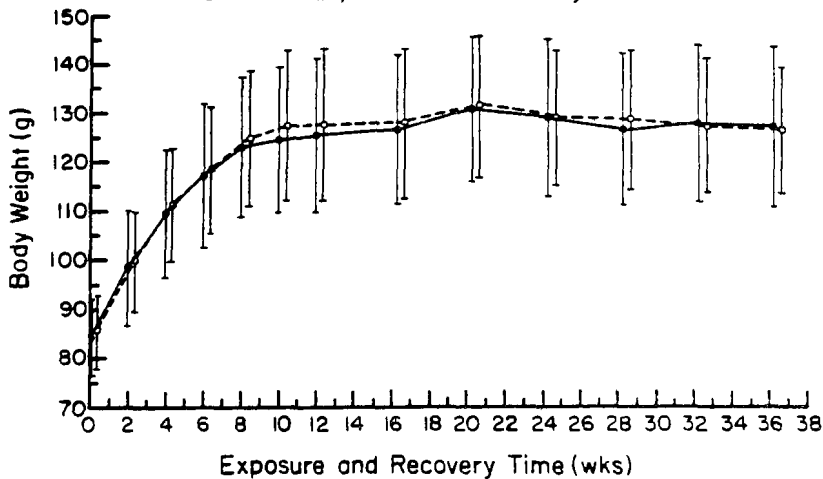
Figure 7. Mean and S.D of body weights of Syrian hamsters exposed (·————·)
to diesel exhaust containing 1500 µg/m³ particulate and controls
(·--------·) during a three-month exposure and a six-month recovery
period.

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COMPARISON OF MOUSE GROWTH CURVES
Group-I
3-Month Exposure and Recovery

Figure 8.   Mean and S.D of body weights of Strain A/J mice exposed (·————·) to diesel exhaust containing 1500 µg/m³ particulate and controls (·--------·) during a three-month exposure and a three-month recovery period.

COMPARISON OF MOUSE GROWTH CURVES
Group-2
3-Month Exposure and Recovery
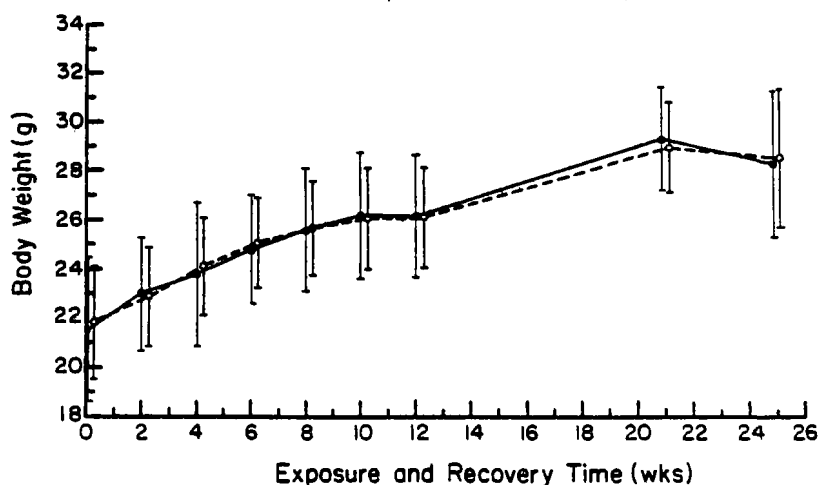
Figure 9.   Mean and S.D of body weights of Strain A/J mice exposed (·————·) to diesel exhaust containing 1500 µg/m³ particulate and controls (·--------·) during a three-month exposure and a three-month recovery period.

COMPARISON OF MOUSE GROWTH CURVES

Group - 3

3-Month Exposure and Recovery
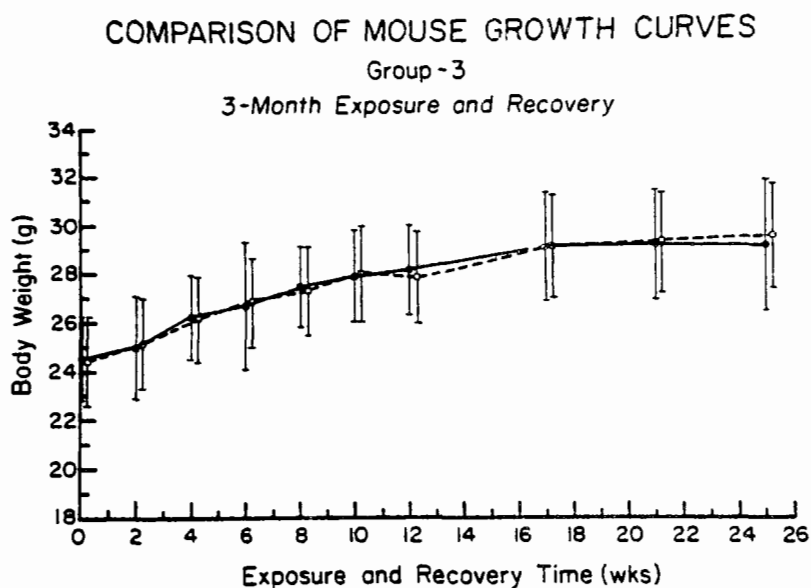
Figure 10. Mean and S.D of body weights of Strain A/J mice exposed (·————·) to diesel exhaust containing 1500 $\mu g/m^3$ particulate and controls (·————·) during a three-month exposure and a three-month recovery period.

COMPARISON OF MOUSE GROWTH CURVES

Group - 4

3-Month Exposure and Recovery
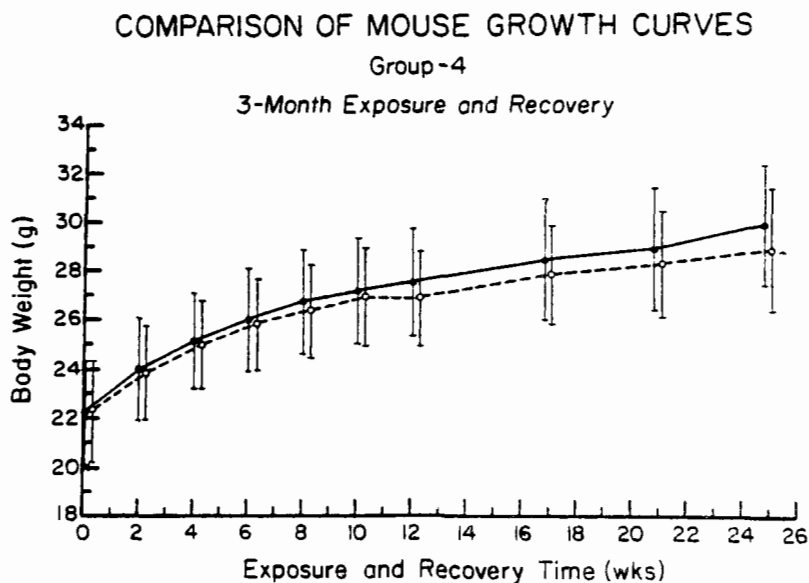
Figure 11. Mean and S.D of body weights of Strain A/J mice exposed (·————·) to diesel exhaust containing 1500 $\mu g/m^3$ particulate and controls (·————·) during a three-month exposure and a three-month recovery period.

TABLE 1

EFFECT OF EXPOSURE TO DIESEL EXHAUST (1500 $\mu g/m^3$ PARTICULATE) FOR THREE MONTHS ON
ORGAN WEIGHTS AND ORGAN-TO-BODY WEIGHT RATIOS IN MALE FISCHER 344 RATS[a]

	Body Weight (g)	Liver		Kidneys		Spleen		Heart	
		g	g/100 g	g	g/100 g	g	g/100 g	g	g/100 g
Control	314.0	10.84	3.46	2.07	0.65	0.61	0.20	0.88	0.28
n=26	±19.0	±0.76	±0.22	±0.14	±0.04	±0.06	±0.02	±0.09	±0.03
Exposed	315.1	10.65	3.42	2.09	0.66	0.61	0.19	0.87	0.28
n=29	±22.3	±1.04	±0.25	±0.16	±0.04	±0.07	±0.02	±0.12	±0.04

[a] Values are Mean ± S.D.

TABLE 2

EFFECT OF EXPOSURE TO DIESEL EXHAUST (1500 μg/m^3 PARTICULATE) FOR THREE MONTHS
ON ORGAN WEIGHTS AND ORGAN-TO-BODY WEIGHT RATIOS IN SYRIAN HAMSTERS[a]

	Body Weight (g)	Liver		Kidneys		Spleen		Heart	
		g	g/100 g	g	g/100 g	g	g/100 g	g	g/100 g
Control	121.1	4.26	3.51	0.95	0.79	0.13	0.12	0.51	0.42
n=30	±18.8	±0.88	±0.35	±0.11	±0.10	±0.05	±0.03	±0.08	±0.08
Exposed	126.2	4.65	3.67	1.00	0.81	0.15	0.12	0.53	0.43
n=30	±18.6	±1.11	±0.60	±0.19	±0.13	±0.05	±0.04	±0.06	±0.07

[a] Values are Mean ± S.D.

Organ Weights/Organ-to-Body Weight Ratios

Organ weights (liver, kidneys, spleen and heart) and organ-to-body weight ratios of control and experimental Fischer 344 rats and Syrian hamsters at the termination of the three-month exposure period are shown in Tables 1 and 2, respectively. There were no significant differences at the 0.05 significance level in any of the organ weights or organ-to-body weight ratios in either species when means of exposed animals were compared with control means using a two sample t test[10].

Lung weights and lung-to-body weight ratios of control and exposed Fischer 344 rats and Syrian hamsters are shown in Table 3. In both species, both absolute and relative (related to 100 g body weight) lung weights were slightly higher in animals exposed to diesel exhaust than in control animals. These differences were not significant at $p \leq 0.05$, using a two sample t test[10], except for the difference in relative lung weights between control and exposed rats. Increased lung weights after exposure to 1500 $\mu g/m^3$ particulate diesel exhaust have been reported previously, and were not the result of water accumulation[11].

TABLE 3

EFFECT OF EXPOSURE TO DIESEL EXHAUST (1500 $\mu g/m^3$ PARTICULATE) ON LUNG WEIGHTS AND LUNG-TO-BODY WEIGHT RATIOS IN MALE FISCHER 344 RATS AND SYRIAN HAMSTERS[a]

	Body Weight (g)	Lung Weight (g)	Lung/Body Weight (g/100 g)
RATS[b]			
Control n = 15	307.4 ± 20.1	1.03 ± 0.08	0.33 ± 0.03
Exposed n = 15	296.4 ± 21.7	1.07 ± 0.08	0.36 ± 0.03[d]
HAMSTERS[c]			
Control n = 15	121.9 ± 17.4	0.54 ± 0.07	0.45 ± 0.05
Exposed n = 15	125.9 ± 13.4	0.59 ± 0.08	0.47 ± 0.06

[a] Values are Mean ± S.D.
[b] Exposure duration 13 weeks
[c] Exposure duration 10 weeks
[d] Significantly different from control, $p \leq 0.05$

Strain A/J Mouse Pulmonary Adenoma Response

The results of the Strain A/J pulmonary adenoma response study are shown in Table 4. Lungs of 458 control and 485 exposed animals were examined for adenomas. This difference in the number of control and exposed lungs resulted from the loss of lobes from several lungs during their storage in fixative. The mean number of tumors per mouse was 0.38 in the control group and 0.45 in mice exposed to diesel exhaust. These values were not significantly different at $p \leq 0.05$ when compared, using a two sample t test[10]. Both the control and exposed values are somewhat higher than the 0.28 mean number of spontaneous tumors in untreated Strain A mice at nine months of age reported in a review by Shimkin and Stoner[9]. The prevalence of adenomas in the control group was 31.4% and 34.2% in the exposed group. These values were not significantly different at $0.25 < p < 0.75$ when compared, using the Chi Square test[10]. In contrast to the control and exposed animals, the positive urethane control group had a 100% incidence of adenomas and a mean number of 22.6 tumors per mouse.

Pathology

Results of macroscopic and microscopic examinations of lungs of animals exposed for three months to diesel exhaust containing 1500 $\mu g/m^3$ of particulate were consistent in the three species. Gross examination revealed gray to black discoloration of the lungs and mediastinal lymph nodes. The pigment was deposited both diffusely and in focal accumulations, producing a grayish overall appearance of the lungs with scattered, denser black areas. The deeply black-pigmented lymph nodes indicated at least partial clearance of the particulate from the lungs via the lymphatics to regional lymph nodes.

Microscopic examination revealed no anatomic changes in the upper respiratory tract (Figure 12). There was no deposition of particulate and the mucociliary border was normal in appearance. In the lungs, particulate was observed diffusely deposited throughout (Figure 13). Most of the particulate was in macrophages but some was free as small aggregates on alveolar and bronchiolar surfaces. The particulate-laden macrophages were often in large accumulations near the entrances of the lymphatic drainage and respiratory ducts (Figure 14). Associated with the larger accumulations, there was a minimal increase in the thickness of the alveolar walls but the vast majority of the particulate elicited no response.

TABLE 4

PULMONARY ADENOMA RESPONSE OF STRAIN A/J MICE EXPOSED TO
DIESEL EXHAUST (1500 $\mu g/m^3$ PARTICULATE) FOR THREE MONTHS

	Number of Mice	Number of Mice According to Number of Lung Tumors/Mouse							Total Number of Tumors	Mean Number of Lung Tumors/mouse[a]	Percent Prevalence
		0	1	2	3	4	13	>13			
Control	458	314	116	24	4	0	0	0	176	0.38 ± 0.03	31.4
Exposed	485	319	133	26	5	1	1	0	217	0.45 ± 0.04	34.2
Urethane (1 mg/g)	18	0	0	0	0	0	0	18	407	22.6 ± 1.90	100

[a] Mean ± S.E.

Figure 12. The mucoliliary border of a mouse nasal turbinate at the end of three-months exposure to diesel exhaust. The cilia are normal and there is no deposition of particulate in the border.

Figure 13. Diffuse deposition of particulate in the lung of a rat at the end of three-months exposure to diesel exhaust. Most of the particulate is in macrophages, some is free in small aggregates.

Figure 14. Large accumulations of particulate-laden macrophages in a rat lung at end of three-months exposure to diesel exhaust. Macrophages are aggregated near entrances of lympahtic drainage and respiratory ducts.

After six months of recovery, the lungs of all three species had considerably less pigment and the pigment was largely in focal accumulations (Figure 15). Intervening tissue was usually pink and normal appearing. The lymph nodes were still deeply pigmented, indicating the continuing process of clearance of particulate by the lymphatics. Microscopically, it was evident that much, if not most, of the pigment had been cleared from the lung. Of the three species, the hamster appeared to have the greatest capability for removal of particulate (Figure 16). The mouse appeared to have the least capability, but, even in this species, most of the particulate had been cleared during the recovery period. Another apparent species difference was the tendency of the rat to form aggregates of particulate-laden macrophages beneath the pleural surface of the lungs (Figure 17). There are lymphatics on this surface and, possibly, the accumulation of macrophages in this area represents the process of particulate removal.

Miscellaneous lesions were observed in a number of organs in each species but the incidence of these lesions was approximately the same in control and exposed animals. There was no indication that exposure to diesel exhaust resulted in pulmonary pathology, other than the accumulation of particulate, or in any pathological changes in other organs.
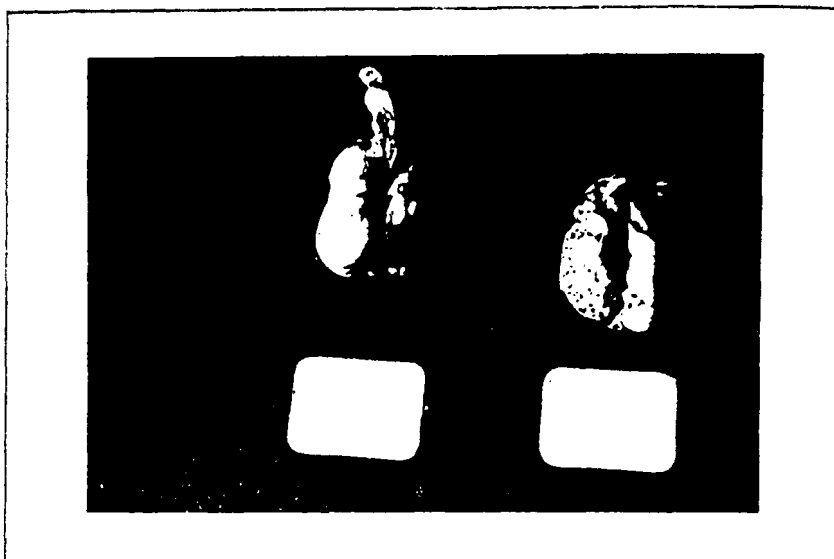
Figure 15. A lung from a control (left) and an exposed (right) Fischer 344 rat
at six-months recovery following a three-month exposure to diesel
exhaust. Most of the particulate was cleared during the recovery
period.

Figure 16. The lung of a Syrian hamster at six-months recovery following a
three-month exposure to diesel exhaust. The very small amounts of
remaining particulate indicate the effective clearance capability of
this species.

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Figure 17. Accumulations of particulate-laden macrophages beneath the pleural surfaces of a rat lung at six-months recovery following a three-month exposure to diesel exhaust. Except for these accumulations, the lung has a normal appearance.

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    nati, Ohio, pp. 465-479.

PULMONARY FUNCTION TESTING OF RATS CHRONICALLY EXPOSED TO DILUTED DIESEL EXHAUST FOR 612 DAYS

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Diesel engine emissions contain particulate matter composed of a multitude of organic compounds, and of a size that may be readily inhaled and retained by the lung. It thus has the potential for interacting with the deep lung, and possibly altering the structure and function of this organ. The study was designed to address the question of whether the chronic inhalation of diluted diesel exhaust may affect pulmonary function. Twenty-five Fischer-344 rats were exposed to diesel exhaust, diluted with clean air at a ratio of 1:15 (particulate concentration = 1500 $\mu g/m^3$) for 20 hrs/day, 5-1/2 days/week, for 612 days. Twenty-five control animals were treated in a similar manner, but exposed to clean filtered air. Noninvasive pulmonary function testing, which produced no apparent harmful effects, were performed on the animals at mean times of 11, 23, 30, 38, 51, 65, and 87 weeks on the exposure regimen. Animals were anesthetized (Halothane, 5%), transorally intubated, and placed in a plethysmograph. Measurements of lung volumes and flows were made while the animals were spontaneously breathing and during forced expiratory maneuvers. Functional residual capacities were computed using the Boyles law principle. All data was normalized by each animal's own forced vital capacity in order to compensate for animal growth and interindividual variability. Through the first year of testing, no significant differences between the two groups were found for any of the measured parameters. During the second year of exposure, seven of the eighteen measured indices displayed a significant difference as indicated by analysis of variance. The normalized functional residual capacity (FRC) and its component volumes – expiratory reserve (ER) and residual volume (RV) – maximum expiratory flows at 40% (MEF_{40}) and 20% (MEF_{20}) of the lung volume remaining, and the forced expiratory volume in 0.1 sec ($FEV_{.1}$) were all greater in the diesel-exposed animals. The normalized inspiratory capacity (IC) was significantly larger in the control group, but the test point of greatest difference for this parameter does not exceed 4%, and at all other test points is 2% or less. The statistical significance of the IC is interpreted as being the result of the very low variability of this parameter, rather than a result of a clinically important change in the pulmonary function. The significantly larger values for relative normalized FRC, ER, and RV in the diesel-exposed animals could be indicative of chronic obstructive lung disease and are similar to the changes seen in other studies in which an emphysema-like condition was induced in rats by intratracheal instillation of elastase, as well as in reported clinical data on chronic obstructive lung disease. However, this interpretation of the changes in the three lung volumes is contradicted by the MEF_{40}, MEF_{20}, and $FEV_{.1}$ values. If chronic lung disease was occurring, these parameters would be expected to decrease in the

diesel-exposed animals compared to the controls. The fact that they increased in the diesel group, suggesting an improvement in airway caliber, is not consistent with what would be expected based on studies reported in the literature for human pulmonary disease. In conclusion, the majority of the measured parameters did not differ significantly between the control and diesel-exposed groups, and while one cannot exclude the possibility that the differences that were observed in this experiment between the diesel-exposed and clean air controls may be attributable to the chronic inhalation of the diesel exhaust, the results are not consistent with documented clinical findings on chronic lung disease.
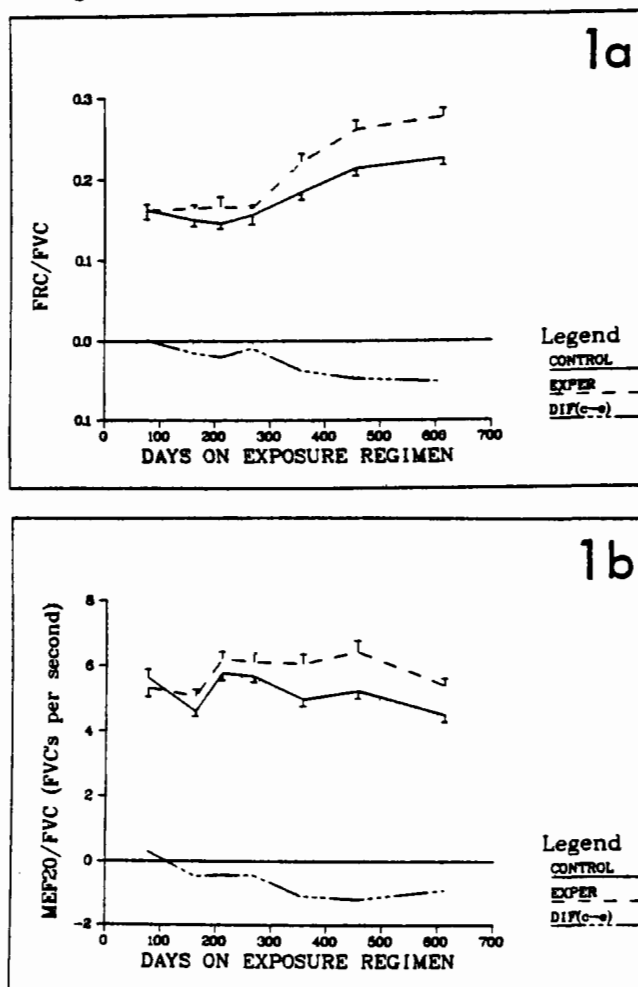
Figure 1a   Functional residual capacity (FRC) of diesel exposed (-----) and control (———) animals as a fraction of each animal's forced vital capacity (FVC). The bottom curve (— - - - —) is the difference between the means of the two groups (control - experimental).

Figure 1b   Normalized maximum expiratory flow rate at 20% of vital capacity (MEF_{20}). Legend same as Figure 1a.

PULMONARY FUNCTIONAL RESPONSE IN CATS
FOLLOWING TWO YEARS OF DIESEL EXHAUST EXPOSURE

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INTRODUCTION:

Both NIOSH and the EPA have responsibilities to assess potential health
effects from exposure to diesel engine exhaust. These responsibilities are
mandated under both the OSH and Clean Air Acts, respectively. While EPA's
responsibility relates to the general population, NIOSH's responsibility is
to the worker, especially those at high exposure risk in the mining and
transportation industries.

The advantages of diesel power are manyfold; however, the prime motivation
for the use of diesels relates to the reduced cost of operation due to longer
engine life, decreased maintenance, cheaper fuel, increased specific energy
(2.4 X that of gasoline), and increased safety. The increase in safety is
related to diesel fuel's lower volatility, flash point with a concomitantly
reduced explosive hazard when compared to gasoline. As a result of our
proximal locations in Cincinnati, the NIOSH, DBBS Cardiopulmonary Laboratory
participated with EPA-HERL Laboratory to apply comprehensive pulmonary
function testing to the cats exposed to the controlled diesel atmospheres.

BACKGROUND:

Evaluation of pulmonary function responses to experimentally controlled
diesel exhaust is primarily confined to investigation of the past few years.
Previous studies are largely epidemiologic and while they are important
guides, they lack qualitative and quantitative characterization, essential
for analytical, dose-response toxicology.

At the last symposium on Health Effects of Diesel Engine Emissions, Gross
reported pulmonary function findings in Fischer 344 rats following 38 weeks
of exposure at 1500 μg/m^3 for 5-1/2 days/week, 20 hrs/day(1). He found no
differences in mechanical properties, lung volumes, or dynamic ventilatory
performance. In a later report presented at the Society of Toxicology meet-
ing (1980), he found higher function residual capacity and maximum expiratory

flow rates at 20% of vital capacity in the diesel exposed group. These results are not interpretable or consistent with dysfunction. His conclusion was that no clinically important alterations were observed after 65 weeks of exposure(2).

In another report, O'Neil et al., reported on functional and morphological consequences of diesel exhaust exposure in mice following 3 months of exposure to a 1:18 dilution (diesel and air) of diesel exhaust with particulate levels of 6.4 mg/m^3. He found no statistically significant findings in lung volume or diffusing capacity(3).

In a third report on functional responses in rodents, Vinegar et al., described significant decrements in vital capacity, residual volume, and diffusing capacity in Chinese hamsters exposed to a dilution of diesel exhaust with a particulate level of 6.4 mg/m^3(4).

In the only larger animal exposure, we reported findings from one year of exposure in cats(5). The exposure was conducted at the Cincinnati EPA, HERL laboratory. A 1:18 dilution diesel exhaust with 6.4 mg/m^3 was used. Our results indicated no response in mechanical properties, lung volumes, distribution, diffusing capacity or ventilatory performance following one year of exposure.

METHODS:

In an effort to enhance functional response characteristics, the diesel exposure was increased by decreasing the dilution ratio from 1:18 to 1:9 after the first year. A summary of exposure is provided in Table I. During this second year, 19 of the original 21 male cats were exposed to 11.7 mg/m^3 particulate, 4.37 ppm nitrogen dioxide, 5.03 ppm sulfur dioxide, and 33.30 ppm carbon monoxide with total hydrocarbons of 7.72 ppm. Twenty male cats served as controls. All cats were young adult males obtained from Liberty Laboratories and were born and maintained in a disease-free environment and inbred for several generations. They were of uniform size (3.63 ± 0.46 kg) and within two weeks of the same age.

Prior to pulmonary function testing, the cats were fasted for one day. The testing followed 18-20 hours of no diesel exposure. On a random schedule, the cats to be tested were anesthesized with ketamine and acepromizine at a dose of 42 mg/kg. Following induction of the anesthesia, an esophageal balloon was placed in the lower third of the esophagus and an 18-22 F endotracheal tube (largest possible) was inserted into the trachea with the aid of a laryngoscope. All testing, except compliance (CL) and resistance (RL) was performed with the cats placed in the prone position in a variable pressure plethysmograph. CL and RL tests were performed with the cats recumbent to facilitate measurement of transpulmonary pressure. Figure I shows the general pulmonary testing situation diagrammatically.

Pulmonary mechanics were obtained from simultaneous volume, flow and transpulmonary pressure. Dynamic compliance (CL_{dym}) was measured from volume and transpulmonary pressure at points of no flow. Average flow resistance ($RL_{ave.flow}$) was measured from change in transpulmonary pressure at equal

volumes, divided by the sum inspiratory and expiratory flow. All mechanics were obtained while the cats were spontaneously breathing (15-25 breaths/min). The pulmonary function tests requiring breathing maneuvers [lung volumes, forced expiratory flows (FEF% Vol), diffusing capacity ($DL_C{}^{18}{}_O$), nitrogen washout (ΔN_2), and closing volume (CV)] were performed using a variable pressure plethysmographic chamber previously described(6). The methods of Brashear et al.(7) and Mitchell et al.(8) were combined to obtain values for $DL_C{}^{18}{}_O$ and total lung capacity (TLC). The calculations for $DL_C{}^{18}{}_O$ were performed according to the methods described by Wagner et al., for $C^{18}O$(9). All gas analyses were done using a respiratory mass spectrometer (Perkin-Elmer MGA1100). Distribution was studied using the single-breath nitrogen washout and closing volume adapted from the human methods described by Buist and Ross(10). All data was tested statistically by nonparametric, Kruskal-Wallis one-way rank analysis of variance.

RESULTS:

Following the first year of exposure, no significant differences were found in mechanical properties, diffusing capacity, uniformity of distribution or ventilatory performance. In contrast to the negative findings following the first year, we now have clearly defined responses at the end of two years. Table II presents all parameters studied for control and exposed cats contrasting the values for one and two years.

The reduction in inspiratory capacity, vital capacity, and total lung capacity with normal values for ventilatory function (mechanics of breathing) indicates that a lesion is present which restricts breathing but does not cause airway obstruction or loss of elasticity. This restrictive disease found in this study is compatible with a diagnosis of pulmonary fibrosis of the interstitial or intraalveolar type. Concurrent status may include chronic inflammation, interstitial edema, or vascular engorgement. Additional support for the diagnosis of interstitial disease is the finding of impaired diffusing capacity. Distribution of this disease appears nonuniform as indicated by the significantly elevated nitrogen washout values for the exposed group.

DISCUSSION:

Pathological description of pulmonary responses to diesel exhaust at similar concentrations has been previously characterized(11,12). The observations include: (1) marked accumulation of black pigment laden macrophage in the interstitum localizing around blood vessels and respiratory bronchioles; (2) hyperplasia of the alveolar lining cells with focal thickening of the interstitium; (3) interstitial pneumonitis; (4) traces of, or no emphysema or peribronchiolitis. While the pathological examination of the cats' lung not complete, the above description is consistent with our physiologic finding of restrictive lung disease.

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Table I. EXPOSURE CHAMBER COMPONENT CONCENTRATIONS, STUDY AVERAGES

		Weeks #1-61	Weeks 62-124
Dilution Factor (air:diesel)	DF	18.16 ± 1.72:1	9.37 ± 1.13:1
Particulate Mass, mg/m^3	M	6.34 ± 0.81	11.70 ± 0.99
Nitrogen Oxides	NO_x		
Nitric Oxide, ppm	NO	11.64 ± 2.34	19.49 ± 3.80
Nitrogen Dioxide, ppm	NO_2	2.68 ± 0.80	4.37 ± 1.19
Sulfur Dioxide, ppm	SO_2	2.12 ± 0.58	5.03 ± 1.03
Total Hydrocarbons, ppn	THCcorr.	4.15 ± 0.97	7.22 ± 0.85
Carbon Monoxide, ppm	CO	20.17 ± 3.01	33.30 ± 2.94
Carbon Dioxide, %	CO_2	0.30 ± 0.04	0.52 ± 0.04

Table II. PULMONARY FUNCTION PARAMETERS COMPARING THE CONTROL
GROUP TO THE DIESEL EXPOSED GROUP AFTER 1 YEAR AND 2 YEARS

Mechanical Properties	One Year Exposed	One Year Control	Two Years Exposed	Two Years Control
$CL_{dyn.}$	23.5 ± 7.2	23.7 ± 9.3	27.5 ± 4.9	26.2 ± 7.1
$RL_{ave.flow}$	10.7 ± 4.6	10.3 ± 4.4	5.6 ± 3.2	5.7 ± 2.3
Lung Volumes				
TLC	415 ± 56.0	449 ± 74.5	428 ± 56.3↓*	484 ± 68.3
FVC	348 ± 43.5	368.9 ± 42.1	369 ± 42.3↓*	410 ± 57.6
FRC	158 ± 35.6	165 ± 42.2	145 ± 26.2↓*	163 ± 36.9
ERV	69 ± 24.6	67 ± 19.0	79 ± 24.0	83 ± 34.5
RV	86 ± 36.9	104 ± 37.7	67 ± 14.3	80 ± 28.2
RV/TLC%	20.3 ± 6.9	22.7 ± 5.9	15.6 ± 1.9	16.4 ± 4.5
IC	279 ± 44.8	301 ± 49.6	291 ± 44.1↓*	328 ± 58.6
Ventilatory Performance				
$FEV_{.5}$%	84.3 ± 8.4	81.6 ± 6.4	86.9 ± 6.1	86.9 ± 5.9
PEFR	1016 ± 185	1042 ± 174	887 ± 98 ↓*	952 ± 110.7
FEF_{50}	728 ± 196	761 ± 160	802 ± 125	864 ± 121
FEF_{25}	490 ± 186.8	481 ± 199.5	518 ± 154	574 ± 153
FEF_{10}	196 ± 107.4	222 ± 156.8	223 ± 109	234 ± 102
$FEF_{40\%TLC}$	486 ± 252.6	557 ± 248.0	586 ± 173	625 ± 213
Diffusion				
DLCO	1.18 ± .43	1.22 ± .40	0.89 ± .27 ↓*	1.01 ± .14
Distribution and Closing Volume				
%N_2/25%/VC	0.32 ± .20	0.29 ± .30	0.39 ± .27 ↑*	0.21 ± .181
CV	25.6 ± 13.4↓*	36.0 ± 16.1	27 ± 17.6	25 ± 19.3

*Statistically significant $P < 0.05$
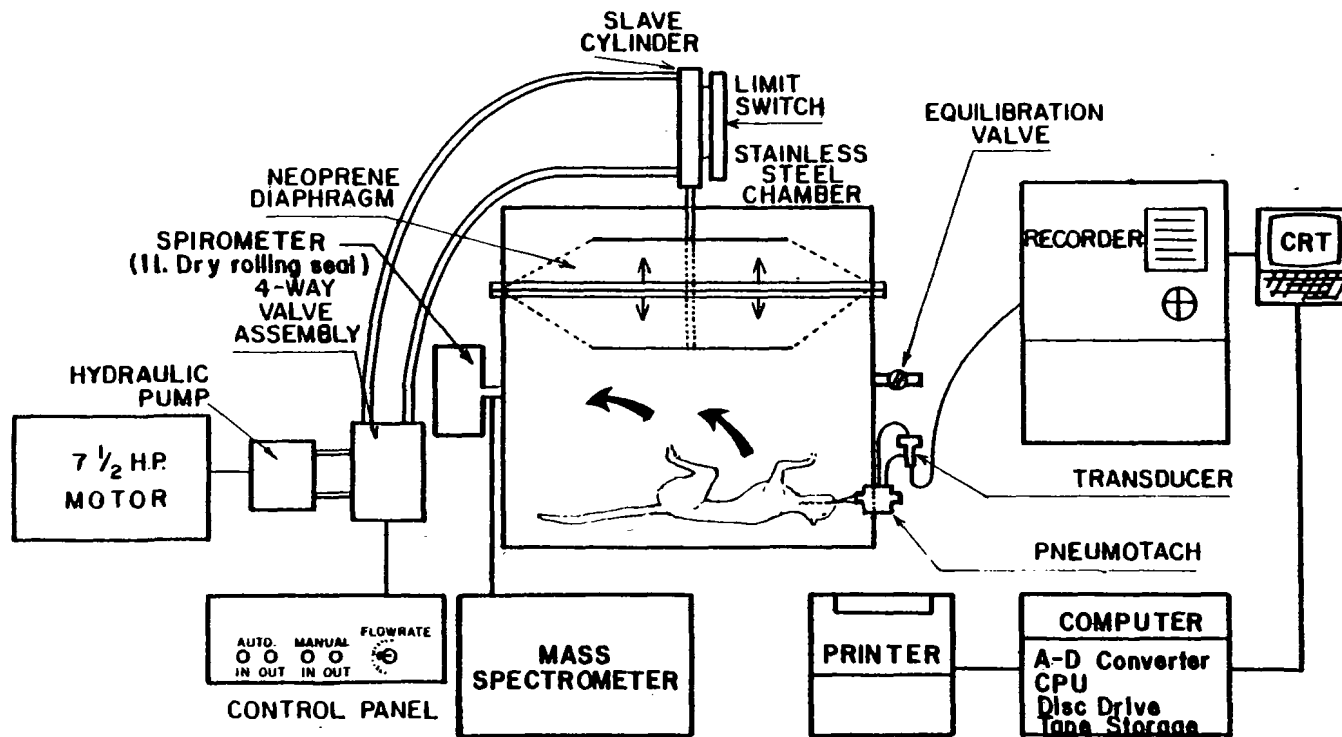
Diagram of Pulmonary Function Lab.

DEPOSITION AND RETENTION OF SURROGATE AND ACTUAL DIESEL PARTICLES

by

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INTRODUCTION

Data on deposition and retention of diesel particles are an important need in assessing their toxicological impact. Deposition and retention experiments were carried out with ^{67}Ga aggregate aerosols (surrogate diesel particles) in Beagle dogs to provide good estimates for human deposition. Experiments were also carried out with these particles in Fischer-344 rats to provide comparative information in a small laboratory animal and also to estimate lung burdens in chronic exposures to diesel exhaust. A method was developed to quantitate actual diesel soot burdens in rats exposed to diesel exhaust. These values were found to be comparable to these predicted from the surrogate particle deposition experiments.

METHODS

Aggregated particles of ^{67}Ga$_2$O$_3$, 0.02 and 0.1 µm volume median diameter (VMD), were produced using heat treatment of ^{67}Ga tetramethyl-heptanedione using methods described previously (1). Ten Beagle dogs from the Institute's colony were exposed for 1/2 hr in a nose-only exposure unit equipped with a plethysmograph for pulmonary function monitoring. Whole body counting and gamma camera analysis were used to measure total amounts of activity deposited and their regional distribution. A total of 144 Fischer-344 rats were exposed 5 hrs/day for either 1 or 3 days in the same style multi-tiered exposure chamber used in the chronic diesel exhaust exposure study. Whole body counting and also sacrifice and tissue counting methods were used to measure deposited radioactivity.

The diesel exhaust exposure system consisted of an Oldsmobile 5.71 diesel engine connected to a dynamometer with the engine load and speed determined by an analog control system. The engine was operated on a 7 mode urban cycle and the fuel was U.S. Department of Energy Reference fuel 8007 (Phillips Chemical Co.). The entire engine exhaust was diluted in a large stainless steel tunnel and then further diluted in 3 stages with filtered air which produced average particulate concentrations of 4150, 990 and 200 µg/m^3. The diluted exhaust was drawn through the 2.2 m^3 exposure chambers (Hazleton Systems, Inc., Aberdeen, MD) at 560 l/min. The exposure schedule was 7 hrs/day, 5 days/week.

Laboratory reared Fischer-344 rats were 12-13 weeks old at the initiation of the study. They were exposed for a cumulative period of 541 hours over 18 weeks. At the end of this period 8 rats from each exposure group were sacrificed. Lungs were removed, homogenized, and centrifuged to produce a cell pellet. This tissue pellet was dissolved in 1 ml H_2O and 2 ml tetramethylammonium hydroxide. The remaining "soot" particles were suspended in 5 ml H_2O. Light absorbance at 690 nm was measured and compared against standards prepared from known weights of diesel particles collected on filters from the dilution tunnel of the inhalation exposure system. For these collections the same engine cycle was used as for the animal exposures.

RESULTS AND DISCUSSION

Table 1 shows the mean total and regional deposition values measured in the Beagle dogs for the 0.02 and 0.01 μm $^{67}Ga_2O_3$ particles. Deposition was higher in all compartments for the 0.02 μm particles. Despite the overall high variability in deposition 9 of the 10 dogs had higher deposition at 0.02 μm than 0.1 μm and the difference was statistically significant ($P < .05$). Although most of the material was deposited in the pulmonary region, deposition in the nasopharyngeal, and tracheobronchial regions was becoming increasingly significant as particle size decreased. Figure 1 shows pulmonary deposition of the 0.02 and 0.1 μm particles is in good agreement with the trends in deposition observed previously in humans (2,3) and Beagle dogs (4) at larger particle sizes. The deposition values are lower than predicted by the ICRP Task Group on Lung Dynamics (5) but are in good agreement with predictions by Yeh and Schum (6) and also Yu (7).

Deposition of the 0.1 μm $^{67}Ga_2O_3$ particles was somewhat lower in rats than had been found in dogs. Lung deposition (bronchial and pulmonary) was estimated to be 15 ± 3%. This estimate was based on measured lung burdens and assuming minute volumes as measured in free standing Fischer-344 rats at this Institute. Absolute lung deposition was found to be 2.4 μg/hr for a particle mass concentration of 1000 μg/m^3. Using these initial deposition values and a measured lung half-life of 75 days for $^{67}Ga_2O_3$, lung burdens could be calculated for various exposure periods. Table 2 shows the predictions for the exposure period and concentrations experienced by the animals in the chronic diesel exhaust exposures.

Measured diesel soot burdens following the 18 week exposure are also shown in Table 2. The observed values were in good agreement at the highest exposure level but overestimated deposition at the two lower levels. Either deposition was higher or clearance was slower at the high exposure level compared to the lower levels.

The degree of agreement between predicted and observed burdens shows that deposition and retention behavior of the surrogate particles is similar to actual diesel particles. The observed long-term retention of $^{67}Ga_2O_3$ in rats was very similar to the 62-day half-time reported by Chan, et al., (8) following acute exposures to ^{14}C-labeled diesel particles. These observations give confidence to extrapolations made from observations with

$0.1 \mu m \ ^{67}Ga_2O_3$ aggregate particles. These data do show that pulmonary deposition is relatively high for diesel particles, and retention times are relatively long.

ACKNOWLEDGEMENTS

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PULMONARY DEPOSITION
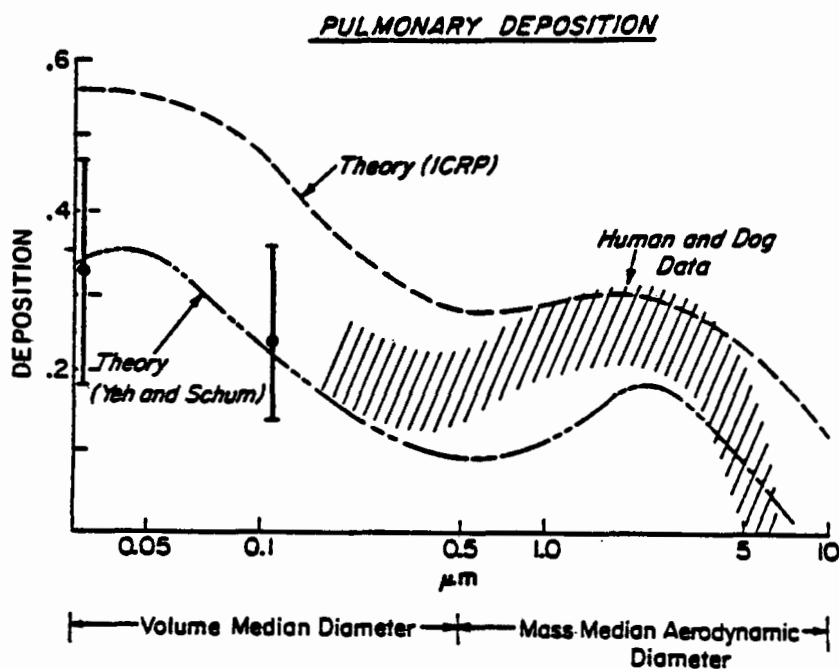
Figure 1.   Comparison of mean pulmonary deposition (± S.D.) of
             0.1 μm irregularly shaped polydisperse aerosols (●) with
             that of spherical monodisperse aerosols. The mid range of
             deposition data (///) taken from human experiments (Lippmann
             1977 (2); Chan and Lippmann, (3)) and dog experiments
             (Cuddihy et al. 1973, (4)) is shown. Also shown are
             theoretical predictions for depositions in humans by ICRP
             Task Group on Lung Dynamics (5) and also by Yeh and Schum (6).

Table 1. Comparison of Total and Regional Deposition of $^{67}Ga_2O_3$ Particles in Beagle Dogs

Compartment	Particle Size	
	0.1 μm	0.2 μm
Nasopharyngeal	7%	9%
Tracheobronchial	7%	12%
Pulmonary	25%	32%
TOTAL	39%	53%

Table 2. Lung Burdens of Diesel Soot in Rats One Day After 18 Weeks Exposure to Diluted Exhaust

Average Aerosol Concentration[a] (μg/m³)	Lung Burden (μg)	
	Predicted	Observed
200 ± 70	100	36 ± 8
990 ± 390	500	224 ± 39
4150 ± 1460	2100	1926 ± 335

[a] ± S.D. of average daily values.

LUNG CLEARANCE OF RADIOACTIVELY LABELLED
INHALED DIESEL EXHAUST PARTICLES

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The fate of inhaled diesel exhaust particles was studied in male Fischer 344 rats using radioactive diesel particles generated from a single cylinder diesel engine and tagged in the insoluble particulate core with radioactive ^{14}C. The particle size, extractability and ^{14}C distribution in the diesel exhaust were characterized at various engine load conditions. At full load, only 1% of the radioactivity of diesel particles was extractable by dichloromethane and the mass median aerodynamic diameter of the particles was 0.12 μm [J. Appl. Tox., 1(2):77-82, 1981]. Radioactive carbon dioxide was removed from the exhaust by a diffusion scrubber prior to exposures via a "nose-only" inhalation chamber. Rapid elimination of the inhaled $^{14}CO_2$ from the blood in the expired air, and urine of test animals indicated that the correction for increased radioactivity due to the inhaled carbon dioxide was necessary only for the initial deposition measurement. The amount of blood and its contribution of $^{14}CO_2$ activity was accounted for in the excised organs.

Test animals were exposed to diluted diesel exhaust at two particulate concentrations with similar total inhaled dose (7000 μg/m³ for 45 minutes, and 2000 μg/m³ for 140 minutes) and had comparable deposition efficiencies. After the exposure, the animals were housed in a clean air environment and the clearance of the radioactively tagged particles was determined over an extended period of time. Up to the period of 28 days after exposure, no significant difference in particle clearance has been observed. This indicates that thus far, the differences in the concentration of inhaled particles did not cause any significant alteration in the alveolar clearance process after a single, short-term inhalation exposure, at least within the studied concentration range.

The retention of inhaled particles in animals exposed to radioactive diesel exhaust at 7000 μg/m³ particulate concentration has been investigated, thus far, to 126 days after the exposure. The particle retention data (Figure 1), analyzed by a curve stripping procedure, indicated three components with approximate half-times of 1 day, 8 days, and 80 days, respectively. The biological meaning of these components can be understood in terms of clearance mechanisms. The first mechanism deals preferentially with particles deposited in the tracheobronchial tree and represents their rapid transport by the mucociliary escalator. The particles are finally cleared through the gastrointestinal tract, and their elimination is clearly documented by the presence of ^{14}C activity in the feces. The second mechanism is interpreted as the transport of material deposited in the most proximal respiratory bronchioles, where only a short

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distance is required for transferring the particulates to the mucociliary escalator. The third mechanism removes the particulate matter from the alveolar region, and the clearance mechanism may involve endocytosis, passive and active absorption, and dissolution or metabolism. The extended data base in continuing studies is expected to provide further information on the presence of additional clearance phase(s) of inhaled diesel particles and to assess their clinical significance.
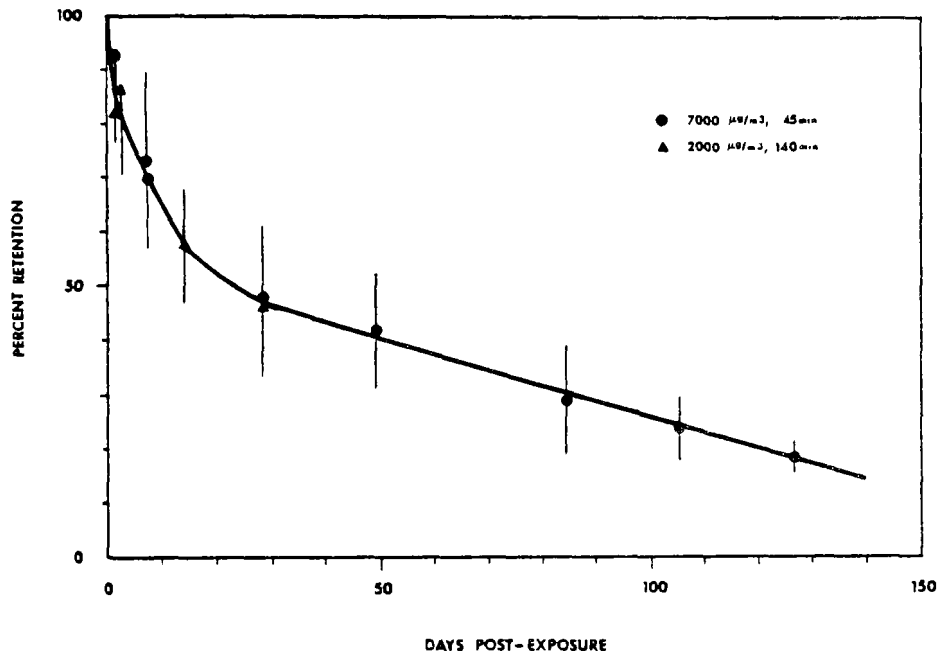
Clearance of inhaled diesel exhaust particles in Fischer 344 rats. The vertical lines represent standard deviations.

COMPARTMENTAL ANALYSIS OF DIESEL PARTICLE KINETICS IN THE RESPIRATORY SYSTEM OF EXPOSED ANIMALS

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One key element in assessing the potential health effects of diesel engine emissions is determining to what extent inhaled particles deposit in the respiratory system and how long they remain before being cleared. The deposition and clearance of diesel particulate is also of interest as an example of a seldom studied interaction between the lung and submicron particles which are assumed to be insoluble. Several investigators have collected data relevant to the deposition and clearance of diesel particulate in animal models. The development of a model of the kinetics of insoluble submicron particles in the respiratory system should provide a basis for organizing, explaining, and comparing these experimental data.

A review of available information on the deposition, transport, and clearance of insoluble submicron particles suggests a model with compartmental divisions illustrated in Figure 1. The model consists of a set of differential equations specifying the rate of transport of particulate mass among compartments. The kinetics are assumed to be first order, since it is the simplest assumption which can be made and since it leads to exponential clearance curves, the type found experimentally.

The major conceptual difference between this model of particulate kinetics in the lung and some previous ones [1,2] is that the model describes a wide range of experimental inhalation conditions, and provides compartments and parameters that apply even to exposures using nearly maximum tolerated doses. Although the ICRP clearance model includes a nasopharyngeal compartment and emphasizes particle dissolution, these features were not considered in the present model because they are irrelevant to the character of insoluble diesel particulates. Inclusion of the "free particulate" compartment does not significantly influence the fit to experimental data, but serves as a reminder that particulate resides on the lung surface for a short time.

General solutions of the model for two types of experiments are presented. In a Type I ("post-exposure") experiment, the animals are exposed for only a short period, and their changing particulate lung burdens are measured starting at the end of the exposure. In a "long exposure" experiment, the animals are exposed continuously or nearly continuously over a long period, and particulate lung burdens are periodically measured.

The major contribution of the particulate kinetics model to the analysis of the experimental data is to point out that the long-term clearance phase may be

interpreted in two ways: it may be clearance of particulate out of the deep lung with a half-time of 62 days as originally interpreted, or it may be a combination of deep lung clearance and sequestering. The amount of particulate in the lungs of chronically exposed animals suggested a linear build-up over time and indicated that after excessive exposure some portion of the deposited particulates may be retained in the lung for longer times. Consequently, the new model emphasizes that the slope of the buildup may be related to the clearance half-time from the "macrophage" to the "sequestered particulate" compartment, to the overall clearance rate from the "macrophage" compartment, and to the deep lung particulate deposition rate.

The application of the model has so far shown that no single set of model parameters fit all the data available from all experimental approaches. The differences may be related to deficiencies in the model or to actual differences in the respiratory system's response to particulate in the different exposure situations.

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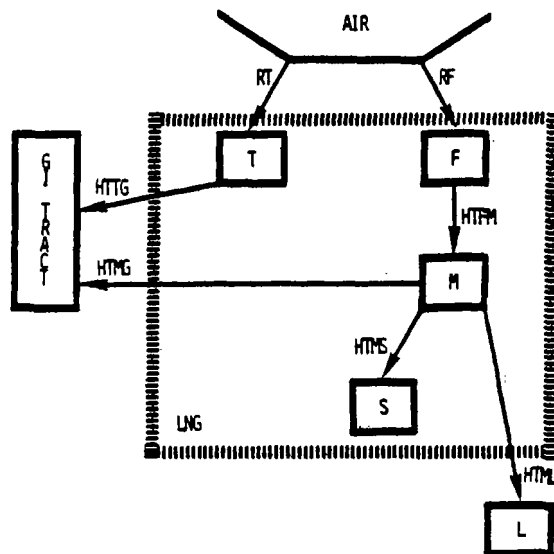
Figure   Compartments and parameters in the model.
          Compartments:
          T = "tracheo-bronchial"   S   = "sequestered particulate"
          F = "free particulate" on   L   = "lymph nodes" draining lung
             deep lung surfaces   G   = "GI tract"
          M = "macrophages" and other   LNG = "total lung"
             scavenger cells

          Parameters:
          RT = deposition rate into T   RF = deposition rate into F
          RT = SDT * C   RF = SDF * C
          SDT = specific deposition   SDF = specific deposition
               rate into T   rate into F
          SDT = DET * VM   SDF = DEF * C
          DET = deposition efficiency   DEF = deposition efficiency
               of compartment T   of compartment F
          C   = concentration of   VM = minute volume
               airborne particulate
          HTxy = clearance half-time from compartment "x" to compartment "y"

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RESPONSE OF PULMONARY CELLULAR DEFENSES TO
THE INHALATION OF HIGH CONCENTRATIONS OF DIESEL EXHAUST

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Bronchopulmonary lavage was used to explore the responses of the pulmonary phago-cytic defense in rats exposed to diesel exhaust at concentratons of 250, 750, or 1500 $\mu g/m^3$ diesel particles for 26 to 48 weeks. Figure 1 shows the quantities of alveolar macrophages, polymorphonuclear leukocytes and lymphocytes obtained by lavage of the respiratory airways (millions of cells in 40 mL). There is no difference in the number or kinds of cells obtained from control and from 250 $\mu g/m^3$ exposed animals after either 6 or 11 months of exposure. However, after exposure to 750 or 1500 $\mu g/m^3$, the number of alveolar macrophages increases by 25-33% and 100-150%, respectively. When the inhaled particulate concentration exceeds 250 $\mu g/m^3$, the quantity of the lavaged alveolar macrophages shows dependence on the inhaled concentration of the diesel particulate after both 6 and 11 months of exposure. In contrast, polymorpho-nuclear leukocytes, which are not observed in lavage fluid from control rat lungs, are obtained in high numbers from rats exposed to 750 or 1500 $\mu g/m^3$ for 11 months or 1500 $\mu g/m^3$ for 6 months. The quantities of lavaged cells are, therefore, dependent primarily on the concentration of exposure and secondarily on the length of exposure.

Since equal amounts of diesel particulate are delivered to the lungs during exposure to 1500 μg DP/m^3 for 6 months or to 750 μg DP/m^3 for 12 months, comparison of the quantities and kinds of cells obtained by lavage points out differences in the response of the phagocytic defense to the inhaled concentrations. When identical amounts of particulates are administered over twice the length of time, the number of alveolar macrophages is increased by 25% above control values, compared to 150% after shorter exposure to a higher concentration. The counts of polymorphonuclear leukocytes are 5.1 ± 1.5 x 10^6 compared to 19.1 ± 4.4 x 10^6 at the shorter time of higher level exposure. This comparison demonstrates that the pulmonary cellular systems respond primarily to the rate of submicron particles entering the lungs, rather than to the total amount of particulate delivered to the lung. Lymphocytes which are not obtained from control rat lungs are lavaged from animals exposed for 11 months to both 750 and 1500 μg DP/m^3, but not to 250 μg DP/m^3. The presence of the nonphagocytic lymphocytes in the lavage fluid may represent a slow immune response to the presence of large numbers of polymorphonuclear leukocytes and particle-filled alveolar macrophages in the lung. The enzyme contents of the acid phosphatase and β-glucuronidase in alveolar macrophages lavaged from control and 250 $\mu g/m^3$ exposed animals were identical. At higher inhaled concentrations, the cellular enzyme content per mL of lavage fluid increased, but due to the variability of the cell counts and types with the continuing diesel exposure, the contribution of each cell type to the total enzyme activity was not determined.

Under the exposure regime used in these experiments (20 hrs/day, 5-1/2 days/week), a threshold rate for response of the phagocytic defense occurs between inhaled concentrations of 250 and 750 µg/m³ diesel particles. The response consists of compensative immigration into the lungs, at first, of alveolar macrophages and later on, also, of polymorphonuclear leukocytes. After excessive exposures (11 months of exposure to 750 or 1500 µg/m³ diesel particles), mononuclear leukocytes (lymphocytes) are also lavaged in high numbers. The probable cause of the observed effects is the continuous phagocytosis of the diesel particles by the macrophage, which results in the cellular accumulation of excessive amounts of "indigestible" carbonaceous particle nuclei. When the macrophage renewal rate in the lungs is too slow to cope with the influx of particles, the macrophages eventually become overloaded. Release of humoral factors from active macrophages may stimulate the recruitment of more alveolar macrophages. Polymorphonuclear and mononuclear leukocytes most probably respond to other not yet known humoral agents from degenerating particle-laden macrophages.
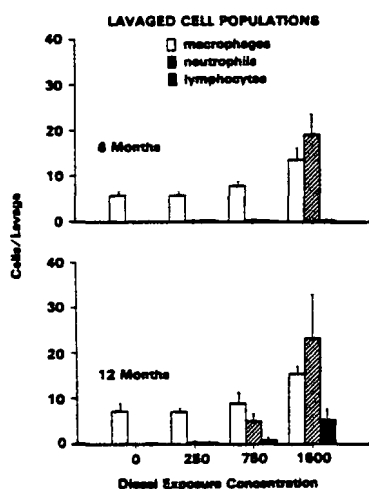
Figure Lavaged cell populations. The proportion of each cell type in lavaged cells was multiplied times the total number of cells obtained from each rat. The numbers were averaged for each exposure group (six rats) and charted as millions of cells in the total lavage volume vs. the diesel exposure concentration. The line shows the standard deviation range of the data.

THE EFFECT OF DIESEL EXHAUST ON CELLS OF THE IMMUNE SYSTEM

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Inhalation of diesel engine exhaust results in the deposition of submicron carbonaceous particles in the respiratory airways. The particles are phagocytized by the pulmonary alveolar macrophages, and are cleared from the respiratory tract via the mucociliary escalator or through lymphatic channels. Lung clearance via lymphatics results in an accumulation of particles in the regional lymph nodes, and literature data suggest that the presence of hydrocarbons or carbonaceous particles in high doses might affect immune functions [1,2].

Two approaches have been used in the present work to determine whether inhalation of diesel exhaust could be immunotoxic. In one series of experiments, dichloromethane extract of diesel particles was injected in massive doses (10-50 mg/kg) intraperitoneally over a 7 day period into C_{57} Bl mice in two separate protocols. First, the splenic lymphocytes were isolated and studied for ability to respond to polyclonal stimulation of B or T cells by E. coli lipopolysaccharide or concanavalin A, respectively. Secondly, dinitrofluorobenzene-induced contact hypersensitivity reaction was measured as a reflection of T cell function by quantifying changes in ear thickness after an irritative challenge. In both experiments, a small deterioration in the immuno-defensive ability of lymphocytes from extract-treated animals was observed. In mitogen response assays, lipopolysaccharide response (LPS) was reduced by about 20% compared to vehicle control groups. Similarly, a 20-50% reduction was seen in concanavalin A (CON A) stimulated cultures. In T cell mediated contact hypersensitivity reaction, all of the treated animals showed decreased ear thickness response. In none of the experiments, however, was a direct dose-response relationship observed. Furthermore, fluctuations in liver weights from experimental animals indicated the possibility that hepatic changes induced by the excessive doses of injected hydrocarbons may be involved in the observed effects.

The approach used in this series of experiments is clearly limited, since 1) by use of high doses of diesel particle extract, the question of bioavailability of hydrocarbon from particles is ignored; 2) the large doses of extract may overwhelm normal defense mechanisms; 3) the route of exposure allows for system distribution of material which may not occur when inhalation occurs; and 4) in the case of mitogen responsiveness, splenic rather than lymph node lymphocytes were studied. Nonetheless, the possibility that the diesel exhaust particle extract administered in high doses may potentially affect the immune system is at least tentatively raised, and should be further verified in inhalation studies.

The second approach was used to detect immunotoxicity of diesel particles by studying lymph nodes, blood and spleen from guinea pigs exposed to diluted diesel exhaust at a particulate concentration of 1500 µg/m^3. In this experiment, immune system organs were studied for shifts in lymphocyte subpopulations counts. Alterations in this parameter have been observed in several forms of human diseases, including active forms of lupus nephritis or chronic glomerulonephritis, inflammatory bowel disease, and other disorders. In addition, exposures to environmental toxins such as lead, poly-brominated biphenyls, and cigarette smoke may also be associated with changes in subpopulation proportions. The data from the present experiments, however, show that no shift in subpopulation occurred in the mediastinal lymph node, the site of primary diesel particle deposition. In spleen and blood, small fluctuations of no more than ± 5% were observed, which is well within the limits of variability described in the literature, and no other significant biological effects were identified.

In summary, small functional differences were seen in lymphocyte responsiveness of $C_{57}Bl$ mice after treatment with diesel particle extracts. However, limitations of the experimental protocol preclude direct extrapolation to possible findings during inhalation exposure to diluted diesel exhaust. In a test system where the more realistic inhalation mode of exposure was employed, no major effects were seen, and more studies are needed to focus on lung immune system reactivity before the immunotoxic potential of diesel particulates deposited in the mediastinal lymph nodes can be definitely assessed.

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INCREASED EAR THICKNESS VS. TIME
(MEAN ± S.E.)
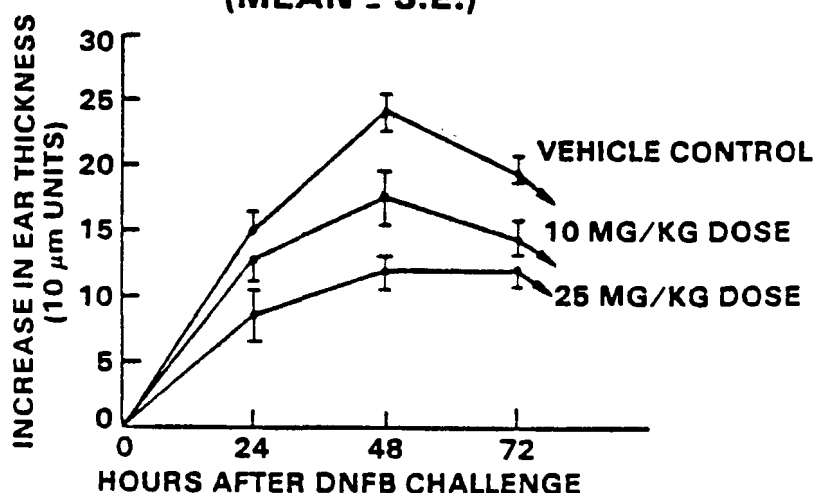
THE PARTICIPATION OF THE PULMONARY TYPE II CELL
RESPONSE TO INHALATION OF DIESEL EXHAUST EMISSION:
LATE SEQUELAE

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Among the earliest cellular responses of the lung to inhalation of diluted diesel exhaust is the focal proliferation of the alveolar Type II cell, the cell responsible for the synthesis of surfactant. This reaction takes place relatively within the same time frame as that for phagocytosis by the alveolar macrophage, and can be seen in rats as early as twenty-four hours post-exposure to a diesel particle concentration of 6000 μg/m^3 [1]. The proliferation is focally within alveoli, sometimes showing several cells in a line. The variation in the staining of their nuclei supports the idea of a fairly rapid proliferation, although mitoses have not been seen. There is no evidence in our hands that this proliferative response is of a reparative nature secondary to damage of the Type I cell. The Type II cells release considerably increased amounts of surfactant, which accounts for a morphologic change in the phagocytic alveolar macrophage which now takes on a more foamy appearance. The transition between the early macrophage (one-day exposure to diesel at 6000 μg/m^3 to that of 6 weeks' exposure) reveals the gradual accumulation of surfactant material. Some of the phagosomes can be seen to contain recognizable myelin figures of Type II provenance changing to a more filamentous form, suggesting an unraveling of the more compact tightly-wound phospholipid. The mechanism of accumulation of cholesterol is certainly not clear, although plate-like crystalline structures can be easily identified within the phagosomes, suggesting cholesterol ester formations. The excess cholesterol could well have its origin from the phagocytosed surfactant material which then at some later stage is de-esterified to form the familiar elongated acicular structures of cholesterol. This foamy cell is apparently more sluggish, and tends to accumulate near the terminal bronchioles where further Type II cell activity is elicited.

With prolonged exposures to 9 weeks at 6000 μg/m^3 and 1500 μg/m^3 for two years, cholesterol also begins to accumulate within the phagosome. Later, the crystals grow and tend to become extracellular. This can be demonstrated by both light and electron microscopy. Build-up of the cholesterol deposits in these late stages has been found to be associated with increased collagenosis of the septal wall in which mast cells are also present. The reaction of mast cells to the release of extracellular cholesterol is also obscure. One could speculate that the heparin of the mast cell is involved in activation of lipoprotein lipase in an attempt to clear the excess of lipid release. In some way, the amine component of the mast cell is also released at this time to provoke the laying down of collagen. In addition, there is an apparent intimate association between laying-

down of extracellular cholesterol, lipids, septal mast cells and collagenosis; the collagenosis is, however, focal and the integrity of the septal architecture is preserved. Occasionally, focal cholesterol granulomas have been observed. These are quite similar in appearance to those seen after prolonged inhalation exposure to Sb_2O_3 [2] and marihuana smoke [3]. The phenomenon of "benign" focal alveolar collagenosis seems to be a consequence of high particulate burden that stimulates an increased production of phospholipids, and not directly of the effect of the diesel particle per se. The observation that diesel particulates, sequestered in the thoracic lymph nodes for up to two years, do not provoke a fibrotic reaction and supports this contention.

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SECTION 4

PULMONARY TOXICOLOGY AND BIOCHEMISTRY

RESPONSE OF THE PULMONARY DEFENSE SYSTEM TO DIESEL PARTICULATE EXPOSURE

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INTRODUCTION

In 1977, the U.S. Environmental Protection Agency reported that organic solvent extracts of diesel particles were mutagenic in bacterial assays,[1] and indicated that the increased penetration of the diesel engine into the fleet of light–duty vehicles could result in an increased frequency of lung cancer in the U.S. population during the years 1985-2000. Several predictions of the expected magnitude of lung cancer excess have been offered during the last years (Table 1), but the wide differences in the quantitative estimates of the expected effects indicate clearly the high level of uncertainty of the theoretical predictions. In sharp contrast, the negative epidemiological studies of the British Medical Research Council conducted on London bus garage workers between 1955 and 1974, as well as the recently published results of long-term inhalation animal studies seemed to assure that the growth of the passenger car population equipped with diesel engines and of diesel particulate concentrations in the urban air would not, according to the current state of knowledge, threaten public health.

The apparent discrepancy between the theoretical prediction based on the suggestive evidence of the simplified bench tests and the real life situation is difficult to explain. First, it is important to note that positive mutagenic tests were observed only after all adsorbed hydrocarbons had been stripped by powerful organic solvents and assayed in the test in the form of extracts. It has been questioned whether it is scientifically appropriate to use an organic solvent to extract hydrocarbons adsorbed on the core of the diesel particles when the *in vivo* biological activity of the entire particle in the living organism is to be assessed. The capacity of such solvents to solubilize organic matter is many times stronger than anything found in the human body, and therefore cannot simulate mechanisms by which biologically active compounds are released for interaction with sensitive cells of the respiratory system.[2]

TABLE 1.   ESTIMATED ANNUAL EXCESS IN U.S. LUNG CANCER FATALITIES
DUE TO LIGHT-DUTY DIESEL VEHICLES (95% UPPER CONFIDENCE LIMITS)

	Predicted Annual Increase
Thorslund[3], EPA 1981	1151
Albert,[4] R., EPA 1979	455
Harris[5], J., NAS 1980	< 30 – 45
Cuddihy,[6] R.G., LITRI 1981	< 30

Using the same laboratory method, Siak et al[7] and Brooks et al[8] have clearly demonstrated that when extraction fluids were used which are compatible with the internal environment of the human body, mutagenic activity was significantly reduced and represented only a small fraction of the effects reported for organic extracts (Figure 1).
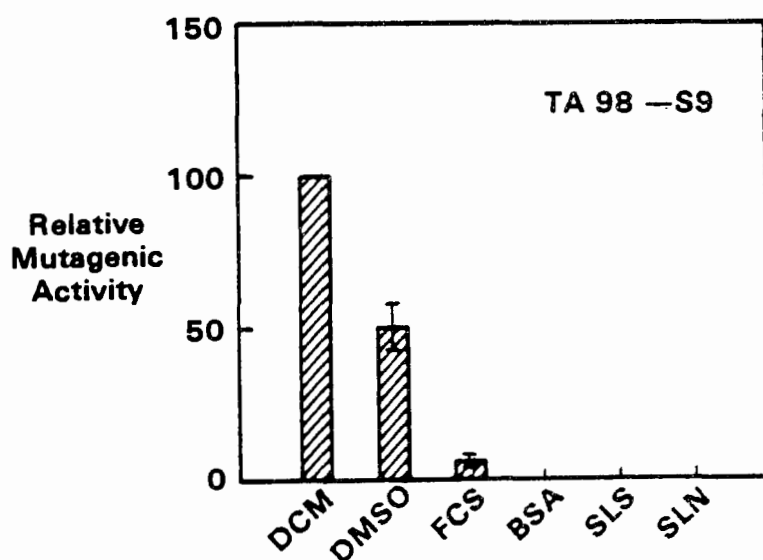
Figure 1 .   Comparison of the mutagenic activities of diesel particulate extract DCM (dichloromethane), DMSO (dimethyl sulfoxide), FCS (fetal calf serum), BSA (0.5% bovine serum albumin), SLS (simulated lung surfactant), and SLN (saline). From Siak et al. (1979)[7]

Thus, while all the findings obtained by using organic solvent extracts from diesel particulates may be of significant scientific value, they are not valid predictors of potential health effects of diesel particulates inhaled by the human respiratory system and, unless the availability of the chemical compounds adsorbed on the surface of diesel particulates for the biological fluids of the human body is considered in the assessment process, estimates of lung cancer excess due to diesel emissions will remain arbitrary and unrealistic.[2]

Second, the highest estimates of increased incidence of lung cancer [Thorslund 1981, Albert 1979] are, unfortunately, not based on laboratory or epidemiological evidence of diesel emission effects at all; they have been derived from unsupported and speculative assumptions "that the carcinogenicity of diesel engine exhaust in units of particle-bound organics, extracted from the exhaust and oven emissions have the same potency per unit mass,"[9] or that a ratio of the relative carcinogenic potencies can be obtained from comparative laboratory tests.[10] The apparent fallacy of the assumption lies mainly in the fact that it presumes the activity of one single representative of the polycyclic organic matter, namely benzo[a]pyrene, is a common denominator responsible for the carcinogenic aggressivity of both coke oven as well as diesel emissions. However, a simple comparison of both pollutants clearly indicates that benzo[a]pyrene concentrations in the diesel particulates are lower by at least two orders of magnitude (Table 2) and that a large fraction of the mutagenic activity of diesel particulates is attributable to the presence of powerful nitroaromatic mutagens, rather than to benzo[a]pyrene alone.[11,12] In addition, the epidemiological data for coke oven emissions are crude and a number of assumptions or corrections must be made before they can be used in the risk assessment process.[13]

TABLE 2. AVERAGE CONCENTRATIONS OF BENZO[a]PYRENE IN SOLUBLE COKE OVEN EMISSIONS AND DIESEL PARTICULATE EXTRACT

Coke Oven Emissions (Jackson et al., 1974)*	5,135 ± 2.019 µg B[a]P/g CTVP
Diesel Particulate Extracts (from 16 FTP tests on 10 Oldsmobile engines - Williams, 1981)**	98.71 ± 143 µg B[a]P/g Ext

*Jackson, et al., Am. Ind. Hyg. Assoc. J., 35(5):276-81.
**Williams, R. (1981), personal communication

Third, results reported from the long-term animal experiments[14] indicate clearly that despite massive exposures and excessive accumulation of particles in the lung, no increased

risk for tumor formation was found in the reported studies and no significant changes in pulmonary function and structure have been detected to date.

Pulmonary defense mechanisms were found effective in removing inhaled particulates and preventing their contact with the sensitive cells. This may provide the final explanation of the apparent discrepancy between the positivity of laboratory bench tests and negative effects in chronic exposures of large human populations or animal inhalation studies. Experimental work on the mechanisms responsible for particle deposition, retention and clearance from the respiratory system has been reported separately, but the role of the cellular defense mechanisms, primarily of the pulmonary alveolar macrophage, represents an equally important if not more potent process which may defend the organism against biological activity of the deposited particles. Research programs in our laboratory concentrated, therefore, on the analysis of the pulmonary cellular mechanisms as a potential explanation of the existing discrepancy.

RESULTS

Pulmonary alveolar macrophages belong to the line of mononuclear phagocytes which are widely distributed in the body, and constitute an important part of the defense mechanisms of the respiratory system by clearing the tissues and fluids of particles such as bacteria or microscopic foreign bodies which penetrate into the organism. After ingestion of particulate material, living matter of microorganisms is degraded, but insoluble particles may be retained inside the phagocytic cell and, along with the macrophage, cleared from the organism via the mucociliary escalator.

LaBelle and Brieger[15] demonstrated that a highly positive correlation exists between the amount of the inhaled dust cleared from the lung and the number of phagocytic cells counted in a unit of lung tissue or bronchopulmonary lavage, thus indicating that the early clearance of dust is mediated by phagocytes. The inhaled particles evoke various responses from the alveolar macrophages. Inhaled cigarette smoke increases the number of lavageable alveolar macrophages,[16,17] the cell diameter,[18] cellular protein content,[19] and the activities of several enzymes.[20]

Strom[21] described the results of chronic experimental exposure of rats to diesel particles at concentrations which range from a nominal value of 250 to 6000 ug diesel particles per cubic meter of air. Male Fischer 344 rats, (*Rattus norvegicus*) six weeks of age, were exposed to diluted diesel exhaust at concentrations of 0, 250, 750, 1500 and 6000 ug/m^3 diesel particulate per cubic meter in the diesel exposure program at the GMR Biomedical Science department.[14] After 6 months and 1 year of exposure. groups of 6 animals each were removed and anesthetized with sodium pentobarbital (50 mg/kg body weight) administered intraperitoneally. The lungs were lavaged *in situ* using Hank's balanced salt solution (HBSS) which was prepared without calcium and magnesium. The

trachea was exposed and cannulated using a 14 gauge Teflon catheter, held in place with three annular ligatures. Before lavaging the lungs, the abdominal aorta was transected to exsanguinate the animal, and the diaphragm was punctured to deflate the lungs. The accessible airways were lavaged seven times with 6 mL aliquots of HBSS at 37° with nominal recovery of 40 mL of solution.

In the case of unexposed animals, all the lavaged cells are usually alveolar macrophages, with a yield of 5 to 7 million cells per animal. However, the cells from exposed animals varied in cell type and cell counts. Figure 2 shows the populations of cells obtained as a function of diesel particle concentration during long-term exposures.
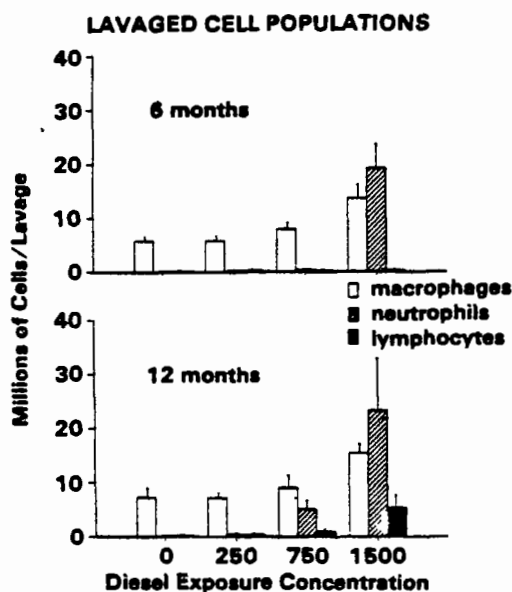
Figure 2. Total cell counts obtained from individual lavaged animals in various exposure groups. Mean ± standard deviation. From Strom, K.A. (1981)[21]

It is important to point out that at the lowest exposure concentration (250 μg/m^3), as well as in the control, the cells from the exposed animals were entirely alveolar macrophages, with no difference in the cell counts in the lavage fluid. At the higher concentrations of 750 and 1500 μg/m^3, the counts of alveolar macrophages increased proportionally with the exposure concentration; the results were identical for alveolar macrophages at both 6 and 11 months of exposure. Obviously, in the long-term exposure the particulate enters the lungs at a rate proportional to the inhaled concentrations, and the normal turnover of alveolar macrophages in the lung can handle the entering mass of

particulates below concentrations not exceeding 250 $\mu g/m^3$. At higher mass influx rates, the resident macrophages escalate the rate of phagocytosis. It is expected that humoral factors are produced and released during phagocytosis, increasing further immigration of mononuclear phagocytes or recruitment of additional phagocytic cells from local resources. The cellular pulmonary defense is stimulated by higher inhaled concentrations to a larger extent than when low concentrations are inhaled. Strom[21] observed that the response in the form of significantly increased macrophage counts is much larger after exposure to 1500 $\mu g/m^3$ for 6 months than after inhalation of 750 $\mu g/m^3$ for 1 year, although the total mass of deposited particulate burden was identical. He postulated that the number of lavaged macrophages is proportional to the mass influx of particulate, rather than to the actual diesel particulate burden in the lung. This suggests that there may be a threshold for the rate of mass influx of diesel particulate into the lung above which there is increased recruitment of alveolar macrophages. Under the exposure regimen employed in the experiments (20 hrs/day, 5.5 days/week), the threshold rate was between the inhaled concentrations of 250 and 750 $\mu g/m^3$ diesel particulate.
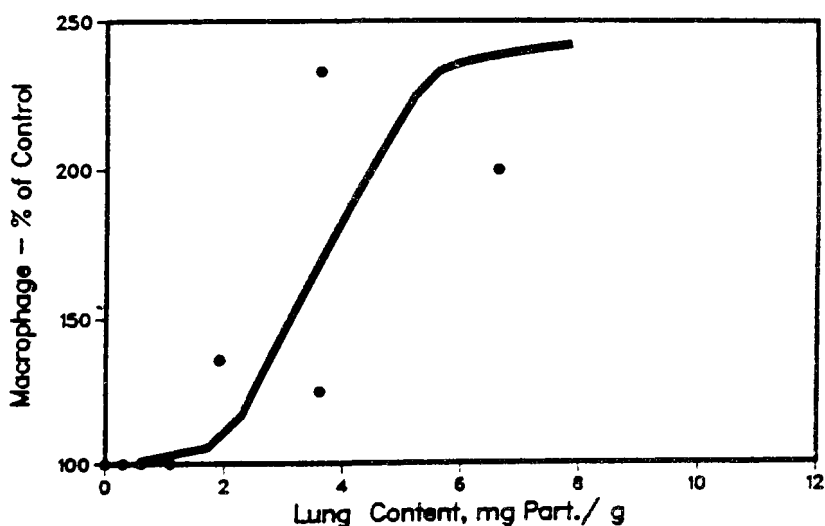
Figure 3. Dose-response curve of the macrophage counts in the bronchopulmonary lavage fluid versus lung content of the diesel particles in long-term exposed animals. Data from Strom, K.A. (1981)[21]

Apart from the question of whether the primary stimulus for recruitment of alveolar macrophages comes from the specific role of the exaggerated particulate influx or the presence of excessive particulate burden in the lung, the threshold character of the defense reaction is obvious, even when the macrophage counts are plotted against the amounts of particulates accumulated in the lung with exposure (Figure 3). The type of curve indicates a distinct dose-effect relationship, and confirms clearly that prolonged exposure to high concentrations provokes defense mechanisms which do not exist at lower levels of exposure.
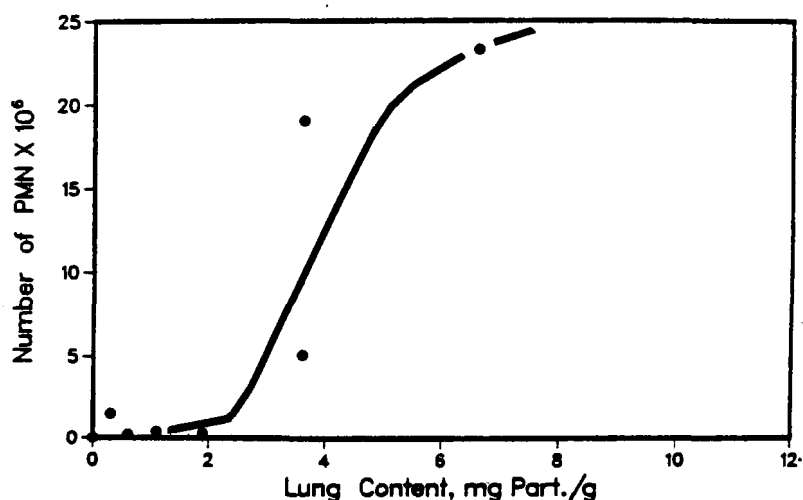
Figure 4. Dose-response curve of the polymorphonuclear leukocyte count in the broncho-pulmonary lavage versus lung content of diesel particles in long-term exposed animals. Data from Strom (1981)[21]

In addition, a similar character of response occurred in the counts of polymorphonuclear leukocytes in the lavage fluid (Figure 4). Both responses occur relatively late in the exposure process, and must be differentiated from the similar increases in the number of macrophages and polynuclear neutrophils described after intratracheal instillations of large loads of particulates.[15,22] However, the character of the primary response to intratracheal administration of particulates is transient, and the cell counts return to normal levels after several days.[22] In contrast, the response to long-term high-level exposure seems to be permanent, and is proportional to the inhaled concentrations and/or duration of the exposure.

Strom[21] also offered the explanation that the primary response of phagocytic cells including polymorphonuclear leukocytes in the acute insult is provoked by the inadequacy of the macrophages to remove the particles from the alveolar surface, and indicated that this would be an unlikely explanation of the polymorphonuclear response in the chronically exposed rats for three reasons. In the first case, the pulmonary defense probably has equivalent capacity to produce more macrophages as well as polymorphonuclears in response to inhaled particulate. Nevertheless, after exposure to 750 $\mu g/m^3$ for 6 months and 1 year, the macrophage counts do not increase, but the number of leukocytes does. Second, morphologically the polymorphonuclears appear in the lung primarily among the diesel particle-laden clusters of macrophages, rather than freely roaming the alveoli as observed after intratracheal administration of particulates. In contrast, the leukocytes seem to represent a specific response to the aggregated macrophages in the late phase of the exposure, rather than to the presence of particulates themselves. Third, in long-term exposures, the leukocytes do not rapidly decline upon cessation of the exposure as they do after the acute insult. After 750 $\mu g/m^3$ exposure for 1 year, significant counts of polymorphonuclears can be obtained by lavage even 16 weeks after the exposure has ended.

It is important to note that a similar biphasic response of the pulmonary defense system was also observed in the reaction of the alveolar cells. White and Garg[23] investigated the lungs of rats exposed to diluted diesel exhaust at concentration levels of 6000 $\mu g/m^3$ for periods from one day to nine weeks using exposure methods previously described.[14] They observed a highly significant scattered increase of Type II cells without any accompanying necrosis of the endothelial (Type I) cells after only 24 hours of exposure (Figure 5). Later on in the exposure (4 weeks), the authors identified that many of the alveolar macrophages with particles became foamy and aggregated in alveoli near terminal bronchioles, as well as near other relatively immobile structures such as vessels and the pleura. By 9 weeks, there was continued increase of diffusely-placed macrophages and accentuation of the aggregated formations of fused phagocytes. The septa of the alveoli that made up these complexes were slightly thickened and showed a positive stain for reticulin which some authors consider to be a precursor form of collagen. Specific stains for collagen were negative, however. At the same time, the Type II cells lining the alveoli of the complexes were again more numerous in apparent proportionality to the amount of aggregated macrophages (Figure 6).

Obviously, like the immediate response of phagocytic cells, the alveolar Type II cells also react transiently to the initial insult of the particulate influx into the alveolus. Later on, another proliferation of Type II cells occurs, however, in response to the formed aggregates of the macrophages. This effect cannot be found in the unexposed subjects, and apparently do not exist at the lower levels of exposure. In the literature, similar "dust macula" or focal occurrence of alveoli with thickened septa has been described in coal

Figure 5. Lung - 6000 µg/m^3, 24 hour exposure. Note several Type II cells (arrows).
Macrophages contain diesel particles (arrow head). Periodic-acid Schiff. 250x From
White, H.J. and Garg, B.D. (1981)[23]
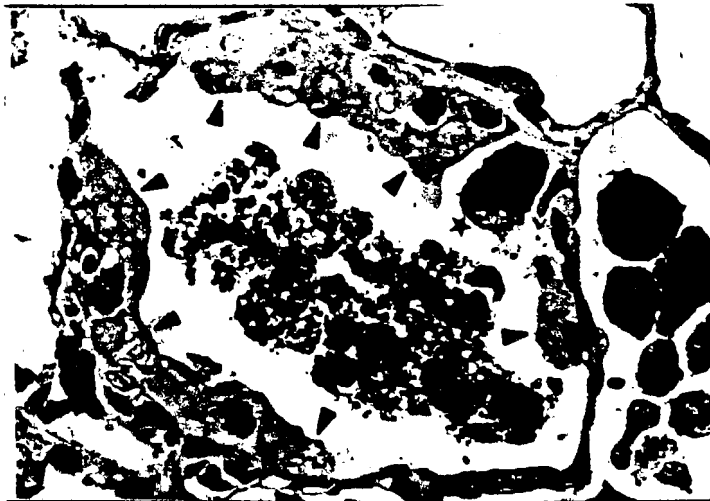
Figure 6. Lung - 6000 µg/m^3, 6 weeks, alveolus containing mass of fused macrophages,
single macrophages(*). Note marked Type II proliferation (arrow head) apparently
secondary to fused macrophage mass. Hematoxylin-eosin. 160x From White, H.J. and
Garg, B.D. (1981)[23]

workers' pneumoconiosis.[24,25] The term "macrophagic alveolitis" was used when referring to the lesion in the lung of the guinea pig exposed to pure coal dust.[26]

The exposure–dependent appearance of these complexes was confirmed in the second study[27] when White and Garg examined the histologic changes in the lungs of animals that had been exposed for greater lengths of time up to two years to concentrations below 6000 $\mu g/m^3$, but still at levels more than one hundred times higher than expected average roadside concentrations in the year 2000. In the chronic experiment, one hundred and fifty adult Fischer 344 male rats (*Rattus norvegicus*) were divided into four equal groups. Animals of group one were exposed to filtered room air, and rats of groups 2, 3, and 4 were exposed to 250, 750, and 1500 $\mu g/m^3$ of diluted diesel exhaust, according to the methods described by Schreck et al.[14] Three rats from each group were sacrificed immediately at the end of 5, 10, 15, 25, 35, and 45 weeks, one year, 18 months, and two years of exposure. In addition, three rats exposed to 750 $\mu g/m^3$ for one year were sacrificed after 35 weeks recovery.

Again, a general increase in diesel particulate deposition was observed in the lungs by light microscopy that was roughly proportional to the product of dose and time. Thus, animals exposed to 250 $\mu g/m^3$ for 5 weeks showed the least accumulations, while those animals exposed to 1500 $\mu g/m^3$ for two years showed the maximal deposition. Shorter periods of time and lower levels of exposure also produced a smaller number of distinct foci of deposition formed by the local aggregation of macrophages with ingested diesel particles. In general, the foci tended to locate near terminal bronchioles and other relatively fixed structures, including vessels and the pleura.

The occurrence of aggregates of macrophages can be described as another form of the cellular defense system: the macrophages which for some reason were unable to leave the peripheral airways became immobilized, but still contained and stored the phagocytized diesel particles, thus preventing their more intimate contact with the sensitive cells of the respiratory system. The reasons for the augmentation of the Type II cells in the directly adjacent alveolar walls cannot be explained at this time. Perhaps the presence of macrophages with particles stimulates their proliferation in order to increase production of lung surfactant. It is completely unknown if the presence of excessive amounts of surfactant in the alveolus leads to the aggregation and clustering of the macrophages. However, preliminary data on the increased concentrations of surfactant phospholipids in the lavage fluid which coincide with the presence of aggregated macrophages may indicate a specific role of the surfactant in this reaction.[28] In this regard, Papahadjopoulos et al[29] have reported *in vitro* evidence of enhanced fusion of cells in the presence of phospholipids.

Certainly, the containment of diesel particles inside macrophages can explain why - except for the functional stimulation of the Type II cell we do not see any specific reaction of the alveolar tissue early in the exposure. It was only after excessive amounts

of particles had been accumulated in the lung – or after the macrophage clusters containing the particles had become stationary in subpleural or peribronchial alveoli for an extended period of time, that the first alveolar wall reaction could be seen. White et al[27] described that in animals exposed to 1500 µg/m^3 for one year, late changes in the appearance and structure of the clustered macrophages can occur. Their cell borders become less distinct, diesel particulates become less contrasting, and give the cytoplasm a more homogeneous foamy appearance which is no longer PAS positive. At approximately the same time, the first changes in the adjacent pulmonary tissue were also observed. Not only was there laying down of reticulin, but focal septal collagenization was reported, as seen by Masson stain and birefringence under polarized light. The picture, while still focal, was more obvious in animals exposed for two years than in those observed after one year (Figure 7). Only by exception was there any loss of parenchymatous integrity; i.e., although there was focal septal thickening, the alveolar structures were not obliterated.

Two other histologically distinct findings accompanied the septal fibrotic changes which were prominent only where intra-alveolar diesel material had accumulated in the macrophage clusters. Lipid-processed, Oil Red O-stained tissue was positive for neutral lipids in these areas, which also showed fine granular and needle-like structures on polarization. Where such polarizable material was noted, the fibrotic septa were found to contain mast cells. These cells are readily recognized by the presence of metachromatic purple granules in the cytoplasm on staining with toluidine blue buffered to pH4 (Figure 8).

As mentioned above, occasionally small foci of more complete fibrosis with a tendency to obliterate the alveolar pattern were seen in a few animals exposed to 1500 µg/m^3 for 18 months or longer (Figure 9). Similar changes were seen in a singular group of animals exposed to 750 µg/m^3 for one year and allowed to recover for 32 weeks before sacrifice. The lesions, in addition to their pronounced delimited fibrotic aspect, also contained large acicular clefts resembling those seen in atheromatous lesions, birefringent in polarized light, and presumably representing cholesterol. Along with the Masson-positive collagen fiber and cholesterol accumulations, mast cells and histiocytes were prominent in the areas of these lesions. When they occurred in the 750 µg/m^3 exposed group, the rest of the lung showed little, if any, fibrosis. Later on, the cholesterol accumulation became quite localized, usually in the subpleural areas where, in the extreme situation, discrete focal lesions were formed that appear identical with the so-called "cholesterol granuloma."

Probably, the proliferation of Type II cells with increased production of surfactant and exaggerated pouring of phospholipids into the alveolus in response to high exposures of respirable particulates is directly responsible for the local accumulation of lipids. Activated alveolar macrophages ingest not only the inhaled particulates, but also the released phospholipids. The authors further speculated that phagocytosis of the lipids makes the macrophages less motile, causing them to aggregate. At this point, local Type II cells
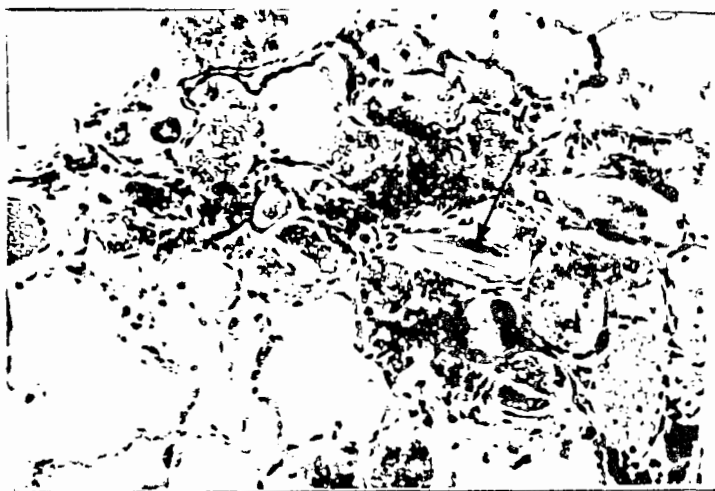
Figure 7. Diesel particulate exposure to 1500 µg/m^3 for 18 months. Focal deposition of needle-like crystalline material (arrow) resembling cholesterol in areas of diesel accumulations. Note fibrotic reaction around cholesterol crystals with focal obliteration of alveolar pattern. Hematoxylin-eosin. 40x From White, H.J. and Garg, B.D. (1981)[27]

Figure 8. Diesel particulate exposure to 1500 µg/m^3 for 2 years. Portion of alveolus with large extracellular acicular formation (short arrow), surrounded by foamy macrophages. Note mast cells (long arrow), and septal collagen (broken arrow). Electron micrograph. 2400x From White, H.J. and Garg, B.D. (1981)[27]

react by cell proliferation resulting in further release of alveolar surfactant, and switching the metabolism of the macrophage toward the precipitation of lipids in the form of cholesteryl palmitate. The final release of free cholesterol, first intracellularly, and later extracellularly in the alveolus promotes the penetration of mast cells into the septa and stimulates the local production of collagen within the septum, but not within the alveolus (Figure 10). It is this condition which White et al[27] would term a "benign fibrosis" as opposed to those conditions which injure the alveolar lining cells and provoke a fibrotic reaction within the alveolus ("fibrosing alveolitis" or "malignant" fibrosis).

The capacity of the macrophage response in the phagocytosis of the invading particulates is otherwise overwhelming. Rudd and Strom[30] developed a method for the direct measurement of the amount of diesel particulate in tissue, and reported that in guinea pigs (*Cavia porcellus*) exposed to diesel particulate for 22 weeks, the amount of particulate in the lung rose proportionally to the exposure concentration. In order to explain the results, they examined the lungs of exposed animals in order to discover the reservoirs for deposited particulates, and again identified that the main sequestering site of the particulates is the alveolar macrophage. The authors reported that through an as yet unknown mechanism, the dust-filled macrophages aggregate in or near terminal bronchioles and the pleural surface, and remain there for as long as two and one-half years after the exposure. They further noted that although this mechanism may be advantageous in clearing particle-laden cells from most of the respiratory surface, it does not promote the actual clearance of particulate from the lungs.

In a complementary study, Siak and Strom[31] addressed the question of how the phagocytes handle the biological activity of the ingested particulates. Young male Fischer 344 rats (*Rattus norvegicus*) were exposed to diluted diesel exhaust at a concentration of 6 mg/m^3 for three days (20 hrs/day). Alveolar macrophages were obtained by bronchopulmonary lavage immediately after exposure and at 1, 4, and 7 days thereafter. Macrophages from forty animals were pooled for each data point, sized and counted. The alveolar macrophages were concentrated by filtration on pre-washed fiberglass filters and dried at room temperature to constant weight. The filters were extracted with dichloromethane in a Soxhlet apparatus for 4 hours (20-25 solvent cycles). The *Salmonella typhimurium* strain TA98 was used for mutagenicity assay. For thin layer chromatography, Whatman LK6 plates were used and the developing solvent was toluene:hexane (5:1).

Figure 11 compares the mutagenic response of macrophage extracts from exposed rats as detected in the Ames microbial assay with the extracts from diesel particles collected from the inhaled air. Although the macrophage extract obtained from exposed animals immediately after exposure gave a positive response, the data show that intracellular material extracted from macrophages interferes with the mutagenic response of diesel
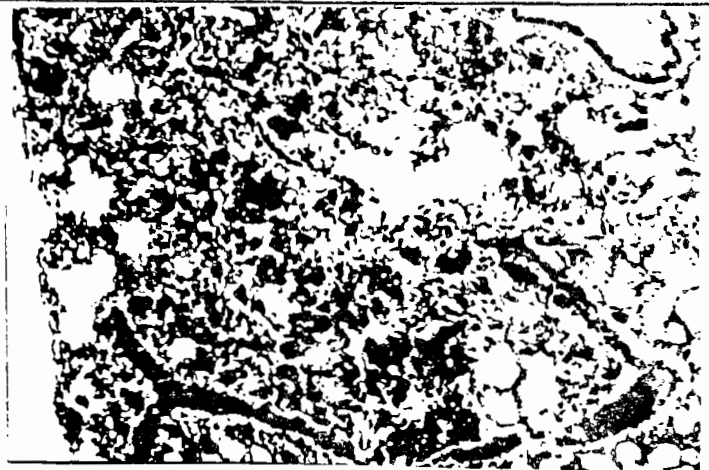
Figure 9. Diesel particulate exposure to 1500 µg/m^3 for two years. The increased collagen material in the alveolar septa polarizes. Note that the process is focal. Masson stain with polarized light. 16x From White, H.J. and Garg, B.D. (1981)[27]
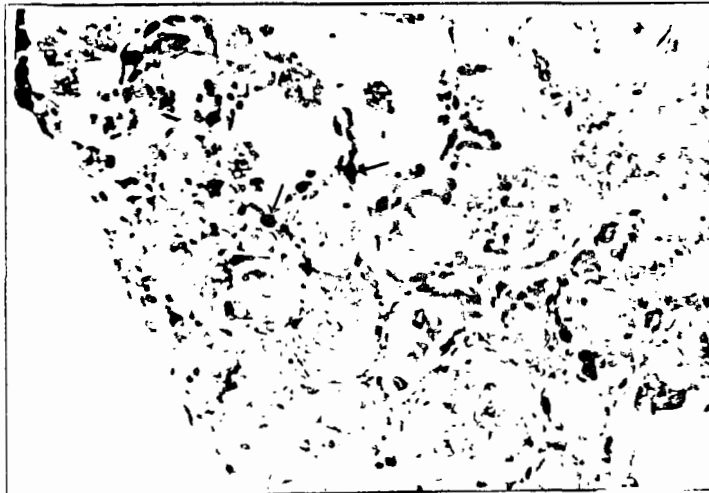
Figure 10. Diesel particulate exposure to 750 µg/m^3 for one year - 32 weeks recovery. By polarized light, alveolar collagen and intra-alveolar lipids are accumulating in subpleural area. Note accompanying septal mast cell reaction (arrow). Toluidine Blue (pH$_4$), polarized light. 40x From White, H.J. and Garg, B.D. (1981)[27]
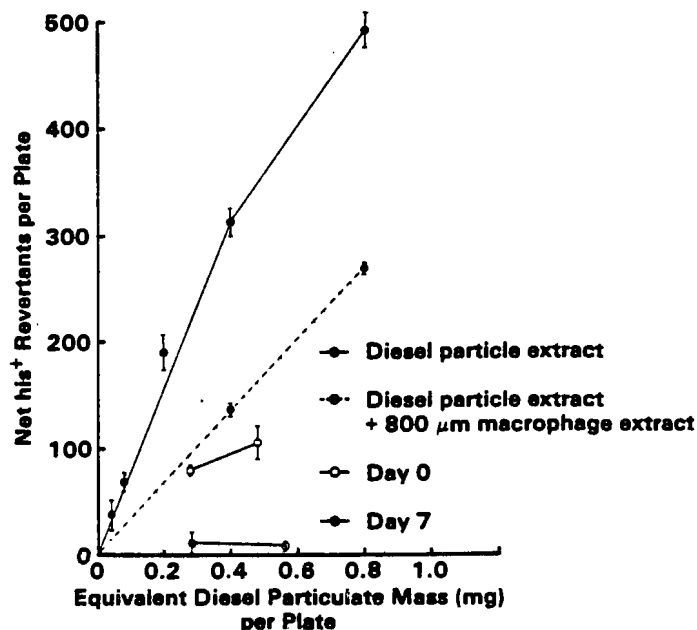
Figure 11. *Salmonella typhimurium*(TA98) mutagenic activity response of diesel particle extracts from alveolar macrophages on day 0 and day 7 after exposure. From Siak, J-S. and Strom, K.A. (1981)[31]

particle extract in the Ames assay (Figure 11). However, in spite of the interference, mutagenic activity of macrophage extracts obtained from the exposed animals was only detected at day 0 and day 1 after exposure; after that, no mutagenic activity could be detected (Figure 12). The interaction between alveolar macrophages and the extractable mutagenic components of inhaled diesel particles was further corroborated by chromatographic analysis. In a parallel way, the thin layer chromatography UV fluorescence banding patterns of the extracts, which were prepared from macrophages lavaged from exposed rats immediately and one day after the exposure, were similar to extracts of particles from inhaled air, whereas the extracts of macrophages on days 2, 3, 4, and 7 after exposure have lost the banding patterns through as yet unknown mechanisms.

Alveolar macrophages are capable of metabolizing polycyclic aromatic hydrocarbons[32,33] and previous work in our laboratory demonstrated that mammalian liver enzymes activate the bacterial mutagenic activity of l-nitropyrene and of diesel particle extract under specific laboratory conditions.[34] Therefore, an enzymatic transformation of the extractable organic compounds of diesel particles by macrophages may be one of the
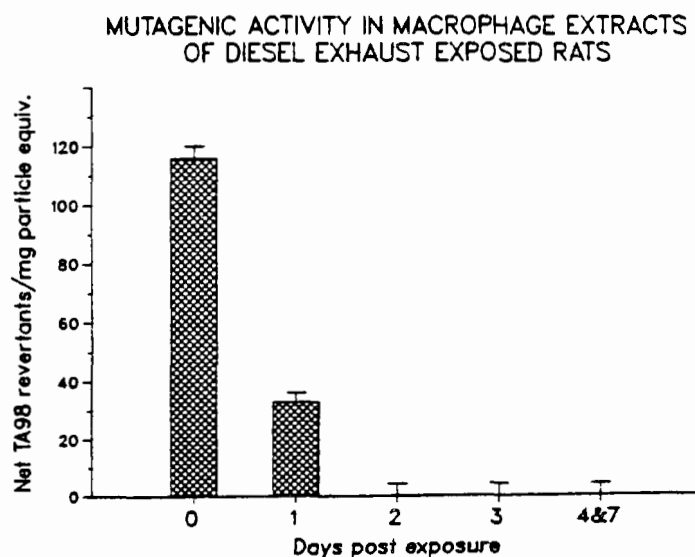
Figure 12. Changes in the mutagenic activity of diesel particle extracts obtained from lavaged pulmonary alveolar macrophages at different times after the exposure. From Siak and Strom (1981)[31]

possible mechanisms involved. Another possible mechanism is the solubilization of the extractable organics from diesel particles by phospholipids from the lung surfactant and by other cellular components of the macrophage. The soluble complexes may diffuse into other tissues, and/or bind to other cellular constituents which render them unextractable by the method employed. Further *in vivo* and *in vitro* experiments are required to provide a better understanding of the mechanisms involved, but the results thus far demonstrate that the insoluble particulates stored for a prolonged period of time in alveolar macrophages represent virtually an innocuous material which may have long lost most of its biological activity.

The lack of biological activity of diesel particulates deposited in the respiratory tract was further documented by the work of several laboratories. Chen et al[35] investigated the effects of long-term inhalation of diluted diesel exhaust on aryl hydrocarbon hydroxylase activity and cytochrome P450 content in lung and liver microsomes in male Fischer-344 rats (*Rattus norvegicus*) and compared them with the results obtained after intraperitoneal and intratracheal administration of organic solvent extracts of hydrocarbon from the diesel particulates. Surprisingly, a decrease instead of an enzyme induction was observed in lung microsomal aromatic hydroxylase activity of animals after the full 9 months of exposure to diesel exhaust at the particulate concentration of 1500 $\mu g/m^3$. The observations were confirmed by other investigators.[36,37] In contrast, 1.4- to 9-fold increases in aromatic hydroxylase activity were observed in liver and lung microsomes of rats pretreated by intraperitoneal doses of particulate extract, which were 10-15 times higher than the most conservative estimate of the deposited lung burden (25-125 mg/kg BW).[35] Furthermore, direct intratracheal administration of the diesel particle extract[38] required doses as high as 6 mg/kg body weight before the activity of the induced enzyme in the lung was barely doubled (Figure 13). The induction was slow and occurred selectively in lung only (Figure 14), indicating that diesel particulate extract probably does not absorb easily into the lung circulation, and is not distributed to other organs.[39] The data suggest that the absence of
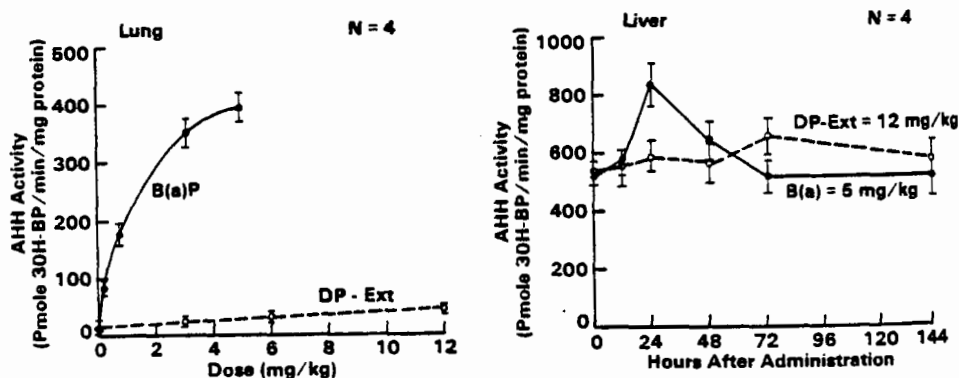
Figures 13 and 14. Aryl hydrocarbon hydroxylase (AHH) in the lung and liver tissue after intratracheal administration of diesel particulate extract (DP-Ext) or benzo[a] pyrene (B[a] P). From Chen, K-C. and Vostal, J.J. (1981)[37]

the enzyme induction in rat lung exposed to diesel exhaust is caused either by the inavailability of hydrocarbons for distribution in the body or by their presence in insufficient quantities for enzyme induction. Available data, therefore, indicate that the inhaled diesel particles would not be capable of inducing aromatic hydroxylase in the lung unless the total deposited dose in the lung reaches approximately 6-8 mg of the particle extract per kilogram of body weight. Since the extractable portion represents only 10-15% of the total particulate mass, the required pulmonary deposits of diesel particles in a 70 kg man would be excessive to become a significant step in promotion of a potential neoplastic process.

Published data on a similarly negative immune response of the lymphoid tissues in the respiratory system to the presence of deposited particles are also in good agreement with the observation of the lack of biological activity of the diesel particles during the prolonged inhalation exposures. The inactivity of the sequestered particles is in sharp contrast with the laboratory demonstrations, that the diesel extract, when administered alone in excessive doses, produces positive effects in the immune response. Dziedzic[40] administered massive doses of dichloromethane extract of exhaust particles (10-50 mg/kg, three times over 7 days, intraperitoneally) to mice (*Mus musculus*), and measured splenic lymphocyte response to the mitogens lipopolysaccharide or concanavalin A. Mitogen responsiveness was determined by isolating spleens, making a suspension of lymphocytes in medium RPMI 1640 plus 10% fetal calf serum and antibiotics (penicillin 100 U/mL, and streptomycin 100 μg/mL) and culturing cells in flat bottom microtiter wells in the presence of an optimally stimulating dose of lipopolysaccharide or concanavalin A. The cells were pulsed with tritiated thymidine, and the uptake of radioactivity was used as an index of
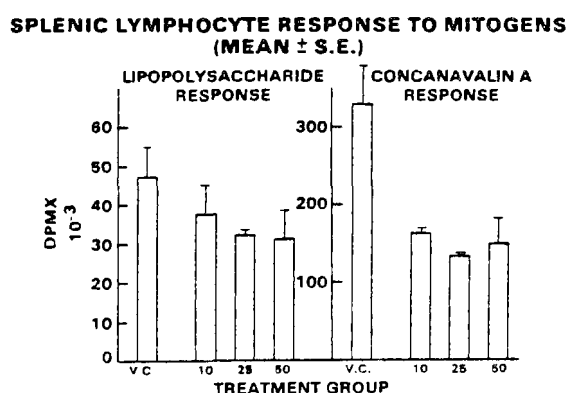
Figure 15. Splenic lymphocyte response to B-cell mitogen lipopolysaccharide or T-cell mitogen conconavalin A after intraperitoneal injection of diesel particulate extract. VC = vehicle control; 10, 25, 50 mg/kg dose. Mean ± S.E. From Dziedzic, D. (1981)[40]

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response. The trend toward decreasing responsiveness in extract-injected animals can be seen in Figure 15. In a separate experiment, T cell responsiveness of mice similarly injected with extract to a contact hypersensitivity reaction was studied. In this

INCREASED EAR THICKNESS VS. TIME
(MEAN ± S.E.)
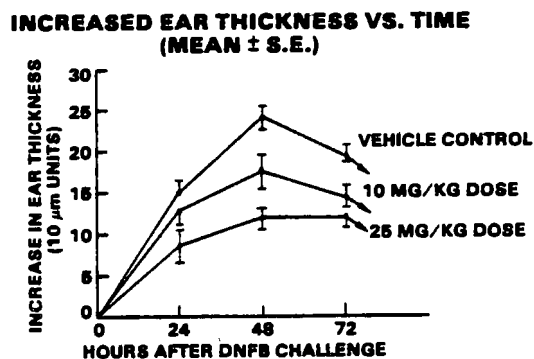
Figure 16. Ear thickness response to the sensitization challenge of dinitrofluorobenzene (0.5%) after intraperitoneally administered diesel particulate extract. From Dziedzic D. (1981)[40]

experiment, groups of mice were sensitized with a 0.5% solution of dinitrofluorobenzene (DNFB) on a previously shaved abdomen. After four days, they were challenged on their left ears with 35 μL of the same solution. Right ears were treated with vehicle alone. The increase in ear thickness at 24, 48, and 72 hours after challenge indicated a decreased ability to respond in the extract-treated animals (Figure 16).

DISCUSSION AND CONCLUSIONS

What appears to be evident from these results is that in contrast with low level exposures, inhalation of diesel particulate at high exposure levels provokes two immediate responses -focal proliferation of alveolar Type II cells and increased numbers of phagocytosing macrophages. When the exposure is excessive, foamy macrophages with particulates aggregate in focal areas of the lung. In the weeks or months that follow, they are accompanied by a localized secondary proliferation of Type II cells. Both of the responses can be identified as an expression of the efforts by the respiratory tract to localize and neutralize the invading particulates. The reaction can therefore be classified as an effective pulmonary defense mechanism which can prevent the contact of particulates with the parenchyma of the pulmonary system. Only after extreme levels of exposure, the focal

accumulation of macrophages leads to an increased deposition of cholesterol, in the form of extra-cellular intra-alveolar deposits, which provoke local quasi-pathological cellular changes resulting from the exaggerated protective response.

The alveolar macrophage not only effectively prevents more intimate contact of inhaled particles with the sensitive cells of the respiratory system, but is capable of deactivating the biological aggressivity of the chemical materials adsorbed on their surface. Even if a prolonged storage of the inhaled particles would, therefore, occur in the respiratory system, it would primarily represent deposits of relatively inert material, which might be more an indicator of past exposure rather than an index of a clinically significant biological hazard.

The biological inactivity of the particulate deposits is well illustrated by the negative response of the inhaled particulates in the induction of metabolizing enzymes as well as by the minimal immunological reaction and lack of significant functional or structural effects resulting from long-term animal exposures to high concentrations of diesel particulates. This happens in spite of positive responses observed after direct administration of materials obtained by stripping the adsorbed hydrocarbons by powerful organic solvent from the surface of diesel particulates in the laboratory test tube. The living organism obviously has effective cellular defense mechanisms which can protect against the invasion of foreign materials and prevent the occurrence of adverse health effects *in vivo* despite the fact that they may have been predicted by the complex chemical analysis and biological testing of the materials *in vitro*.

It may be therefore concluded that the accumulated experimental data offer a plausible explanation why both the epidemiological studies or extended animal inhalation experiments with high concentrations of diluted diesel exhaust did not reveal clinically significant changes despite the reported positivity of the laboratory screening tests indicating serious concerns for adverse health effect hazard. It is more important that the experimental data indicated a substantial protective role of the pulmonary defense mechanisms despite conditions of extremely exaggerated exposure. It can therefore be expected to be fully capable of protecting the general population against adverse health effects of the wider use of diesel engines on our roads, particularly in view of the expected much lower particulate levels in the ambient air.

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INVESTIGATION OF TOXIC AND CARCINOGENIC EFFECTS OF DIESEL EXHAUST IN LONG-TERM INHALATION EXPOSURE OF RODENTS

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INTRODUCTION AND OBJECTIVES

Approximately 2 1/2 ago a long-term study using Syrian golden hamsters was begun taking as its basis a five months inhalation study using three different dilutions of Diesel engine exhaust[1].

The primary objective of this life time exposure study was to investigate as to whether a carcinogenic or a syncarcinogenic effect could be induced in the respiratory tract of the golden hamsters by inhalation of either the diluted total exhaust from a Diesel-engine or the same exhaust void of particulate matter. In addition to the testing for a potential carcinogenicity of the Diesel exhaust, a variety of data on clinical chemistry and hematology were collected at certain intervals. Furthermore, several tests of pulmonary function were performed. They were carried out with rats which were exposed to the exhaust along with the hamsters.

EXPERIMENTAL FACILITIES AND STUDY DESIGN

The exhaust gas for our experiments was produced by a 2.4 liter Daimler-Benz Diesel engine. The engine was operated at a constant load of 16 kW and a uniform speed of 2400 r.p.m. The fuel used in the engine was a European Reference Fuel with a sulfur content of 0.36 %.

The exhaust gas pipe was divided into two lines. One line passed the emissions directly from the exhaust pipe to the mixing chambers, while the other line sent the exhaust first through a centrifugal seperator in order to remove all particulate matter from the ex-haust. Both, the genuine exhaust and the non-particulate exhaust were then diluted in the mixing chambers with clean, dry,

refrigerated air at a ratio of one part exhaust to seven parts of air. The previously reported 5-months study had indicated that this was an optimum concentration for a life time study that was to avoid acute toxic effects.

The inhalation chambers, had a volume of about 250 liters. They were horizontally ventilated with approximately 5 m^3 of diluted exhaust per hour. Special baffles and perforated diaphragms were placed between the exposure area and the inlet and outlet tubes, respectively. They provided a uniform distribution of the aerosol flow in the exposure chambers. In each chamber, 24 hamsters were held on 2 levels of wire cages.

The actual concentrations of a selected number of exhaust components were either measured continuously, or monitored in certain intervals. One set of measurements was made right in the inhalation chambers and another set was obtained in the conduits approximately 5 m upstream of the chambers. Inside the inhalation chambers, the average particle concentration of the diluted exhaust was 3.9 mg/m^3. The mass median aerodynamic diameter of the exhaust particles was o.1 μm.

The concentration of several gaseous components contained in the exhaust, such as CO, CO_2, SO_2, total hydrocarbon, methane and nonmethane hydrocarbons, NO, NO_x, NO_2 and O_2, were present in comparable concentrations in both, the chambers receiving total exhaust and the ones receiving the exhaust without particles (Fig.1). The measurements taken within and upstream of the chambers indicated that no major changes in concentration occurred on the way from the mixing chambers to the inhalation chambers. The concentration of all gaseous exhaust components measured in these locations were sufficiently low so that SO_2 and NO_2 concentrations would not exceed 3 and 1 ppm, respectively, and no acute toxic effects on the experimental animals were expected.

The sulfur content of the soot particles in the exposure chambers was measured by way of x-ray fluorescence spectroscopy, which gave a value of 37 mg/g of soot. In addition to this measurement, quantitative analyses were carried out for 14 specified polycyclic aromatic hydrocarbons (PAH) which were found to be bound to the soot particles. No details will be given here about the extraction of the PAH.These processes have been described in

in some earlier publications of our institute[2].

	MEASUREMENT			
	IN THE CHAMBERS		BEFORE THE CHAMBERS	
	A	B	A	B
CO [ppm]	18,0 (± 4,4)	18,5 (± 4,9)	17,0 (± 3,7)	19,0 (± 5,1)
CO_2 [Vol%]	0,52 (± 0,13)	0,54 (± 0,15)	0,47 (± 0,11)	0,47 (± 0,13)
SO_2 [ppm]	2,8 (± 1,7)	3,1 (± 1,8)	4,2 (± 2,9)	3,8 (± 2,2)
C_NH_N [ppm]	7,9 (± 3,3)	9,3 (± 4,6)	4,9 (± 2,7)	4,9 (± 2,4)
CH_4 [ppm]	2,6 (± 1,8)	3,0 (± 2,2)	1,0 (± 0,3)	1,0 (± 0,3)
$C_NH_N - CH_4$ [ppm]	5,0 (± 2,5)	5,9 (± 3,0)	3,9 (± 2,4)	3,8 (± 1,9)
NO [ppm]	17,2 (± 5,9)	16,5 (± 5,8)	16,2 (± 5,5)	16,5 (± 6,4)
NO_x [ppm]	19,2 (± 6,1)	18,6 (± 5,8)	17,8 (± 6,4)	17,9 (± 7,2)
NO_2 [ppm]	1,0 (± 1,5)	1,2 (± 1,7)	0,8 (± 1,2)	0,3 (± 0,3)
O_2 [Vol%]	20,0 (± 0,7)	19,5 (± 0,6)	20,0 (± 0,7)	19,1 (± 0,5)
PARTICLES [mg/m³]	-	3,9 (± 0,5)	-	-

A: EXHAUST WITHOUT PARTICLES   B: TOTAL EXHAUST

Fig. 1. Component concentrations of the diluted Diesel exhaust

Figure 2 shows in the last column the concentration of 14 PAHs
extracted from the diesel soot as compared to the concentration in
samples of airborne dust taken from 4 different cities in the
Federal Republic of Germany (Fig.2). The PAH concentrations given
in ug/g of dust or soot indicate that, except for fluoranthene
(FLU) and pyrene (PYR), the concentration of PAH is substantially
higher in airborne dust than in Diesel soot. However, the PAH
concentrations in airborne dust are average values over a collec-
ting period of one year (1979/80) during which the collecting
filter was changed every two weeks. In case of the Diesel soot,
on the other hand the collecting period was only about 8 hours and
the concentrations of NO_2 and SO_2 were observed to be higher in the
dilute exhaust than in the city air. Thus, on account of the
different sampling conditions, a comparison of the PAH
concentrations in Diesel soot and airborne dust is of rather
limited value.

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PAH \ SAMPLE	1. Town	2. Town	3. Town	4. Town	DIESELEXH.
FLU -	26.1	21.5	38.5	17.0	134.6
PYR -	21.9	19.6	35.9	13.1	65.8
BaF -	7.1	6.1	12.2	2.4	5.4
BbF	11.0	11.4	20.8	5.6	5.3
BaA +	36.7	35.6	51.0	17.8	9.8
CHR +	80.9	80.2	101.6	46.2	25.7
BFL +	138.7	141.3	165.4	94.1	22.2
BeP -	59.4	58.3	69.5	42.0	14.1
BaP +	27.2	27.0	42.0	14.7	7.0
PER -	6.3	5.9	7.3	3.9	-
IP +	36.6	37.2	42.7	23.5	13.4
DBA +	14.6	11.2	13.5	7.2	-
BghiP -	50.7	44.5	56.5	34.8	21.4
COR -	24.0	19.2	26.9	18.2	12.5

+ = CARCINOGENIC, - = NON-CARCINOGENIC IN ANIMAL EXPERIMENTS

Fig. 2. The PAH content (14 PAH) of airborne particulate matter and of Diesel exhaust particles (in μg/g dust or soot).

Based on the PAH concentrations measured in the inhalation chambers, an estimate can be made as to the inhalation impact of PAH on the experimental animals. Assuming that the hamsters have a respiratory minute volume of 1oo ml, then a daily exposure period of 8 hours for 5oo days in 2 years would amount to 24 m^3 of inhaled diluted exhaust. Estimating the deposition rate of inhaled particles at some 4o % which is conservatively high and taking the average particle concentration at 4 mg/m^3, then, at most, about 4o mg of soot would be deposited in the lungs of the experimental animals. With reference to a measured benzo(a)pyrene (B(a)P) concentration of 7 μg/g of soot, this would represent a B(a)P uptake of about 27o ng, if the availability of the B(a)P bound to soot particles is not limited. Apparently, this estimate

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suggests that less than 1 /ug of B(a)P and similar amounts of most of the other PAHs considered here may be deposited in the lungs of the hamsters. Therefore, it seems that the quantity of carcinogenic PAHs inhaled by the experimental animals is much too small, and the maximum available latency period of about 2 years based on the normal life span of the experimental animals is much too short to make any negative result on carcinogenicity really conclusive. In other words, by way of inhalation of dilute Diesel enginge exhaust alone, the animal experiments will most likely not permit a firm and definite conclusion with regard to a carcinogenicity in human beings.

Since it is rather unlikely, that unsophisticated straight-forward inhalation tests with dilute exhaust gas will ever give statistically significant proof for the existence or non-existence of a carcinogenic effect, our lifetime experiment used the follow-ing animal model: In order to observe subtle changes in tumor incidence rates, it is desirable to work with an increased tumor frequency. An enhanced basic tumor incidence rate in the respiratory tract can actually be induced by intratracheal or subcutaneous administration of carcinogenic dibenzo(a,h)anthracene (DB(a,h)A)or, for instance, diethyl nitrosamine (DEN). Then, in case there is an actual additive or synergistic effect of the ex-haust, the basic tumor incidence rate will increase if the Diesel engine exhaust is inhaled simultaneously.Thus, without using extremely large numbers of animals, the probability of detecting a significant change in the tumor frequency is greater, if these changes occur along the steep slope of the sigmoidal dose response curve rather than along the shallow initial slope of that curve. This may be effected by having a sufficiently enhanced basic tumor rate instead of the low spontaneous incidence rate. In the steep slope range of the curve, relatively small changes in dose.should result in relatively large changes in tumor frequency.

The hamsters were kept in wire cages. Due to space limitations, each compartment held 3 animals. The temperature in the inhalation chambers was kept at 24-25°C and the humidity was 55-65 %. The animals were exposed for 7-8 hours/day and 5 days/week. During the exposure periods, all feed was removed from the cages in order to prevent oral uptake of Diesel soot.

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As can be seen in the experiment protocol, 18 groups of at least 48 animals were used in the inhalation program (Fig.3).

ITR. INST. / S.C.	CLEAN AIR	TOTAL EXHAUST	EXHAUST WITHOUT PARTICLES
WITHOUT	48	48	48
DB(a,h)A 6 MG (20 x 0.3 MG IN 0.02 ML)	48	48	48
DB(a,h) A 2 MG (20 x 0.1 MG IN 0.15 ML)	48	48	48
PYRENE 3 MG (20 x 0.1 MG IN 0.15 ML)	48	48	48
DEN 1.5 MG/KG BW	72	57	48
DEN 4.5 MG/KG BW	48	60	48

DB(a,h)A = DIBENZ(a,h)ANTHRACEN   DEN = DIETHYLNITROSAMINE

Fig. 3. Number of animals in the different groups with and without additional treatment (i.tr. - intratracheal, s.c. - subcutaneous)

There were three exposure atmospheres: clean air, total exhaust and exhaust without particulate matter. For each exposure type, there were 5 treated animal groups. Two of these groups received subcutaneous injections of 1.5 or 4.5 mg DEN/kg body weight at the beginning of the experiment. Two other groups received o.1 and o.3 mg of DB(a,h)A by intratracheal instillation once a week for 2o weeks. The control group corresponding to the animals treated with DB(a,h)A was instilled once a week for 2o weeks with pyrene, a non-carcinogenic PAH. Another group received no additional treatment.

RESULTS
Median lifetime of the hamsters

The experimental animals employed in this study were female Syrian golden hamsters from a breeding farm in Frankfurt-Hoechst, West-Germany. At the beginning of the experiment, the animals were approximately 8 weeks old.Inspite of an apparently unfavorable condition of keeping the hamsters in wire cages, the median

experimental lifetime, that is the time where 5o % of the animals
had died, was still 72-74 weeks for both, the controls and the
animals exposed to the emissions (Fig.4). This corresponds to an
actual average lifetime of 8o-82 weeks. Contrary to expectations,
the longest average lifetime did not occur among the untreated
controls, but with the hamsters treated with DB(a,h)A.
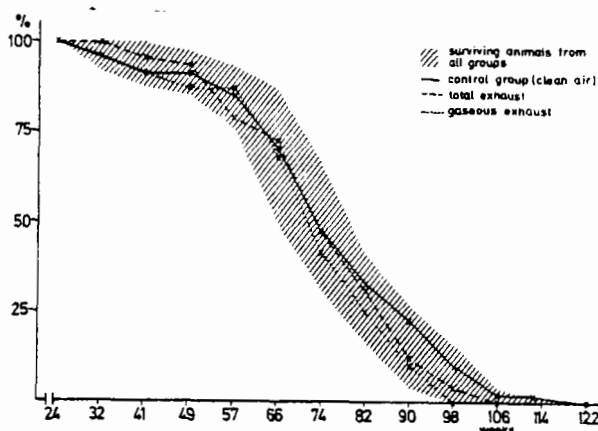
Fig. 4. Surviving hamsters at different times after the start of
the experiment (in %).

Clinical chemistry and hematology
    For the animals not receiving any special pre-treatment, several
hematological parameters as well as a number of enzyme activities
and metabolic products were determined following the 29th, the
42nd, the 55th and the 75th week of the experiment. The different
values at various points in time represent in each case an aver-
age value from 14-2o animals. Lines were drawn through these data
points to yield curves for the individual parameters. The graphs
of the hematological (Fig.5,6) and clinical chemical data (Fig.7,8)
of the control animals and the animals exposed to the total ex-
haust give an example.
The following hematological and clinical chemical parameter were
determined; erythrocytes (ERY), hemoglobin (HB), hemoglobin/ery-
throcytes (HB_E),hematocrit (HCT), mean cellular volume (MCV),

HAEMATOLOGY AFTER 29,42,55 AND 75 WEEKS
CONTROL GROUP
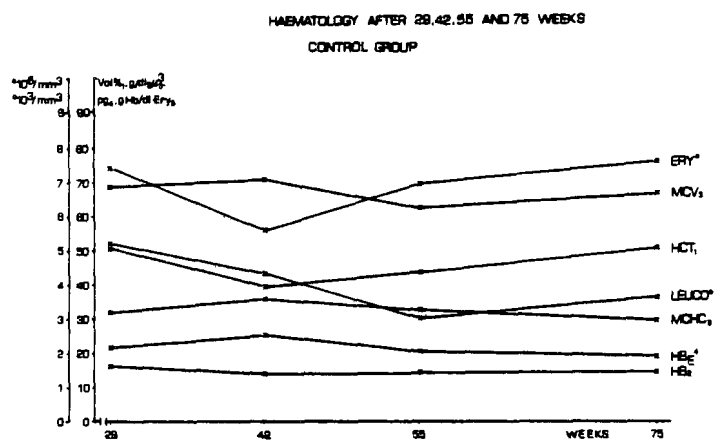
Fig. 5. Haematological data of the control group

HAEMATOLOGY AFTER 29,42,55 AND 75 WEEKS
TOTAL EXHAUST
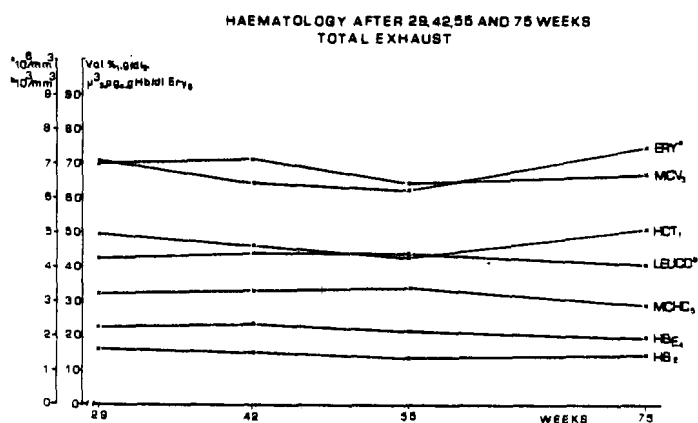
Fig. 6. Haematological data of the group inhaling total exhaust
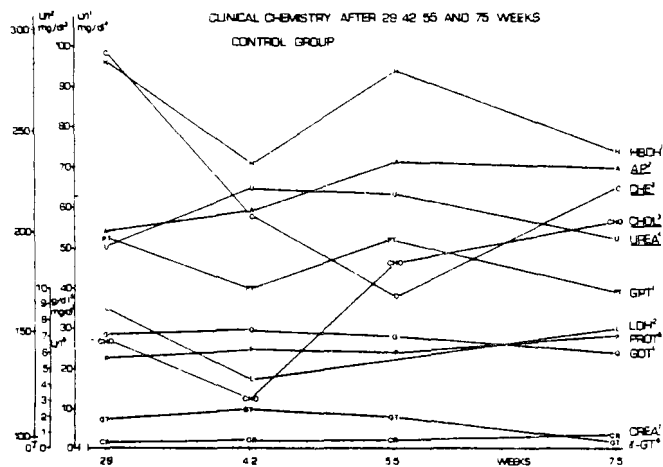
Fig. 7. Clinical chemical data of the control group
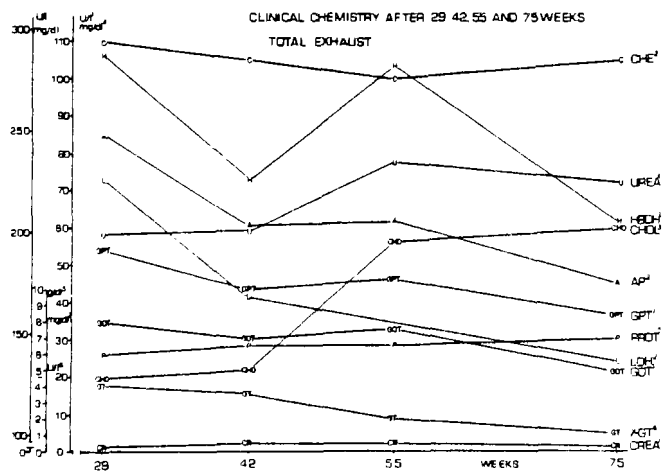
Fig. 8. Clinical chemical data of the group inhaling total exhaust

mean cellular hemoglobin concentrations (MCHC), leucocytes (LEUCO), glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), cholinesterase (CHE), lactic dehydrogenase (LDH), alkaline phosphatase (AP), cholesterol (CHOL), gamma-glutamyl transferase (gamma-GT), alpha-hydroxybutyrate dehydrogenase (alpha-HBDH), blood urea, creatinine (CREAT).

For both, the controls and the exposed animals, it was noticed that several parameters, such as CHE, AP and alpha-HBDH, showed values of great variations, which did not always go in the same direction. At the same time, from about the 3oth or 4oth experimental week on, the controls and the test animals, alike, showed increasingly serious degrees of amyloidosis of the kidneys, adrenals, liver and spleen. Usually the kidneys were affected the earliest and most markedly. In addition, there were liver cysts (enlargements of the bile ducts). Therefore, it appears justified to attribute the observed fluctuations of the biochemical parameters mainly to endogenous organ changes. In view of the fact, that such endogenous organ changes were not detected during the first 3o weeks of the exposure, only the values for the 29th experimental week are analysed here for potential changes due to exposure.

For the animals exposed to the exhaust emissions the results indicate that, after 29 weeks, significantly enhanced values occur for enzymes such as GOT, LDH and AP. The same goes for gamma-GT which was not included in the graphs because of a different scale (Fig. 9). These results give reason to suspect that the inhaled Diesel emissions may have an effect on the liver. The significantly elevated values for blood urea in the animals exposed to Diesel emissions may indicate an impairment of the renal function, but the low creatinine values do not support this interpretation.

Electron-microscopical studies employing ultra-thin sectioning techniques on livers of hamsters exposed to exhaust emissions show, among other things, the degenerative changes of structure in the mitochondria of the hepatocytes[3]. The increase in the mitochondrial and microsomal enzymes, as indicated by our GOT and gamma-GT values may be considered a result of this type of damage to cells or organelles.

The hematological studies in the 29th experimental week revealed
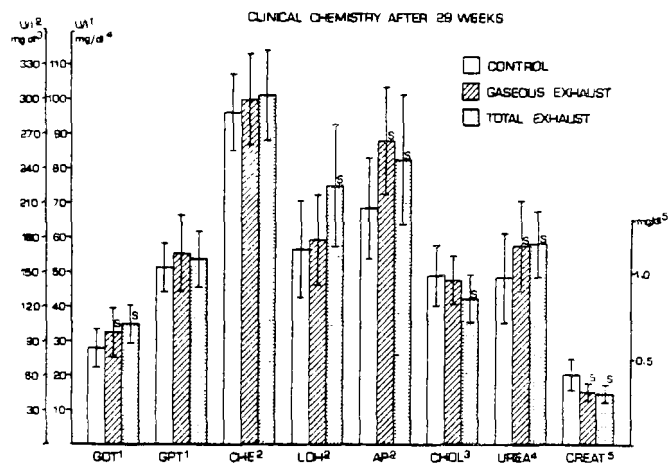
Fig. 9. Some clinical chemical data determined for 2 exposed and 1 control group after the 29th week of the experiment
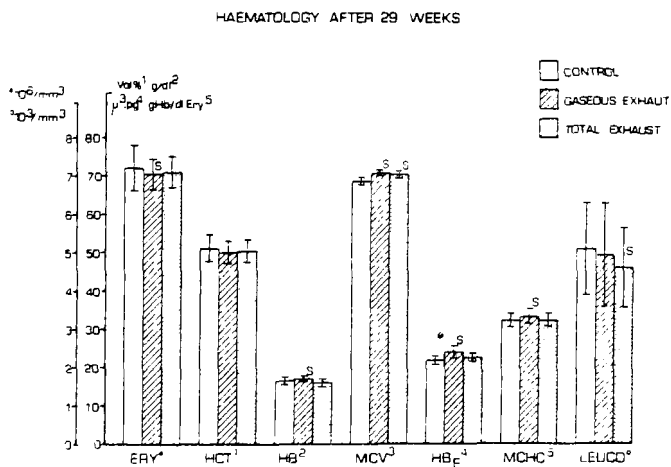
Fig. 1o. Some haematological data determined for 2 exposed and 1 control group after the 29th week of the experiment

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a low erythrocyte count which was accompanied by an increase in erythrocyte volume and a reduced leucocyte count in the animals inhaling Diesel exhaust (Fig. 1o).

Histo-pathology of the respiratory tract

The histo-pathological examination of the respiratory tract yielded the following findings: A lung tumor was found in only 2 out of all of the hamsters used in the experiment. One of these animals belonged to the group that received a total dose of 6 mg DB(a,h)A and was exposed to the particle-free Diesel emissions. The other animal was found in the group which had inhaled the total exhaust and had received an additional injection of 1.5 mg DEN/kg body weight. These animals died after experimental exposures of 75 and 67 weeks, respectively.

According to the independent results of our co-author Friedrich Pott, intratracheal instillation of DB(a,h)A will induce a high degree of lung tumors in the golden hamsters which are available from the Central Institute for the Breeding of Laboratory Animals (TNO) in the Netherlands[4]. By comparison, the animals used in our exhaust inhalation study, which were obtained from the breeding farm of the Hoechst Company, West Germany, appear to be substantially less sensitive to DB(a,h)A. In view of the fact that the same dose and method of application was used, it must be concluded that these two lines of hamster show genetic differences regarding the sensitivity to the carcinogenic action of DB(a,h)A. At present, a suitable experiment is in progress to check this conclusion. This may explain why we failed to produce any enhanced basic tumor rate in the hamsters treated with DB(a,h)A.

A more common observation were some discrete proliferative changes in the lung, 6o % of which were described as adenomatous proliferations. If these cases are expressed in per cent of the total number of investigated animals per experimental group, one arrives at the following result: In all experimental groups exposed to the total exhaust emissions, independent of any additional treatment, the number of animals exhibiting definite proliferations in the lung is significantly higher than for the corresponding experimental groups exposed to particle-free emissions or clean air

(Fig. 11). Furthermore, the percentage of hamsters exhibiting proliferative growth in the lung is particularly high for all experimental groups receiving intratracheal instillations. Nevertheless, even in case of the hamsters treated by instillation, the highest incidence of proliferative growth is always observed in those animals exposed to the total exhaust emissions. In this case, proliferation is seen much more frequently in the experimental groups treated with DB(a,h)A than in animals instilled with pyrene.

With the DEN-dosages used in our study, that is 1.5 and 4.5 mg/kg body weight, basic tumor rates of 13.4 and 44.7 %, respectively, were induced in the larynx/trachea region of the control groups. These tumors proved exclusively to be papillomas. Papillomas have also been observed in inhalation studies with pure B(a)P[5]. In the case of the higher DEN-dosage the tumor incidence increased significantly to 66 % and to 7o.2 % by inhalation of particle-free exhaust and total exhaust, respectively. The difference between the two increased tumor rates however, is not significant (Fig. 12).

The animals which were treated with 1.5 mg DEN/kg bodyweight and exposed to the exhaust emissions, also exhibited a tendency toward increased tumor incidence rates. However, there was no significant difference in the tumor incidence among the three groups of different exposure atmosphere. As expected, the tumor incidence curves for the smaller DEN dosage groups are substantially lower and first papillomas were observed about 2o weeks later than in case of the high DEN dosage.

Among all the experimental groups not treated with DEN, only 5 animals were observed with papillomas in the larynx/trachea region. These 5 animals all belong to the three experimental groups treated with (DB(a,h)A. No animal without special carcinogenic treatment, exhibited any tumor in the respiratory tract, no matter what the inhalation exposure was.
The high DEN dosage which induced a basic tumor incidence rate of 44.7 % in the controls, caused an additional increase of the tumor frequency in conjunction with the total exhaust emissions as well as with the gaseous components alone. Further inhalation studies involving Diesel and gasoline engine emissions are presently under way in our institute to explore in greater detail as to whether this effect is reproducible and significant for a co- or syn-
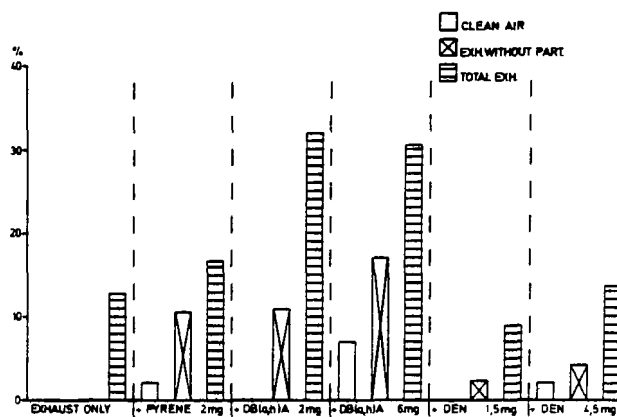
Fig. 11. Percent of hamsters with focal proliferations in the lung.
There are 5 treatment groups (Pyren,DB(a,h)A,DEN) and 1 group
without additional treatment
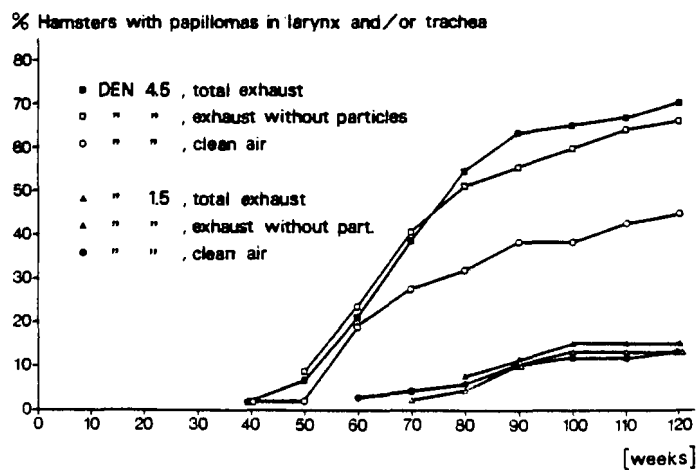
Fig. 12. DEN-treated hamsters with papilloma in larynx/trachea ex-
pressed as a percentage of the animals per group at the start of
the experiment. DEN 4.5 = 4.5 mg/kg b.w.;DEN 1.5 = 1.5 mg/kg b.w.

carcinogenicity of Diesel exhaust, or may be obtained with any
other inhalation burden containing some gaseous components such
as NO_2 and SO_2. It will further be checked whether the effect will
also appear in combination with other carcinogens affecting the
respiratory tract. The basic tumor incidence rate of 13.4 %, as
induced by the small DEN dosage, may possibly not have fallen into
the steep slope range of the dose dependent tumor frequency curve.
This would explain why no statistically measurable change in the
tumor incidence rate could be observed.

Pulmonary function tests

    The pulmonary function tests have been conducted on female Wistar
rats, which were exposed to the exhaust for up to 24 months under
the same conditions as the hamsters.

    After an exposure period of approximately 18 months, these rats
were subjected to tests in order to determine the lung clearance
rate for a hematite iron oxide aerosol ($^{59}Fe_2O_3$). The iron was
radioactively labelled and the insoluble aerosol was inhaled for
a short period of time so that a fraction would deposit in the
respiratory tract. At various times after the inhalation of the iron
oxide aerosol, the activity over the thoracic lung area was measured
and recorded as a percentage of the activity measured on the first
day. After being exposed to the $^{59}Fe_2O_3$-aerosol the animals con-
tinued to inhale the Diesel exhaust. As can be seen from the de-
creasing exponential curves for the activity measured between the
15th and the 7oth day following the iron oxide inhalation, the
long-term alveolar clearance is definitely impaired by the total
exhaust emissions and also, to a somewhat lesser degree, by the
gaseous phase of the exhaust (Fig. 13,14) The biological halflife
of the iron oxide deposit in the lungs of the animals exposed to
the non-particulate components of the exhaust is about 4o % longer
than in case of the controls. For the animals exposed to the total
exhaust emissions, the halflife is nearly twice as long as in the
controls.

    For the rapid phase of lung clearance, the biological halflife
was not measured because the short-term clearance which is
primarily determined by the bronchial clearance mechanism, is
practically completed within 15 days after iron oxide exposure and
could not accurately be measured on account of the small numbers

CLEARANCE OF $^{59}Fe_2O_3$ FROM RAT LUNGS

x diesel exhaust
$T_{1/2} = 92.4 \pm 14.3$ days
$Y = 58.4\, e^{-0.0075t}$
$R^2 = 0.8387$

o control group
$T_{1/2} = 46.5 \pm 4.2$ days
$Y = 47.7\, e^{-0.0149t}$
$R^2 = 0.9400$
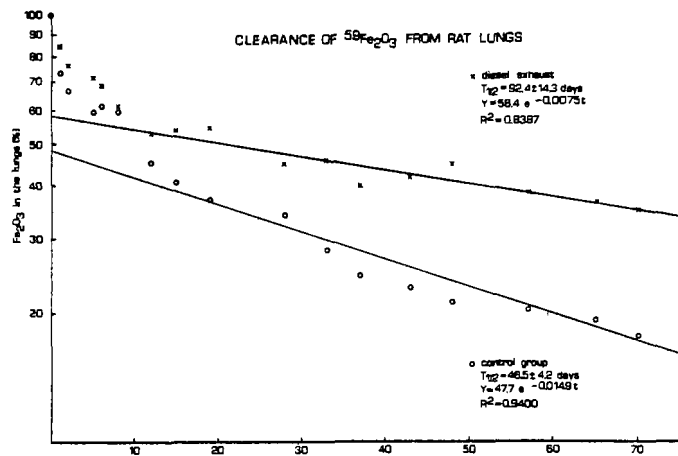
Fig. 13. Lung clearance of $^{59}Fe_2O_3$-particles in rats exposed to total Diesel exhaust.

CLEARANCE OF $^{59}Fe_2O_3$ FROM RAT LUNGS

x diesel exhaust without particles
$T_{1/2} = 81.8 \pm 5.8$ days
$Y = 40.7\, e^{-0.0085t}$
$R^2 = 0.9615$

o control group
$T_{1/2} = 57.3 \pm 5.3$ days
$Y = 51.7\, e^{-0.0121t}$
$R^2 = 0.9359$
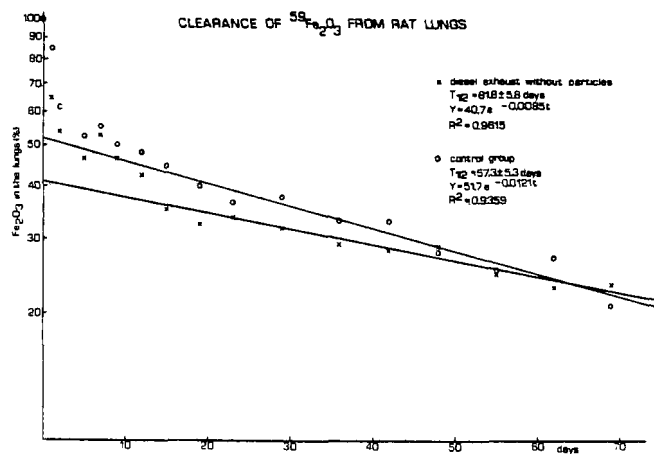
Fig. 14. Lung clearance of $^{59}Fe_2O_3$-particles in rats exposed to Diesel exhaust without particles.

of animals involved. However, it can be seen from the distribution of the measured values within the first 15 days that animals exposed to the particle-free emissions show a better degree of bronchial clearance activity than the corresponding control animals. The animals exposed to total exhaust emissions exhibit a lower bronchial clearance activity than the controls.

No significant changes of respiratory rate, respiratory minute volume or compliance and resistance, as measured with a whole body plethysmograph, were observed in any of the test animals. This applies to exposures for over 2 years to the gaseous exhaust components as well as to the total emissions (Fig.15)

	Control	Gaseous exhaust	Total exhaust
Respiratory rate breath/min.	49,6 ± 12,2 (50,6 ± 12,2)	47,6 ± 22,6 (44,9 ± 21,2)	57,5 ± 18,0 (56,4 ± 17,8)
Tidal volume ml	2,6 ± 0,51 (2,6 ± 0,43)	2,9 ± 0,61 (3,1 ± 0,90)	2,6 ± 0,64 (2,5 ± 0,52)
Minute volume ml	127,5 ± 32,1 (132,0 ± 22,5)	138,3 ± 74,5 (131,2 ± 52,8)	147,5 ± 39,5 (137,9 ± 40,7)
Intrapleural pressure cm H₂O	4,0 ± 0,77 (4,7 ± 0,92)	4,6 ± 1,1 (6,1 ± 2,0)	4,0 ± 0,97 (4,1 ± 1,0)
Compliance ml/cm H₂O	0,82 ± 0,25 (0,71 ± 0,14)	0,79 ± 0,15 (0,82 ± 0,31)	0,84 ± 0,27 (0,78 ± 0,26)
Resistance cm H₂O/ml·sec⁻¹	0,14 ± 0,03 (0,16 ± 0,03)	0,19 ± 0,16 (0,25 ± 0,18)	0,14 ± 0,05 (0,17 ± 0,09)
Breathing time quotient T exp./ T insp.	0,53 ± 0,14 (0,58 ± 0,19)	0,53 ± 0,15 (0,72 ± 0,25)	0,60 ± 0,13 (0,56 ± 0,16)
Heart rate Pulse/min.	317 ± 41,5 (323 ± 37,7)	334 ± 43,5 (324 ± 42,0)	305 ± 32,9 (309 ± 33,5)

with acetylcholin inhalation ( )
Acetylcholin concentration = 0,3%
Exposure time about   1/2 minute

Fig. 15. Pulmonary function test of anesthetised rats after 2 years exposure with total Diesel exhaust or gaseous exhaust without particles.

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Even when briefly inhaling an acethylcholin aerosol prior to the measurements of pulmonary function, the test animals did not react more sensitively than the control animals. It thus may be concluded that, even after long term inhalation of dilute Diesel-engine exhaust, a significant impairment of the mechanical pulmonary functions is not observed.

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MORPHOMETRIC ULTRASTRUCTURAL ANALYSIS OF ALVEOLAR LUNGS OF GUINEA PIGS
CHRONICALLY EXPOSED BY INHALATION TO DIESEL EXHAUST (DE)

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INTRODUCTION

Increased usage of diesel engines is expected in the future to meet energy
needs for industrial, home and recreational needs. Concern about possible
health effects of diesel exhaust (DE) is reasonable. Most studies have focused
on cancer risk but there is presently no statistically significant difference
in tumor incidence in controlled laboratory animal experiments[1].

Diesel emissions have a gaseous phase similar to emission controlled gasoline
engines. But, it is established that diesel engines emit 50 to 80 times more par-
ticulates (DEP) than do gasoline engines[2]. Most if not all of the particulates
are in the respirable range with particulate size near 0.3 μm^3.

In assessment of the health risks of DE, it seems especially pertinent to
understand the lung's response to a chronic burden of DEP. The physiologic and
pathologic impacts of DE exposure on the lung can be defined by a systematic
ultrastructural approach. Our group began such a study three years ago in coop-
eration with the General Motors Biomedical Laboratory. We are studying the
effects of acute and chronic inhalation of DE (250 - 6000 μg DE/m^3) in Fischer
rats and Hartley guinea pigs exposed for periods up to 2 years. Several publi-
cations are anticipated as necessary to adequately describe our results and
make interpretations of the massive data bank. Preliminary reports have been
published[4-6]. The present report documents the impact of DE on pulmonary ultra-
structure of alveolar lung of guinea pigs subjected to chronic exposure of DE
from 2 weeks to 2 years. Morphometric approaches are used to quantify selected
lung parameters.

MATERIALS AND METHODS
Animals

Male Hartley guinea pigs (about five weeks old) from Charles River Breeding
Laboratory, were housed at the General Motors Biomedical Research Laboratory
(GMBL), Warren, MI for an initial quarantine period of two weeks prior to enter-
ing the controlled exposure regimens at GMBL[7]. Individually caged pigs were

placed in either a clean air environmental chamber or chambers receiving freshly diluted diesel engine exhaust to achieve particle concentrations of either 250, 750 μg, 1500 μg or 6000 μg/m^3. Chamber temperature was 22 $\pm$ 2°C with 56 $\pm$ 6% relative humidity. During cleaning periods the animals were rotated to ensure equal exposures. Food and water were always available.

Fasted guinea pigs (3 per exposure group plus age matched controls) were delivered at Wayne State University for sacrifice, dissection and tissue processing for the ultrastructural studies. These 64 animals were assigned code numbers which were unknown to investigators.

Exposure conditions and characteristics of DE. The animals were exposed by inhalation to either clean air or DE air for 110 hr/week.

The DE exhaust was emitted by a production 1978 Oldsmobile 5.7L, four cycle, indirect injection diesel engine. It was run at steady speed and load to simulate a 65 km/hr (40 mph) cruise situation. Amoco type 2D federal compliance fuel and Amoco 200 30W lubricating oil were used. Well dispersed DE particulates were delivered uniformly to the inhalation chambers in an air flow of 2.8L/min. The mass median aerodynamic diameter of the DE aerosol was 0.19 $\pm$ 0.03 μm with 88 $\pm$ 5% of the mass in particles smaller than 1 μm[7]. Details of exposure conditions and monitoring of particulate and gas concentrations have been published[7]. The average particulate mass concentrations were within 2% of the target dose value.

Animal and tissue preparation. Guinea pigs were anesthetized with an intraperitoneal injection of sodium pentobarbital. A tracheotomy was done and a plastic cannula (Abbott Butterfly -19 set without needle) was secured in the trachea. After collapsing the lungs by letting air enter the thoracic cavity, the intact lungs were reinflated and fixed in situ by instillation of pH 7.4 cacodylate buffered 1% glutaraldehyde of 300 mOsms. This fixative fluid was instilled at a pressure of 20 cm H$_2$O above the hilum to achieve a normal expansion of the lungs. The trachea was tied to insure preservation of the intrapulmonary fixative volume and the still intact lungs were removed from the chest and placed in fresh fixative. Lung volumes were determined after removal of extrapulmonary structures, using volumetry following the submersion method of Scherle[8]. Lungs remained submerged in glutaraldehyde for 18-24 hr prior to selection of tissue samples for microscopy.

Tissue slices (0.5 x 1 cm) were taken for the scanning electron microscopy (SEM) study which were used to establish the alveolar parenchymal lung volume[6]. Specimens were prepared according to published procedure.

The transmission electron microscopy (TEM) specimens (cubes 0.2 cm thick) were cut from tissue slices of the right middle lobe and left lower lobe. An admixture of cubes represented dorsal and ventral parts, exclusive of pleural edges. At least 5 cubes from each animal were selected randomly and processed by conventional procedure[6]. Semi-thin sections (0.5 - 1.0 μm) and ultrathin sections (about 60 nm) were cut using a diamond knife. The semi-thin sections were stained with methylene blue and viewed in a light microscope for orientation purposes; eg., to be certain the specimen was mostly alveolar lung and not conducting airway or large blood vessels. Ultra thin sections, doubly stained with uranyl acetate and lead citrate, were examined and photographed in an electron microscope operated at 50 kV.

Details regarding morphometry. Evaluations were made on micrograph data banks developed from systematic photography of one nearly perfect section from each of five randomly selected blocks of alveolar lung per animal (Fig. 1). A minimum of 100 photographs was taken per animal using two levels of initial magnification (X 2000 and X 4000) which were enlarged to final magnifications of X 5000 and X 10000 for use in point counting, intersect counting and linear measurements.

For determinations of volume density and surface density of the major tissue components a plastic overlay (Weibel coherent multipurpose test system of 168 test points[9]) was placed over the X 5000 micrographs. The distribution of points falling on structures and the surface intersects with certain cell surfaces were counted and recorded using a Zeiss MOP 3.

We used Hally's method of relative standard error as a guide to the minimum number of points to count for an accurate estimate of the volume density of the cell type expected least often[10]. To achieve a relative standard error of 5%, if the cell type of interest (eg., alveolar macrophages) comprises 2% of the total cells, a total point count of 19800 is required. But a 7600 total point count should accurately reveal distribution when the cell type is 5% of total cells.

Mean caliper diameters for all nuclear types were calculated based on the modeling of cell nuclei as oblate spheroids. The method of Cruz Orive[11] was used for this purpose. The major and minor axis of each complete nuclear profile was measured using the Zeiss MOP 3. With this information, the mean caliper diameter ($\bar{D}$) for each type of cell nucleus was estimated by the formula:

$$\bar{D} \sim \frac{\pi}{2} \, n \left[\sum_{i=1}^{n} \frac{1}{M_i} (1 - y_i^2)^{5/2} q(y_i \mid 0, -2, 0) \right]^{-1}$$

where: $y_i^2 = 1 - \left[\dfrac{m_i}{M_i}\right]^2$

m_i = the major axis of the ith elliptical profile

M_i = the minor axis of the ith elliptical profile

n = the number of profiles measured

$$q(y_i \mid 0,-2,0) = \dfrac{2}{\pi \sqrt{1 - y_i^2}} \int_0^{\pi/2} (1 - y_i^2 \sin^2\theta)^{-5/2}(1 + 2y_i^2 \sin^2\theta)\,d\theta$$

The above integral for q was evaluated using four point Gaussian Quadrature.
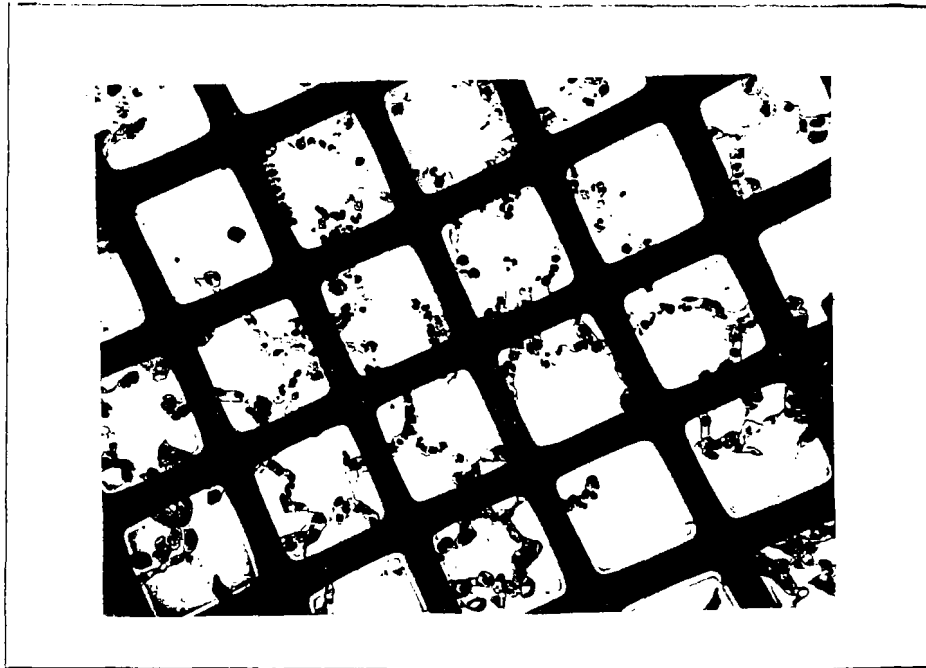
Fig. 1. Low power view of an area of an electron microscope grid bearing a thin section of alveolar lung. Random photographs are taken to develop the micrograph data base for morphometry. Note that neither large blood vessels nor terminal airway are present. From about 40 grid views of this perfect section of lung, 15-20 low power micrographs are taken at random per block of tissue.

Harmonic mean thickness and diffusion capacity were determined using another plastic overlay (square lattice of 6 horizontal and 8 vertical lines). This was placed over the X 10000 micrographs for direct linear measurements (Zeiss MOP III) of the length of the intercept line crossing from the epithelial to the endothelial surface and from the endothelial surface to an erythrocyte surface.

Formulae used for morphometry calculations are listed. Derivation of formulae and complete discussion of rationale for applying these to analyzing lung are given by Weibel[9].

Volume Density (V_{vi}) P_i/P_t

Surface Density $(S_{vi}) - 2\ I_i/L_t$

Arithmetic Mean Thickness $(\tau_i) = Z \cdot P_i / 2 \cdot I_i$

Harmonic Mean Thickness $(\tau_{hi}): \dfrac{1}{\tau_{hi}} - \dfrac{3}{2n} \sum_{i=1}^{n} \dfrac{1}{L_i}$

Diffusion Capacity $(D_L): \dfrac{1}{D_L} \quad \dfrac{1}{D_t} + \dfrac{1}{D_p} + \dfrac{1}{D_e}$

Identification of symbols: P_i (number of points falling on item of interest), P_t (total points); I_i (number of intersections with item); L_t (test line length); Z (line length in Weibel multipurpose test system based on an equilateral rhombus); L_i (length of intercept); D_t (diffusion capacity tissue); D_p (re plasma); D_e (re erythrocyte).

Computations were run on the Wayne State University Computer (Michigan Terminal System Operating System), which was also used for raw data storage, primary and secondary parameter computation, manipulation and storage. Confidence limits for quantitative evaluation procedures were computed using the Student's t-test.

RESULTS

Histopathology. Even upon gross inspection of the lungs it was apparent which animals had received DE exposure. Such lungs were dusty grey in color with punctate blacker regions. By light microscopy the most notable sign of DE exposure was the scattering of pigmented macrophages which were occasionally clustered at ends of terminal bronchioles. Pigmentation was also observed in lymphatics near bronchiolar-alveolar junctions. There was an increase in such pigment deposits related to both duration and exposure dose. By 6 months at 750 μg DE there was beginning fibrosis in regions of macrophage clusters and evidence of focal epithelial type 2 cell proliferation. Occasional eosinophils were present although tissue reaction was not prominent. One incidental adenoma

occurred in an animal exposed for 24 months to 250 μg DE. Bronchial and bronchiolar epithelium appeared normal.

Ultrastructure of alveolar lung. The general organization of the lung was not appreciably altered. Alveolar epithelium retained tight junctions. Two cell types of the alveolar parenchyma (epithelial type 1 cells and macrophages) and one type of granulocytic leukocyte (eosinophils) phagocytized DEP. The DEP was stored within phagosomes of alveolar macrophages (Fig. 2). Occasionally a macrophage (reactive monocyte) with multilobulated nucleus was noted.
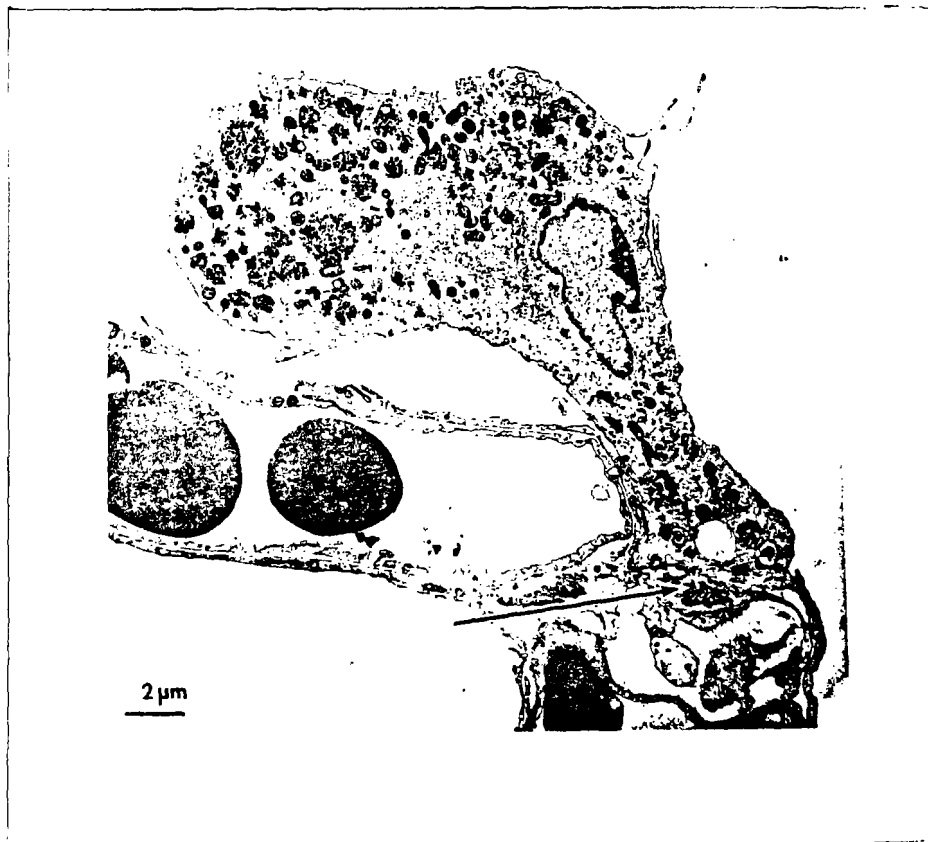
Fig. 2. Alveolar macrophage with DEP contained within phagocytic vesicles. Also note (arrow) DEP in Epi type 1 cell from 3 month 1500 μg DE exposure.

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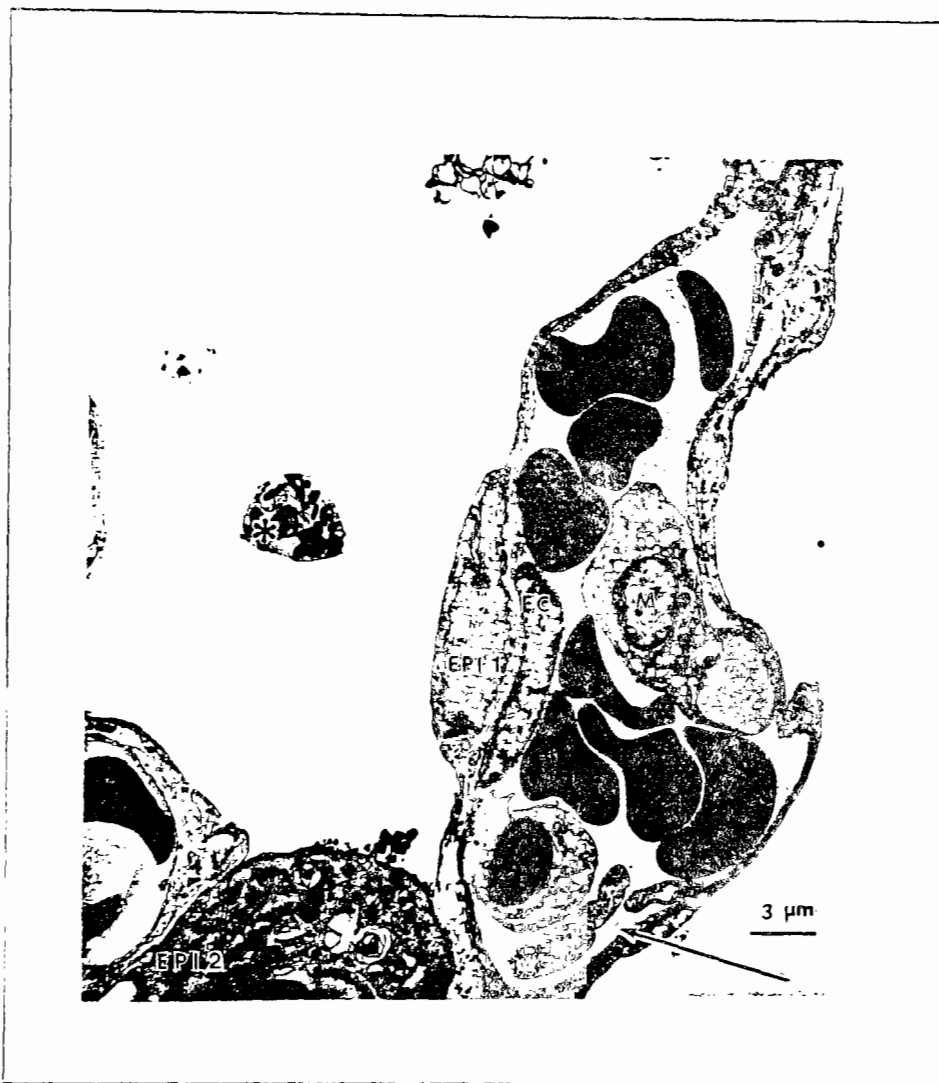
Fig. 3. Micrograph from 3 month 750 µg DE exposure has an eosinophil* in air-
space but the lacy constituents around the specific granules are indicative of
granule degeneration. Note Epi 1 and Epi 2 cell comprise alveolar wall and that
an endothelial cell (EC) nucleus shows in right hand capillary. Mononuclear (M)
cells within this capillary have phagocytized RBC. Platelets (arrow) also are
present. The debris in upper right alveolar space is secretory product from
Epi 2 cells.

Eosinophils emigrated into the alveolar airspace (Fig. 3). Occasionally they contained DEP within phagosomes. Increasing numbers of epithelial type 1 cells (Epi 1) contained DEP, illustrating a DE dose dependency (Fig. 2,4,5). Neither epithelial type 2 cells (Epi 2) nor endothelial cells took up DEP.
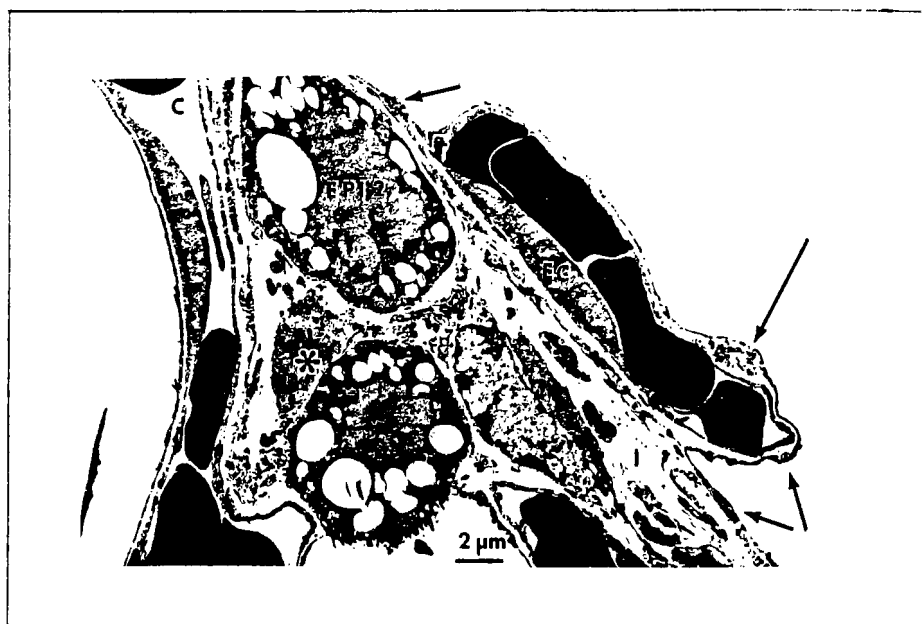
Fig. 4. Epi 1 uptake of DEP (arrow) in a specimen from 24 month 250 μg DE exposure. Interstitial cell* also has DEP. Symbols: EC=endothelial cell, I= interstitium, C=capillary.

Especially notable at DE exposures greater than 250 μg was the increase in cellular composition of the interstitium in contrast to the age matched controls (Fig. 2,4). Fibroblasts, monocytes, eosinophils, plasma cells and macrophages were identified more often within the perivascular and peribronchiolar interstitium than in alveolar walls. Interstitial macrophages sequestered DEP within membrane lined vesicles (Fig. 4); more often this was seen in the higher dose/duration animals. DEP was not seen scattered among the fibrillar components of the interstitium.
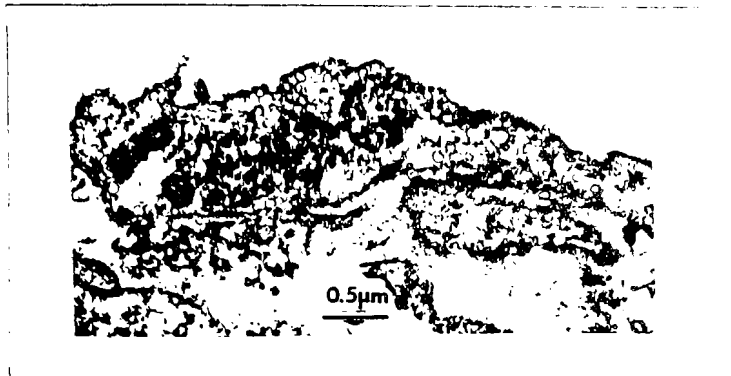
Fig. 5. Uptake of
DEP by Epi 1 in
alveolar lung of
a 2 week expo-
sure to 750 µg
DE/m^3.

Hypertrophy and proliferation of Epi 2 cells was observed in certain alveoli walls (Fig. 6) even by 2 week exposure to 750 µg DE/m^3. With increasing DE dose/duration, Epi 2 cell clusters occurred in some alveoli. Excessive secretory products also were noted in alveolar spaces (Fig. 3) but were particularly prominent after 1500 and 6000 µg DE.

Physiologic features of animal groups. Experimental groups and their age-matched, generally concurrent, control groups had approximately equivalent body weights and lung volumes. For example, for the 12 mon sets, body weights were 1041 $\pm$ 140, 1044 $\pm$ 81, 1146 $\pm$ 121 and 1083 $\pm$ 85 g while lung volumes were 19.0 $\pm$ 1.9, 21.7 $\pm$ 2.4, 23.4 $\pm$ 2.4 and 23.7 $\pm$ 3.4 for respectively the control, 250 µg, 750 µg and 1500 µg DE groups. These physiologic parameters as well as certain of the morphometrically determined lung parameters are age related.

Harmonic mean tissue thickness (τ_{ht}) of the air-blood barrier. The τ_{ht} did not change appreciably for control guinea pigs of 2 mon to 52 weeks of age. The mean value of these controls was 0.552 $\pm$ 0.09 µm. Significant (p < 0.05) changes in τ_{ht} occurred in the DE exposed animals primarily through 6 mon.

Arithmetic mean tissue thickness (τ_t) of the air-blood barrier. Exposure to 750 µg DE/m^3 resulted in a significant increase (p < 0.05) in τ_t at all sacrifice times, to 1 year but τ_t dropped from a peak of 2.46 $\pm$ 0.43 µm to 1.67 $\pm$ 0.18 µm. In a similar way the 1500 µg DE set decreased from a peak τ_t of 2.56 $\pm$ 0.30 at 6 mon to 1.88 $\pm$ 0.34 µm after 18 mon exposure.

Morphometric Diffusion Capacity (D_L). As expected D_L varied in relationship to body weight and age of the guinea pigs; for example the 6 wk old pigs D_L = 0.09 $\pm$ 0.13, the 19-22 wk pigs D_L = 1.66 $\pm$ 0.18, the 30-34 wk pigs D_L = 3.2 $\pm$1.1,

the 46–48 wk pigs D_L = 3.84 ± 0.46, and 60 wk pigs D_L = 3.7 ± 0.46. DE exposure appeared to increase D_L about 37%.
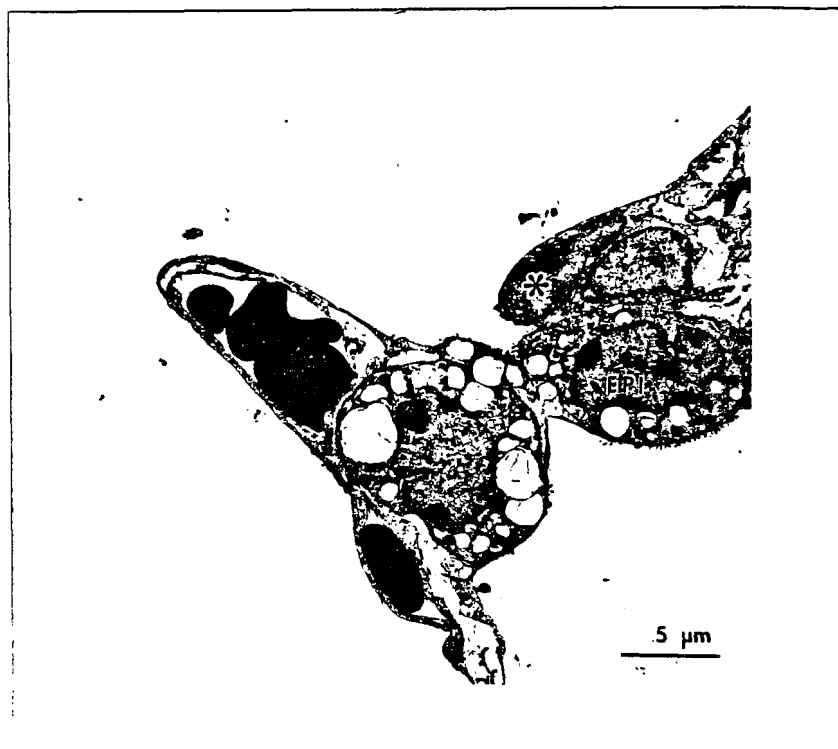
Fig. 6. From an animal exposed for 12 months to 750 µg DE, note proliferation of Epi 2 cells with their microvilli and secretory vesicles (EM preparative artifact leached out the lamellar bodies). On the right upper alveolar wall is a transitional epithelial cell* which is classed as an Epi 1 cell because it does not show distinctive features of Epi 2 cells.

Nuclear diameters. The mean caliper diameters were computed as specified previously. Four thousand nuclei were measured with 2090 controls and 2023 from DE exposed animals. The dimensions for controls were 7.22, 7.17, 7.56, 7.60 and 6.18 µm for Epi 2, endothelial cells, Epi 1, alveolar macrophages and interstitial cells respectively. The 250, 750 and 1500 µg DE sets were not significantly different through 1 year. However, endothelial cell nuclei appeared smaller in the 4 mon 6000 and 18 mon 1500 µg DE sets as well as in 21 and 24 mon animals. From the mean caliper diameters one can calculate absolute volumes and numerical density for the parenchymal cell types.

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Morphometric studies on the alveolar lung parenchyma. From point and inter-sect counts the fractional volumes, surface areas and numerical densities were established for each cell or tissue compartment of the alveolar lung. The percentage differences between controls and the 750 µg DE/m^3 sets are shown in Tables 1-3.

TABLE 1
EFFECTS OF DIESEL EXHAUST (750 =6/M^3) EXPOSURE ON MORPHOMETRIC PARAMETERS OF CELLS IN ALVEOLAR LUNG

ALVEOLAR LUNG CELL TYPES	PERCENTAGE CHANGE FROM CONTROL AFTER 750 =6 DEP EXPOSURE				
	2 WK	3 MON	6 MON	9 MON	12 MON
ENDOTHELIAL CELLS					
V (ml/cm^3)	+ 45.7	+ 21.2	0.0	+15.1	- 3.6
S (cm^2/cm^3)	+ 21.9	+ 22.2	+25.0^a	+23.9	- 6.0
N (N/cm^3)	+ 64.6^A	+ 36.8^A	+ 4.6	+45.7^A	+ 39.6^B
EPITHELIAL TYPE 1					
V (ml/cm^3)	+ 24.1	+ 15.6	+20	-10.3	0.0
S (cm^2/cm^3)	+ 31.9	+ 6.4	+14.9	+29.9	+ 1.7
N (N/cm^3)	+ 44.4^A	- 33.3^A	-21.4	+44.4	+ 44.4^B
EPITHELIAL TYPE 2					
V (ml/cm^3)	+ 75.0^B	+113.6^B	- 3.6	+46.8^B	+ 62.5
S (cm^2/cm^3)	+118.4	+126.0^B	-28.4	+18.0	+ 12.3
N (N/cm^3)	+227.8^A	+105.5^A	- 6.9	+96.9^A	+108.0^A

A,B STUDENT'S "T" TEST ESTABLISHED SIGNIFICANT DIFFERENCES IN THE PRIMARY DATA AT A = P < 0.05 AND B = P < 0.01.

TABLE 2
(INFLUENCE OF DIESEL EXHAUST INHALATION ON INTERSTITIUM & ALVEOLAR MACROPHAGES OF ALVEOLAR LUNG

EXPOSURE SPECIFICS TIME-DE$_{µ6}$	PERCENTAGE CHANGE FROM CONTROL					
	INTERSTITIUM				ALVEOLAR MACROPHAGES	
	V_{TI}	V_{NCI}	V_{CI}	N_{CI}	V_{AM}	N_{AM}
2w-750	+ 48.8^a	+ 44.4^a	+ 52.0^a	+127.8^a	+ 85.1	+132.0^A
3w-750	+ 26.1	+ 29.6	+ 21.0	+ 6.5	+ 25.0	- 47.7^A
3w-1500	- 21.7	- 58.8^a	0.0	- 2.2	+ 77.8	+ 19.3
4w-6000	+ 68.7^B	+ 15.8	+146.3^A	+214.3^A	+300.0^A	+141.1^A
6w-750	+ 13.3	+ 33.3	- 4.2	- 16.0	+ 1.3	- 35.6
6w-1500	+186.7^A	+195.2^A	+108.2^A	+266.0^A	+312.5^A	+344.0^A
9w-250	+ 33.3^A	+ 75.0^A	0.0	+ 2.3	+150.0^A	+152.9^A
9w-750	+ 35.5^B	+ 60.0	+ 16.0	+ 12.8	+ 14.3	+193.0
12w-750	+ 58.5^A	+ 23.8	+108.0^A	+ 53.3^A	+125.0^A	+171.0^A
18w-1500	+ 60.0^A	+ 57.2	+ 75.0^B	+ 64.1^A	+567.0^B	+595.0^A
24w-250	0.0	- 4.3	- 3.3	- 10.2	+ 75.0	+ 34.1

A,B STUDENT'S "T" TEST ESTABLISHED SIGNIFICANT DIFFERENCES IN THE PRIMARY DATA AT A = P < 0.05 AND B = P < 0.01

Even as brief a DE exposure as 2 weeks increased tissue volume, (V_T), over the age matched controls. For the 750 µg DE exposure sets, V_T increased 36% after 2 weeks, 46% after 3 mon and remained near 35% through the year's exposure. The DE inhalation of 1500 µg resulted in a significant increase (p < 0.01) in V_T to 112% at 6 mon and still exceeded controls by 81% after 18 months exposure. Four months exposure to 6000 µg DE evoked a 68% increase in V_T. In contrast, 250 µg DE sets were not significantly increased at 9 or even 24 mon exposures.

Subdivision of the tissue into the individual cellular components reveals some interesting changes. For the 750 µg DE sets, volume density (V), surface density (S) and numerical density (N) were significantly increased for Epi 2 cells but a time related reduction occurred (Table 1). A similar though not as spectacular change occurred with endothelial cells. A different pattern of response by interstitial cells occurred with the early increase followed by reduced volume density from 3 through 9 mon but significantly increased by 12 mon exposure (Table 2). The non-cellular interstitium (NCI) was not significantly changed during the exposure.

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Considering the interstitial responses to DE further, the 1500 µg DE sets showed increased volume density after 6 mon followed by a time related reduction, that after 18 mon exposure still exceeded control values (Table 2). The 6000 µg DE/m^3 exposure for 4 mon resulted in a 69% increase in total interstitium (V_{TI}) with the significant change being an increase of 146% in the fractional volume of cellular interstitium (V_{CI}). When nuclear diameters were taken into account, this value translated into a 214% increase in numerical density of interstitial cells.

TABLE 3

MORPHOMETRIC CHANGES IN MAJOR LUNG CELLS:
COMPARISON OF CONTROLS WITH THE 750 µg DE SETS

ALVEOLAR LUNG	2 WK		3 MON		6 MON		9 MON		12 MON	
	C	DE	C	DE	C	DE	C	DE	C	DE
EPITHELIAL TYPE 2										
TOTAL NO. X 10^6	18.4	58.0^A	18.6	37.4^A	29.0	26.8	32.2	63.4^B	25.5	51.7^A
AVG. VOL..,µM^3	875±125	476±100^A	1183±621	1257±610	966±239	1007±280	885±216	647±137	941±272	760±113
AVG. SURFACE AREA,µM^2	49± 16	107± 29^B	50± 23	113± 72	95± 28	68± 25	89± 15	105± 20	106±24	119± 26
% TOTAL LUNG CELLS	11.7	18.5	12.3	20.8	17.3	17.2	20.3	25.9	20.5	25.3
% ALV. SURFACE COVERED	7	11.1	6.8	13.4	5.0	8.4	10.6	9.9	11.4	12.5
INTERSTITIAL CELLS										
TOTAL NO. X 10^6	51.3	115.7^A	46.5	48.6	49.8	68.5	43.1	53.3	30.2	45.9^A
AVG. VOL..,µM^3	487±113	328± 60^B	419±124	473± 95	484± 63	445± 83	476± 28	544±141	391± 96	545±163
% TOTAL LUNG CELLS	32.8	36.5	30.9	27.0	29.7	26.8	27.2	21.8	24.3	22.6
ALVEOLAR MACROPHAGES										
TOTAL NO. X 10^6	4	9.3^A	8.8	4.6^A	9.9	7.3	4.2	12.3	6.9	18.7^A
AVG. VOL..,µM^3	1175±535	935±591	1000±427	2391±1131^B	768±280	1055±364	881±344	1301±611	1203±503	941±235
% TOTAL LUNG CELLS	2.6	2.9	5.8	2.6	5.9	4.8	2.6	5.0	5.6	9.2
ENDOTHELIAL CELLS										
TOTAL NO. X 10^6	64.8	107.0^A	55.3	75.1^A	64.9	68.5	69.9	101.8^A	52.6	74.7^B
AVG. VOL..,µM^3	548± 82	383± 59^A	597±115	633±162	468± 61	445± 84	476± 28	374± 39^B	525± 73	363± 34^A
AVG. SURFACE AREA,µM^2	470±572	573± 73	559±134	683±146	840±110	630± 69^B	772± 62	932± 67^A	837± 80	787± 71
% TOTAL LUNG CELLS	41.4	33.7	36.8	41.8	38.8	44.0	44.0	41.7	42.4	36.6
EPITHELIAL TYPE 1										
TOTAL NO. X 10^6	18.0	26.3^A	21.3	14.1^A	13.9	11.2	9.3	13.5	8.9	12.7^B
AVG. VOL..,µM^3	1622±237	1361±187	1502±325	2624±482^A	1835±229	2680±516^B	3160±305	2348±235^B	2921±513	2055±128^B
AVG. SURFACE AREA,µM^2	648± 97	855±194^B	686±150	730± 99	870± 92	740± 81	747± 60	952± 62^A	821± 95	835± 66
% TOTAL LUNG CELLS	11.5	8.4	14.2	7.8	8.3	7.2	5.9	5.6	7.2	6.3
% ALV. SURFACE COVERED	93	88.9	93.2	86.6	90.1	91.6	89.4	90.1	88.6	87.5

^{A,B} Student's "t" test is statistically significant at A = p < 0.05 and B = p < 0.01. Primary data are given as Mean ± S.E.M. C = control.

The fractional volume and numerical density of alveolar macrophages were elevated over control values after most DE exposure doses and durations (Table 2). The greatest increases occurred after 1500 and 6000 μg DE exposures.

Selected absolute changes in morphometric parameters for the responses of parenchymal cells to the 750 μg DE challenge are given in Table 3. A striking feature for all cell types was the increased cellularity. Since comparisons were with age-matched controls these findings appear DE and not age related changes although the latter are documented in Table 3. During DE exposure Epi 2 cells doubled or tripled the control numbers while their cell volumes fluctuated and they generally covered a greater surface area of the alveolar wall than control cells. The total number of Epi 1 cells/cm^3 was not reduced by this exposure regimen. Interstitial cells, a heterogeneous group of different cell types, were particularly increased after the 2 wk exposure; eg., about 116 million/cm^3 occurred to contrast with 51 million/cm^3 in the matched control. Endothelial cells were increased in number and had significantly smaller average cell volumes.

Translation of the numerical density data to absolute pulmonary cell numbers per animal was done. Graphic display of certain data from the 750 μg sets permits contrast of controls and DE exposures for 1 year (Fig. 7).

CHANGES IN TOTAL ALVEOLAR LUNG CELLS
IN GUINEA PIGS EXPOSED CHRONICALLY
TO 750ug DE/m^3
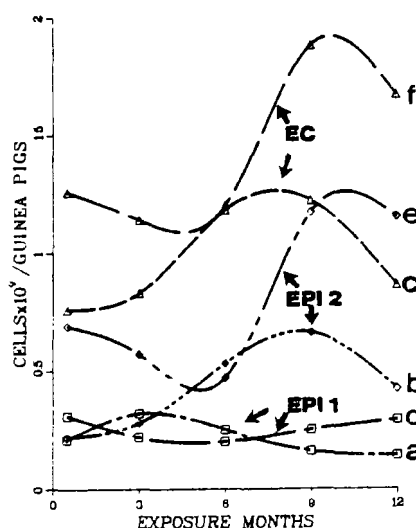
Fig. 7. Age-related changes are shown in the control curves for each cell type; eg. a = Epi 1, b = Epi 2, c = endothelial cells (EC). Effects of DE exposure are designated in the other 3 curves; eg. d Epi 1, e Epi 2 and f = endothelial cells (EC).

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About 230 million Epi 1 cells were in the guinea pig lungs regardless of age up to one year. The DE exposure did not reduce that number significantly. Age related changes occurred in Epi 2 and endothelial cells. The youngest guinea pigs had in their lungs about 220 million Epi 2 and 760 million endothelial cells which numbers reached 660 and 1200 million respectively by 9-10 mon of age but declined somewhat by 12 mon. Cellularity for these two cell types was increased 2 to 3 fold over controls at most times; for example after 9 mon exposure there were about 1200 and 1900 million Epi 2 and endothelial cells respectively. In certain higher DE exposures, namely 1500 µg for 3 mon and 6000 µg DE for 4 mon, the absolute numbers of Epi 1 were statistically reduced over control values. The percentage change was -60% and -24% respectively (Table 4).

Comparison of pulmonary responses to possibly equivalent DE doses

Five sets of the DE animal series had similar total dose-duration exposures although the actual doses and exposure intervals differed. If one assumes these paired sets of animals had equivalent burdens of DEP to respond to and handle, one might expect similar magnitudes of tissue responses in the paired exposures. Comparative data in terms of percentage change in numerical density are given in Table 4. It is apparent, if the macrophage responses are discounted, that in 4 of 5 comparison sets the shorter duration higher doses elicited statistically significant ($p < 0.05$) and greater tissue responses as indicated by underlined values in Table 4. The paired sets of 18m - 1500 µg DE and 4m - 6000 µg sets had a mixed pattern of increased cellularity.

Tolerance of the lung to the 250 µg DE/m^3 inhalation

This concentration seemed relatively ineffective as a stimulus to elicite appreciable alveolar tissue responses. After 9 mon exposure there were approximately 30% increases ($p < 0.05$) in Epi type 1, Epi type 2, endothelial cells and the interstitium over concurrent age matched controls (Tables 2,4). By 24 mon exposure, with the exceptions of the alveolar macrophage and Epi type 1 cell responses, there were no significant differences from controls. The alveolar macrophage responses appeared to be reduced as the exposure continued. On the other hand, the Epi type 1 cell responses were maintained at an increased numerical density near 30%.

Throughout the inhalation exposure (from 3-24 mon), DEP was observed within vacuoles of certain Epi type 1 cells (Fig. 4). Interstitial macrophages also sequestered phagosomal DEP (Fig. 4).

TABLE 4

EVIDENCE OF ADAPTATION IN LONGER DURATION EXPOSURES
TO DIESEL EXHAUST

EXPOSURE CONDITIONS	PERCENTAGE CHANGE IN CELLS/CM^3: DE EXPOSURES COMPARED TO AGE MATCHED CONTROLS				
TIME-DE μg	ENDOTHELIAL CELLS	EPITHELIAL TYPE 1	EPITHELIAL TYPE 2	INTERSTITIAL CELLS	ALVEOLAR MACROPHAGES
9M-250	+32.0	+ 31.2	+ 32.3	+ 2.5	+152.4
3M-750	+ 35.8	+ 33.8	+101.1[B]	+ 4.5	+ 47.4[A]
24M-250	-12.2	+ 31.5	+ 10.9	- 8.5	+ 32.9
9M-750	+ 45.6	+ 45.2	+ 96.9[B]	+ 23.7[A]	+192.8[A]
6M-750	+ 5.5	- 19.4	- 7.6	-16.3	- 26.3
3M-1500	- 12.6[B]	- 60.1[B]	+ 97.8[B]	- 2.6	+ 19.3[B]
12M-750	+ 82.8	+ 42.7	+101.2	+52.0	+171.0
6M-1500	+188.0[A]	+107.2[A]	+255.2[A]	+268.5[A]	+344.4[A]
18M-1500	+ 35.4[B]	+107.6[A]	+143.8[A]	+65.0[A]	+591.1[A]
4M-6000	+ 60.9[A]	- 23.8[B]	+145.8[B]	+213.9[A]	+139.3[A]

A,B STUDENT'S "T" TEST ESTABLISHED SIGNIFICANT DIFFERENCES IN THE PRIMARY DATA AT A = $P < 0.05$ AND B = $P < 0.01$

DISCUSSION

This communication chiefly documents the lung's quantitative responses to a chronic burden of DEP. Exposures to 250 μg DE/m^3 resulted in little or no change in morphometric parameters through 24 mon exposure.

Of course, DEP was seen within the alveolar macrophages and Epi 1 cells in the 250 µg DE sets but was more prevalent at higher DE doses. However, regardless of the carrier cell type, DEP remained sequestered within membrane-enclosing vesicles, contrary to the report of Wiester's group that DEP was in the macrophagic cytoplasm[12]. Standard histologic procedure and light microscopy employed by Wiester's group are inadequate to resolve membrane detail of the phagolysosomes.

The finding that the phagocytized DEP does not escape into the cytoplasmic milieu is a significant difference from events that occur when cytotoxic silica particles, asbestos fibers and fly ash are taken up by macrophages[13-14]. Moreover if DEP was cytotoxic to alveolar macrophages, one would expect their absolute numbers to decrease whereas they increased; eg., after 18 mon 1500 µg DE/m^3 the 591% increase over controls (Table 4) reflects 1300 million macrophages versus 210 million in the control. However, there may be some degree of functional impairment for phagocytosis according to Dr. Shan-te Chen's work, in our laboratory, using broncholavaged macrophages[5,15].

The question of whether or not epithelial type 1 cells are injured by their uptake of DEP at these concentrations is not completely resolved. Several points support the view that the contained DEP is not cytotoxic. 1. The epithelial DEP remained within membrane-bounded vesicles. 2. Tight epithelial cell junctions were maintained. 3. Neither the numerical density nor absolute number of Epi 1 cells were reduced over age matched controls except in the 3 mon 1500 and 4 mon 6000 µg/m^3 sets.

The appearance of DEP within Epi 1 cells is probably a sign of a particulate overload which resident alveolar macrophages cannot adequately handle. Thus Epi 1 uptake of particles may represent a second line of defense against particulates. The mechanisms by which Epi type 1 cells take up DEP is not yet clear but probably involves both macropinocytosis of individual DEP (Fig. 5) and phagocytosis of DEP aggregates. More Epi type 1 cells contained DEP as dose and duration of the exposure increased but the actual magnitude of the response needs to be established. Also, the fate of the Epi type 1 cells containing large vacuoles of DEP is not known. Nor is it known whether the DEP is released from the Epi type 1 cells to the interstitium.

Ultrastructural changes occurred as early as 2 weeks after exposure to 750 μg DE/m^3 illustrating how dynamic and responsive the normal lung can be to an environmental stress.

Thickened alveolar septa were noted for exposures > 250 μg DE/m^3. Increased thickness was due to at least 2 components; 1). the increased mass of the interstitium, largely the result of increased numbers of interstitial cells some of which were macrophages and eosinophils and 2). increased numerical density and hypertrophy of Epi type 2 cells. Although arithmetic and harmonic mean tissue thicknesses of the air-blood barrier were occasionally increased the morphometric diffusion capacity was not adversely affected.

The estimates derived from morphometric analysis lead us to suggest the following conclusions:

1. The 250 μg DE/m^3 elicits insignificant tissue changes, except for alveolar macrophage uptake of DE, through 24 mon exposure.

2. The lung responds rapidly to DE challenges > 250 μg DE/m^3 by increased cellularity of all types of alveolar lung cells.

3. Ultrastructural changes are DE concentration dependent for a single duration of exposure but are not for roughly equivalent doses of DE experienced for different durations.

4. Partial adaptation occurs in normal guinea pigs during chronic exposure to DE illustrating the normal lung's potential for repair and defense.

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BIOCHEMICAL ALTERATIONS IN BRONCHOPULMONARY LAVAGE FLUID AFTER
INTRATRACHEAL ADMINISTRATION OF DIESEL PARTICULATES TO RATS

by

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Male Sprague Dawley rats weighing 180-200 g were given intratracheally 5 mg of diesel particulates (DP) in 0.75 ml saline. Control rats were given saline. Five days after administering the DP and three hours before the rats were sacrificed they were injected with a pulse of 20 μC of ^{14}C-acetate.

The lungs were intubated before they were removed and lavaged 3 times with 5 ml of saline. The combined lavage fluids were lyophylized. The lipids were extracted from the lyophylized lavage fluid with chloroform: methanol (2:1) and its cholesterol (C) and phospholipid (PL) content determined. The radioactivity incorporated into the lipids was determined by separating the lipids by a TLC method and scraping the spots of interest into counting vials.

Pulmonary lavage fluid from the rats given DP contained 4 times more PL, C, and protein than in control rats (Table 1). Total radioactivity incorporated into lecithin was twice that of controls and was 3.5 times greater than the radioactivity found in the other PL studied. The lavaged lungs from the control and experimental rats were lyophylized and homogenized in a chloroform:methanol (2:1) solution. The lipid analysis showed no difference in the PL and C levels between the control and experimental lungs (Table 2).

The fatty acid (FA) profile of the lavage fluid determined by a GLC method indicated a three-fold increase in palmitic acid and arachidonic acid. Stearic, oleic and linoleic acids were not significantly altered (Table 3).

These studies imply that the lipid loading observed in lungs exposed to 5 mg of DP for 5 days are a result of increased deposition of pulmonary surfactant (extracellular lipids) and are not a result of intracellular lipids.

Table 1. Analysis of Pulmonary Lavage Fluid from Rats Intratracheally Exposed to 5 mg of Diesel Particulate

	Phospholipids		Cholesterol		Protein	
	mg	SD	mg	SD	mg	SD
Experimental	1.90	0.48	.539	.040	8.47	0.33
Control	0.49	0.18	.151	.081	2.09	1.04
Student's t	7.82	P < .001	6.37	P < .001	8.25	P < .001

Results expressed as mg of lipids in the total lavage fluid

Table 2. Analysis of Lavaged Lungs from Rats Intratracheally Exposed to 5 mg of Diesel Particulate

	Phospholipids		Cholesterol	
	mg	SD	mg	SD
Experimental	34.9	1.34	15.9	1.02
Control	31.30	10.6	13.1	3.23
Student's t	.453	NS	1.18	NS

Results expressed as mg of lipid per lung

Table 3. Fatty Acid Profile from Lung Lavage Fluid of Rats Exposed to 5 mg Diesel Particulate

	C_{16}	C_{18}	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Experimental	1.77	0.115	0.152	0.152	0.104
SD	.07	.033	.016	.030	.015
Control	0.541	.097	0.152	0.225	.030
SD	0.195	.103	.015	.322	.021
Student's t	8.50	NS	NS	NS	4.61
	$P < .001$				$P < .01$

Results expressed as mg of the fatty acid methyl ester per total lavage fluid

LIPID CHANGES IN LUNG OF RATS AFTER
INTRATRACHEAL ADMINISTRATION OF DIESEL PARTICULATES

by

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Lung, liver and serum from 180-200 g male Sprague Dawley rats were analyzed for various lipids 5 days after the rats were given intertracheally in a saline solution 1 mg of diesel particulates (DP). The lipogenic activity occurring in these rats was studied by giving i.p. 20 μC of ^{14}C-acetate one hour before they were sacrificed.

Phospholipids and cholesterol content of the lungs were significantly increased while that of triacylglycerols were not changes significantly (see Table 1).

Contrariwise to pulmonary lipids, hepatic phospholipids and cholesterol levels were decreased in rats exposed to diesel dust while hepatic triacylglycerol (TG) levels were not significantly altered. Accompanying the loss of hepatic lipids are an increased phospholipid, cholesterol and TG specific activity indicating increased hepatic lipogenesis. To determine if the loss of hepatic lipids were due to their being mobilized to the serum from the liver serum lipids were determined and were found not to be significantly altered. However, the specific activity of serum phospholipids, cholesterol and triacylglycerols were all significantly increased in rats intratracheally given the diesel particulates. A corresponding doubling of radioactivity in pulmonary phospholipids and cholesterol was also detected in these animals.

The results obtained here are similar to those reported earlier for rats intratracheally given silica dust (1-3) and suggest that a particulate insult to the lungs results in the lung producing lipotrophic factors which stimulate the liver to increase lipogenesis and lipid export to the blood. The lung in turn picks up the lipids from the serum and remodels them to meet pulmonary lipid need. To further study this hypothesis, rat hepatocytes were isolated and incubated in a pH 7.0 phosphate buffer containing 2 μC

^{14}C-acetate and several cofactors. To this hepatocyte suspension was added lung slices and the system thence incubated for 2 hours at 37°C.

Phosphatidyl choline (PC) was isolated from each sample by TLC and the PC spots from each TLC scraped into counting vials. The amount of radioactivity incorporated into the PC spots of the 5 samples for each experimental manipulation was averaged and is presented in Figure 1.

This study clearly demonstrated increased lipogenesis above that of the sum of lung slice PCgenesis and hepatocyte PCgenesis. The results from the in vitro studies strongly support the concept that a pulmonary lipotropic factor exists which stimulates lipgenesis in the liver and that these de novo synthesized lipids are utilized in part to maintain lipid homeostasis in the lung.

Table 1. Pulmonary Lipids from Rats Intratracheally Exposed to 1 mg of Diesel Particulate

	Phospholipids		Cholesterol		Triacylglycerols	
	mg	SD	mg	SD	mg	SD
Experimental	51.7	4.64	11.95	1.11	20.00	3.15
Control	30.8	4.66	8.27	0.63	24.52	1.24
Student's t	6.15	P < .001	5.15	P < .005	2.32	NS

Results expressed as mg of lipids per lung

Table 2. Hepatic Lipids from Rats Intractracheally Exposed to 1 mg of Diesel Particulate (mg/g of liver)

	Phospholipids		Cholesterol		Triacylglycerols	
	mg	SD	mg	SD	mg	SD
Experimental	27.9	1.9	3.36	0.10	8.57	1.43
Control	31.3	1.7	4.02	0.11	9.07	0.88
Student's t	2.52	P < .05	8.60	P < .001	.53	NS

Figure 1.

Lecithin Formation in Hepatocytes
and Lung Slices from ^{14}C-Acetate

p < .05

p < .05
F = 23.44

DPM × 10^{-2}

20 —

15 —

10 —

5 —

Hepatocytes + DMSO

Hepatocytes + DMSO + Indomethacin

Hepatocytes + Indomethacin + Lung Slice (200mg) + DMSO

Hepatocytes + Lung Slice + DMSO

Hepatocytes + DMSO + 0.1μg PGE$_1$

Hepatocytes + 0.1 μg PGF$_{2α}$ + DMSO

Hepatocytes + 0.1μg PGE$_{2α}$ + DMSO

200mg Lung Slice + DMSO

200mg Lung Slice + DMSO + 10μg Indomethacin

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BIOAVAILABILITY OF DIESEL PARTICLE BOUND [G-3H-] BENZO(a)PYRENE (^{3}H-BP) AFTER INTRATRACHEAL INSTILLATION

by

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Recently, we have investigated whether pulmonary prostaglandin dehydrogenase (PGDH) activity is affected by long-term exposure to low doses of diesel exhaust (1) and whether high doses (6.0 mg/m^3) for a short period (2-8 weeks) can affect such biochemical functions as (a) blood methemoglobin level, (b) reduced glutathione levels, (c) angiotensin coverting enzyme activity, and (d) mixed function oxidase activity (2, 3). In general these studies have shown that as far as the above set of biochemical parameters is concerned, there exists no particular adverse effect of the diesel exhaust in the exposed animal. Because of these findings and the observation made by Siak et al., (4) that simulated biological fluids elute no significant mutagenic activity from the diesel particles, we have contended that the many polycyclic hydrocarbons, such as benzo(a)pyrene (BP) which are known to be present in the diesel particles (DP), probably remain unavailable to the pulmonary tissue. In order to provide support for this contention attempts have been made to determine the bioavailability of ^{3}H-BP following intratracheal administration of this agent bound to diesel particles in an albumin-saline suspension.

In order to determine the bioavailability of benzo(a)pyrene as bound to diesel particles, ^{3}H-BP (120 µCi/1.79µg/1.0 ml ethanol) was allowed to bind diesel exhaust particles by adsorption. After removal of free ^{3}H-BP by repeated suspension and centrifugation, nearly 90-95 µCi of ^{3}H-BP remained bound to 1 mg of DP. Tightness of the binding was tested by continuously washing 125 µg of ^{3}H-BP bound DP with 6.0 ml either Krebs-Henseleit (K-H) solution or K-H with 3.2% albumin solution or DMSO or dichloromethane for one hour. Results showed the least dissociation in K-H solution (2.1%) and the highest in the presence of dicholoromethane (72%). For the bioavailability experiments, female guinea pigs (Hartley) were lightly anesthetized with ether in preparation for instillation of 1 mg labelled DP. When animals were appropriately anesthetized, diesel particles as suspended in 0.2 ml K-H solution containing 3.2% albumin was introduced slowly into the trachea over a period of 10-15 minutes by inserting a PE10 polyethylene tubing through the tracheal ring and pushing deep inside the broncheal tree. After intratracheal instillation of labelled particles guinea pigs were divided into two groups
Five guinea pigs of one group were immediately sacrificed within 20-25

minutes, while six guinea pigs belonging to the second group were moved to Nalgene(R) metabolic cages, held there individually for collection of urine and feces for 48 hours and then sacrificed for excision of selected organs such as liver, kidney, intestine and collection of blood for measurement of radioactivity.

Results of these studies showed that intratracheal instillation of 1 mg labelled DP did not produce any obvious symptoms in these animals. Furthermore, except for individual difference, no abnormality was noted in feeding and excretory profiles of these guinea pigs. At autopsy, lobar localization of DP was clearly visible as distinct patches of black marks of 1-2 cm diameters. Percentages of radioactivity retained by guinea pig lungs immediately after intratracheal instillation of DP bound to ^{3}H-BP, when calculated on the basis of the administered dose, showed wide variability in that 77-95% of the theoritical radioactivity (90-95 µCi ^{3}H-B(a)P/mg DP) was actually measured in the lungs of these guinea pigs that were sacrificed within 20-25 minutes of instillation. As observed by Henry and Kaufmann (5), in the present study also the discrepancy between the amount of dose actually intended for delivery and the amount actually measured in the lungs after intratracheal instillation could not be accounted for any loss due to regurgitation of the suspension.

Percentages of radioactivity retained by six guinea pig lungs at 48 hours following intratracheal instillation of DP bound ^{3}H-BP, when calculated on the basis of the administered dose, showed a mean disapperance of 42% ± 6 of radioactivity during the 48 hour time period. However, the observation that actual delivered dose was somewhat less than the dose intended for instillation meant that the lungs might have lost much more radioactivity during the 48 hours time interval. It was also noted that at 48 hours radioactivity had distributed widely in that all the organs studied such as liver, kidney and intestine showed about 1-2% as much ^{3}H-BP content per gram in comparison to the radioactivity retained per gram of lung tissue. Furthermore, during 48 hours 25% ± 3 of the radioactivity was excreted in urine and feces in these animals.

In conclusion, these studies show that ^{3}H-BP dissociates from the labelled diesel particles upon instillation in the lungs and appears in urine and feces. This rapid dissociation of ^{3}H-BP from the diesel particles implies that by the existing method of labelling of DP by adsorption with ^{3}H-BP we may not have simulated the forces by which benzo(a)pyrene binds to diesel particles under engine condition.

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THE POTENTIAL FOR AROMATIC HYDROXYLASE INDUCTION IN THE LUNG BY INHALED DIESEL PARTICLES

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Diesel exhaust particles contain trace amounts of a wide spectrum of polyaromatic hydrocarbons (PAH) adsorbed on the surface and, when extracted by an organic solvent, produce mutagenic effects in short-term microbial laboratory tests. Since the mutagenic or carcinogenic effects of hydrocarbons are frequently initiated by a metabolite rather than by the parent molecule, and since the activity of the metabolizing enzymes can be easily increased by preceding administration of powerful inducers, the enzyme induction could theoretically predetermine the potential for the adverse health effects of inhaled diesel exhaust emissions.

The effects of long-term inhalation of diluted diesel exhaust on aryl hydrocarbon activity (AHH) and cytochrome P450 content in lung and liver microsomes were investigated in male Fischer-344 rats and compared with repeated parenteral administration of organic solvent extracts of hydrocarbon adsorbed on the diesel particulate surface during the combustion process. No significant effects of long-term inhalation exposure were observed in liver microsomal AHH activity. The animals were exposed to concentrations of 750 μg m^{-3} or 1500 μg m^{-3} of diesel particulates from a 5.7 GM diesel engine at 20 hours per day, 5-1/2 days per week for up to 9 months, or treated by repeated IP injections of diesel particulate extract dissolved in corn oil, from the same engine at the several dose levels for 4 days. A decrease in lung microsomal AHH activity was found in rats following 9 months of exposure to diesel exhaust at the particulate concentration of 1500 μg m^{-3}. In contrast, 1.4- to 9-fold increases in AHH activity were observed in liver and lung microsomes of rats pretreated by intraperitoneal doses 10-15 times larger (25-125 mg/kg BW) than the most conservative estimate of the deposited lung burden [J. Appl. Tox., 1(2):27, 1981].

Since the intraperitoneal injection of diesel particle extract may not fully represent the activity of PAH deposited on the inhaled diesel particles in the respiratory airways, direct intratracheal instillation (ITI) of various doses of extract was used, and microsomal enzyme induction was investigated in the lung as well as in the liver in order to detect the local and systemic response to hydrocarbons deposited in the respiratory system. Diesel particulate extract or pure benzo[a]pyrene, dissolved in a gelatin-saline solution and used as a reference compound, were administered by ITI at several dose levels. The results show that direct intratracheal administration of the diesel particle extract required doses as high as 6 mg/kg BW before the activity of the induced enzyme in the lung was barely doubled (Figure 1). The induction was slow and

occurred selectively in lung only (Figure 2), indicating that diesel particulate extract probably does not absorb easily into the lung circulation, and is not distributed to other organs. The data suggest that the absence of AHH activity induction in rat lung exposed to diesel exhaust is due to the inavailability of hydrocarbons for distribution in the body and insufficient quantities for enzyme induction. All data seem to indicate that the inhaled diesel particles would not be capable of inducing aromatic hydroxylase in the lung unless the total deposited dose in the lung reaches approximately 6-8 mg of the particle extract per kilogram of body weight. Since the extractable portion represents only 10-15% of the total particulate mass, the required pulmonary deposits of diesel particles in a 70 kg man would be excessive to become a significant step in promotion of a potential neoplastic process.
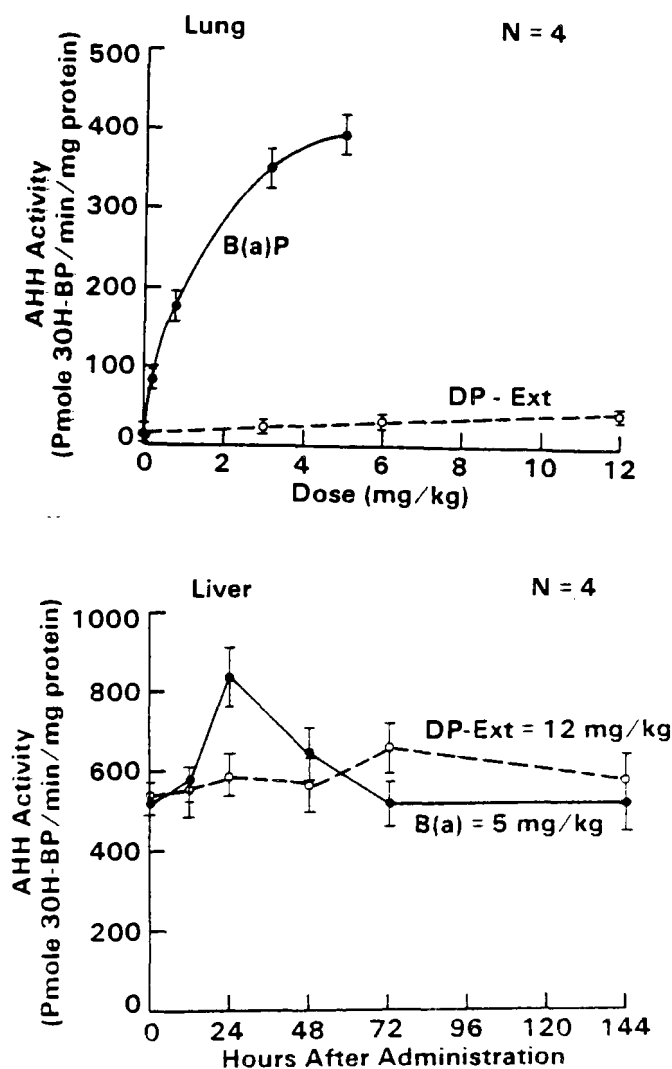
XENOBIOTIC METABOLIZING ENZYME LEVELS IN MICE EXPOSED TO DIESEL EXHAUST OR DIESEL EXHAUST EXTRACT

by

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Diesel exhaust (DE) contains organics, specifically polycyclic aromatic hydrocarbons (PAH), which are mutagenic[1] and also potentially carcinogenic. These PAH, for the most part, must be metabolized via the xenobiotic metabolizing enzymes to become active mutagens and/or carcinogens. The primary functions of these enzymes are to detoxify and/or to make these PAH more readily excretable. Unfortunately, a percentage of the metabolites formed are reactive electrophiles, which can bind to proteins, RNA and/or DNA to cause mutations and/or cancer. Moreover, the body can respond to an environmental PAH assault by increasing the levels of the metabolizing enzymes; thus, potentially increasing the formation of active metabolites and the potential risk of cancer. Therefore, the effects of DE on the xenobiotic metabolizing enzymes levels were determined as one approach to assessing the potential carcinogenic risk from DE exposure.

The inhalation study involved the chronic exposure of Strain A/J male mice 8 hours/day, 7 days/week for periods of 6 and 8 months to clean air or DE diluted to 6 mg/m^3 of particulates at the U.S. Environmental Protection Agency, Center Hill facility in Cincinnati, Ohio[2]. In this study, lung and liver microsomal aryl hydrocarbon hydroxylase (AHH) activities and liver microsomal cytochrome $P_{448/450}$ levels were determined using the slightly modified methods of Van Cantfort (1977) et al., and Omura and Sato (1964), respectively. The results (Table 1) indicated that there were no statistical differences in the liver microsomal cytochrome $P_{448/450}$ levels and liver microsomal AHH activites between clean air and DE exposed mice both at 6 and 8 months. Small differences were noted in the lung microsomal AHH activities, but these are believed to be artifactual differences, due to increases in non-microsomal lung protein present in the microsomal preparations. The only significant differences found were in the weight of the animals' lungs. The DE exposed mice were found to have significantly increased wet lung weights, which could be attributed, in

part, to the deposition of diesel particulates in the lungs and possibly to lung physiological and biochemical changes caused by the DE insult on the lungs.

A follow-up study was conducted to assess the ability of extracted diesel particulate organics to cause changes in the levels of liver cytochrome $P_{448}/_{450}$. This was done to see if changes in these enzymes levels could be produced from intraperitoneal (i.p.) injections of diesel exhaust extract (DEE) given at a maximum tolerated dose to mice. The extract of the diesel particulates was used because it should be fully bioavailable to the body's systems, whereas there are still doubts as to the degree of bioavailability of the DE organics when adsorbed onto diesel carbon particulates and then deposited in the body. The experimental approach involved the i.p. injection of male and female Strain A/J mice with DEE, the positive enzyme inducing compounds phenobarbital (PB, a cytochrome P_{450} inducer) or 3-methylcholanthrene (MC, a cytochrome P_{448} inducer), or the appropriate vehicle controls for two days and sacrificing the animals the third day. Liver microsomal preparations were immediately prepared after sacrificing and the liver microsomal cytochrome $P_{448}/_{450}$ levels were determined. The total doses given per kg body weight were DEE - 500 mg, PB - 160 mg, MC - 40 mg, and vehicle controls (DMSO and saline) - 300 u l. The DEE was derived from the 24-hour soxhlet extraction, without cellulose thimble, of DE particulates collected on teflon coated pallflex TbOA20 type filters, using methylene chloride as an elutant. The extract was then made up to the desired injectable concentration in DMSO via solvent exchange using a nitrogen atmosphere for methylene chloride removal. The results (Table 2) showed that PB and MC did result in the induction of the respective P_{448} and P_{450} enzymes in both sexes as expected. The injected DEE caused significant increases in the liver cytochrome $P_{448}/_{450}$ levels in male but not female mice. This increase, however, was smaller than those seen in the PB and MC induced animals, and the male DEE induced enzymes were found spectrally between where the cytochrome P_{448} and P_{450} enzymes were found for PB and MC, respectively.

It is therefore concluded, from the chronic inhalation and the i.p. injection studies, that enzyme inducing chemicals are present in DE and that the absence of enzyme changes found in the mice exposed to DE via inhalation may be due to 1) the enzyme inducing organics associated with DE were not bioavailable to the body system and/or 2) the inhalation dose was not sufficient to elicit a detectable change in the enzyme levels.

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Table 1. Liver Cytochrome $P_{448/450}$ Levels and Liver Aryl Hydrocarbon
Hydroxylase Activity in Mice Exposed to Clean Air or Diesel Exhaust

	Cytochrome $P_{448/450}$ Level Values = $\overline{X}$ + SEM in (nMoles/mg microsomal protein) *n = sample size		Aryl Hydrocarbon Hydroxylase Activity Values = $\overline{X}$ + SEM (pMoles/min./mg microsomal protein) *n = sample size	
Months Exposed	Control	Diesel Exposed	Control	Diesel Exposed
6	1.52 + 0.073 n = 10	1.54 + 0.066 n = 11	48.41 + 2.40 n = 10	44.08 + 1.26 n = 11
8	1.61 + 0.067 n = 9	1.62 + 0.081 n = 8	50.84 + 2.57 n = 9	49.04 + 1.76 n = 8

*Each sample consisted of pooled microsomes from two mice.

Table 2.   LIVER CYTOCHROME P448/450 LEVELS

Values = $\overline{X}$ $\pm$ SEM in (nMoles/mg microsomal protein)

n = Sample Size

	Saline (control) 100 μl/30gm BW	Phenobarbital 160 mg/Kg BW in 100 μl saline	DMSO (control) 300 μl/30gm BW	Diesel Exhaust Extract 500 mg/KG BW in 300 μl DMSO	3-Methylcholanthrene 40 mg/KG BW in 300 μl DMSO
Males	1.386 + 0.049 n = 4	2.426 + 0.020 n = 4	1.096 + 0.059 n = 8	1.346 + 0.080 n = 6	1.524 + 0.077 n = 6
Females	1.302 + 0.067 n = 4	3.013 + 0.127 n = 4	1.106 + 0.056 n = 7	1.186 + 0.066 n = 5	1.512 + 0.056 n = 6

SECTION 5

MUTAGENESIS

MUTAGENIC ACTIVITY OF DIESEL EMISSIONS

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INTRODUCTION

The initial report that the organics extractable from diesel particles demonstrate mutagenicity in the Ames Salmonella typhimurium assay[1] has now been confirmed by many independent investigators.[2-4] The mutagenic activity in bacteria is characterized as causing frameshift mutations without requiring microsomal metabolism. Recent studies have attributed this bacterial mutagenic activity to the presence of nitrated polynuclear aromatic (NO_2-PNA) compounds in diesel organic emissions.[5,6] Certain NO_2-PNAs (e.g., 1,8-dinitropyrene) are unusually potent frameshift bacterial mutagens, which do not require an exogenous microsomal metabolic activation system for activity;[7,8] they appear, however, to be activated by endogenous bacterial nitroreductases.[7] Concern that bacterial mutagenesis assays may "overestimate" the mutagenic activity of NO_2-PNAs[9] in diesel emissions points to the importance of evaluating the mutagenic activity of these emissions in eucaryotic organisms, mammalian cells, and whole animals.

Mammalian cell mutagenesis bioassays capable of detecting gene mutations, DNA damage, and chromosomal aberrations have confirmed the mutagenic activity of diesel emissions.[10,11] Many of these assays performed with mammalian cell lines (e.g., L5178Y mouse lymphoma cells, BALB/c 3T3 cells, and Chinese hamster ovary [CHO] cells) require the addition of a metabolic activation system containing microsomal as well as other mammalian liver enzymes to metabolize polynuclear aromatic hydrocarbons (PAHs). Very few studies have been published on the activity of NO_2-PNAs or presence of nitroreductases in these systems.

The objective of this paper is to review the mutagenic activity of diesel emissions. The organics extractable from diesel particles, which may constitute 5 to 50% of the mass of these submicron particles, have been most extensively examined in microbial and mammalian cell mutation assays. This paper compares the microbial mutagenicity, mammalian cell mutagenicity, and mouse skin tumorigenicity of these organics. Whole diesel particles, gaseous emissions, and whole exhaust emissions, examined in several different in vivo bioassays for both somatic and heritable mutagenic activity, will also be reviewed.

METHODOLOGY

Mobile source emissions samples

The diesel and gasoline particle emissions used in the microbial and mammalian cell mutagenesis studies reported here were collected using dilution tunnel sampling techniques.[11] The total exhaust from passenger cars or a portion of the exhaust from heavy-duty engines was diluted with filtered air (10:1) prior to collection on 20 in. x 20 in. Teflon-coated Pallflex T68-A20 filters. The mobile sources, fuels, and test conditions are shown in Table 1. The diesel samples were all obtained from vehicles and engines operated on the same lot of No. 2 diesel fuel. The vehicles were operated on a chassis dyna- mometer, using the highway fuel economy test cycle (HWFET) that averages 48 miles per hour in 12.75 minutes over 10.24 miles. The engines were operated on engine dynamometers at steady-state operation.

TABLE 1

MOBILE SOURCE SAMPLES

		Vehicle Description	Fuel	Driving Cycle
Diesel:	Cat	Caterpillar 3304	Diesel No. 2	Mode II[a]
	Nissan	Nissan Datsun 220C	Diesel No. 2	HWFET[b]
	Olds	Oldsmobile 350	Diesel No. 2	HWFET
	VW Rabbit	Volkswagon Turbocharged Rabbit	Diesel No. 2	HWFET
	Mercedes	300 D Mercedes	Diesel No. 2	HWFET
Gasoline:	Mustang	1977 Mustang II-302, V-8 Catalyst and EGR	Gasoline Unleaded	HWFET
	Chev 366	Heavy Duty Chev 366	Leaded	Full-rated load[c]
	Ford Van	6 cylinder, in line van	Leaded	HWFET

[a]Mode II   = 2200 rpm steady state, 85 lb. load
[b]HWFET   = Highway fuel economy cycle 10.24 mi, ave. 48 mph, 12.75 min.
[c]Full-rated = 2300 rpm, 100% load

The particle samples collected on 12 to 16 Teflon-coated filters were Soxhlet- extracted with dichloromethane (DCM) in a 2.3 liter side-chamber extractor for 48 hours. The Soxhlet-extracted organics were filtered using Teflon millipore filters (0.2 μm pore) to remove any remaining particles and concentrated by rotary evaporation under reduced pressure. Aliquots were evaporated to dryness

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under nitrogen and stored frozen in the dark. The samples were dissolved in dimethyl sulfoxide (DMSO) for all of the bioassays except mouse skin tumorigenesis, oncogenic transformation, and mutation in BALB cells where acetone was used as the solvent.

The whole exhaust emissions employed in the _in vivo_ mutagenesis bioassays are described in the references cited for each of those assays.

Bioassays

The mutagenesis bioassays applied to diesel emissions generally included assays for which standard protocols have been developed and validated with a series of individual chemicals. The mutagenesis assays were selected to detect gene mutations, DNA damage, and chromosomal aberrations, as outlined in Figure 1. The bioassays were conducted with coded samples at 5 to 7 doses or concentrations after a preliminary toxicity range-finding test. For those assays where a positive dose response was obtained, the slope of the dose-response curve was determined by the linear regression analysis, except for the _S._ typhimurium plate incorporation assay, where the non-linear model slope was used.[12,13]

GENE MUTATION ASSAYS

Salmonella typhimurium bioassay

The Ames _S._ typhimurium assay measures histidine reversion in a series of tester strains. The _S._ typhimurium plate incorporation test was conducted as described by Ames et al.,[14] with minor modifications as described by Claxton.[15] The modifications included adding the minimal histidine to the plate media rather than to the overlay and incubating for 72 rather than 48 hours. Claxton initially reported the specific activity of five of these mobile source samples calculated from the linear regression analysis at 100 μg of sample.[15] This data and that from the additional samples have been reanalyzed using the non-linear model slope analyses,[12,13] as shown in Table 2.

The extractable organics from the diesel particle samples were all mutagenic without the addition of metabolic activation. Comparison of the mutagenic activity in all five tester strains[16] showed the diesel samples to be positive in TA1538, TA1537, TA98, and TA100, and negative in TA1535. The Caterpillar (Cat) and Volkswagen (VW) Rabbit samples show increased mutagenic activity in the presence of the S9 activation system, whereas the Nissan and Oldsmobile (Olds) samples show decreased activity with S9 activation. The gasoline particle samples were all less mutagenic in the presence of S9 activation.

A. MUTAGENESIS BIOASSAYS
  1. GENE MUTATION ASSAYS
     A. Bacterial
        1. Salmonella typhimurium
        2. Escherichia coli

     B. Mammalian cell
        1. Mouse lymphoma, L5178Y
        2. Mouse embryo fibroblasts, BALB/C3T3
        3. Chinese hamster ovary, CHO

  2. DNA DAMAGE ASSAYS
     A. Yeast
        1. Saccharomyces cerevisiae D3 recombinogenic assay

     B. Mammalian Cell
        1. DNA strand breaks in SHE cells
        2. Unscheduled DNA repair in liver cells
        3. Sister chromatid exchanges in CHO cells

  3. CHROMOSOMAL ABERRATIONS
     A. Mammalian cells
        1. CHO cells
        2. Human lymphocytes

B. CARCINOGENESIS BIOASSAYS

  1. ONCOGENIC TRANSFORMATION ASSAYS
     A. Chemical transformation
        1. Mouse embryo fibroblasts, BALB/ c 3T3
        2. Syrian hamster embryo, SHE

     B. Viral enhancement of transformation
        1. SA7 virus enhancement in SHE cells

  2. SKIN TUMOR INITIATION

Fig. 1. Outline of the bioassays used to examine the extractable organics from
        mobile source particle emissions.

A significant difference was observed between the particle emission rates
(g/mi) and the percent organic extractable matter for the different vehicles.[12]
The diesel cars emitted approximately 100 times more particles per mile than
the unleaded gasoline car. A direct comparision of the mutagenic emission rate
for the cars is best expressed as revertants/mile. Claxton and Kohan[17] have
reviewed the mutagenic emission rates for a number of certification vehicles
and found that the diesel vehicles emitted 45 to 800 x as much mutagenic
activity per mile as the gasoline catalyst vehicles.

TABLE 2

REVERSE MUTATION IN SALMONELLA TYPHIMURIUM

Vehicle Description	Slope: Rev/µg[a] in TA98	
	-MA	+MA
Diesel: Cat	0.30	1.6
Nissan	20.8	15.1
Olds	2.1	1.4
VW Rabbit	5.2	6.1
Gasoline, unleaded:		
Mustang	2.1	8.6
Gasoline, leaded:		
Chev 366	INC	INC
Ford Van	16.8	29.7
Benzo(a)pyrene	NEG	167.9
1-Nitropyrene (>99%)	572	802
1-Nitropyrene (95%)	4234	736

[a]Non-linear model slope, revertants/µg.

Escherichia coli WP2 bioassay

The E. coli WP2 tryptophan reversion assay is very similar to the S. tyhimurium plate incorporation assay[14] using McCalla's E. coli WP2 tryptophan auxotroph (trp) with a DNA repair deficiency mutation (uvrA).[18] Mortelsmans[19] found that the Mercedes diesel sample elicited a reproducible dose-related increase in the number of trypotophan-independent revertants in the absence of metabolic activation. In the presence of metabolic activation, the Mercedes diesel sample was non-mutagenic.

L5178Y mouse lymphoma mutagenesis assay

The L5178Y mouse lymphoma assay of Clive and Spector[20] measures forward mutation frequency at the thymidine kinase (TK) locus. The mouse lymphoma assay was conducted according to the method of Clive et al.[21] by Mitchell et al.[22] and Cifone and Brusick[23] in the evaluation of the mutagenicity of a series of diesel and related environmental emissions. Preliminary dose-range toxicity assays were conducted to select 10 concentrations of each sample that resulted in cell survivals of 5 to 90% of the controls. In the mutagenesis assays, duplicate samples were used for each concentration tested.

In each assay, 6 x 10^6 L5178Y TK+/- cells were treated with the organic extracts in 10 ml for 4 hours while rotating in a roller drum at 37°C.

The mutation frequency was calculated by dividing the number of mutant cells per ml by the number of viable cells per ml at each concentration. Concentrations resulting in less than 10% total relative growth were not used in determining the slope of the mutation response curve for each emission sample, as shown in Table 3.

TABLE 3

GENE MUTATION IN MOUSE LYMPHOMA L5178Y CELLS[a]

Vehicle Description	Slope: -MA	Mutation freq/10^6 cells/μg/ml (r^2)	+MA	(r^2)
Diesel: Cat	0.25	(.96)	0.063	(.78)
Nissan	4.19	(.88)	2.87	(.86)
Olds	1.21	(.95)	1.28	(.93)
VW Rabbit	0.98	(.89)	0.72	(.64)
Mercedes	NEG		1.82	(.87)
Gasoline, unleaded: Mustang	0.38	(.98)	1.09	(.81)
Gasoline, leaded: Chev 366	1.50	(.81)	3.20	(.77)
Ford Van	NEG[b]		5.60	(.90)
Benzo(a)pyrene	NEG		5.42	
1-Nitropyrene (95%)	NEG		39.3	

[a]Assay performed with 6 x 10^6 cells in 10 ml for 4 h.
[b]Highly toxic at less than 10 μg/ml.

All the diesel samples were mutagenic in the mouse lymphoma assay, and except for the Mercedes sample, all the diesel samples showed that the mutagenic activity was greater in the absence of the metabolic activation system. All the diesel organic samples were also more cytotoxic in the absence of metabolic activation than in its presence.[22] The maximum increases in mutation frequency (2 to 4 times the spontaneous frequency) occurred at concentrations ranging from 20 to 300 μg/ml. The gasoline catalyst Mustang sample was more mutagenic and cytotoxic in the presence of metabolic activation than in the absence of the activation system. Polycylic aromatic hydrocarbons such as benzo(a)pyrene (B[a]P) are not mutagenic in this assay without the addition of the S9 metabolic activation system. Preliminary evaluation of 1-nitropyrene (95%) in this assay

suggests that it also requires an exogenous metabolic activation system for activity.

BALB/c 3T3 mutagenesis assay

The BALB/c 3T3 mutagenesis assay was developed by Schechtman and Kouri[24] to measure simultaneously both mutagenic activity and morphological transformation. Forward mutation is measured using ouabain resistance.[25] Cells (1-2 x 10^6) were exposed in suspension for 2 hours with increasing concentrations of the diesel organics dissolved in acetone. Curren et al.[26] assayed the Caterpillar, Nissan, and Oldsmobile diesel samples, and the Mustang gasoline sample in the BALB/c 3T3 mutagenesis assay. Although several individual doses of the diesel sample, did induce a significant increase in ouabain-resistant mutants, none of the samples induced a dose-dependent increase in mutation frequency. A majority of the concentrations tested appeared to be above the limit of solubility as evidenced by insoluble material in the assay. This problem had not previously been encountered when DMSO was employed as a solvent with a similar sample. Curren et al.[26] assumed that all seven doses tested may have been similar due to the solubility limits. They combined all of the mutant colonies observed for a sample and divided it by the total number of surviving cells to determine a mutation frequency for the dose range tested. Using this method of analysis, both the Nissan diesel sample and Mustang gasoline sample were highly mutagenic ($p < 0.05$) both without and with metabolic activation. The Oldsmobile diesel sample showed approximately a twofold increase in mutation frequency, which was not significantly different from the solvent control, and the Caterpillar diesel sample showed no increase in mutation frequency.

Chinese hamster ovary mutagenesis assay

The CHO assay measures forward mutation at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus using 6-thioguanine resistance.[27] The CHO assay was conducted with modifications by Casto et al.[28] to evaluate the mutagenic activity of the diesel and gasoline samples. Cells (1.5-2 x 10^6) were treated with increasing concentrations of the organics dissolved in DMSO for 24 hours. These assays were only conducted in the absence of metabolic activation in two to three separate experiments. Re-analysis of the combined data with cell survivals above 10% using linear regression analysis showed a relatively weak to negative response for the Caterpillar, Oldsmobile, and Mustang samples. The samples that would be considered positive were the Nissan and VW Rabbit with activities of 0.16 ($r^2 = 0.73$) and 0.091 ($r^2 = 0.46$) mutation

frequency/10^6 cells/µg/ml, respectively. Li and Royer[29] also have reported that the extractable organics from a series of diesel cars was generally very low in mutagenic activity in CHO cells with a slight increase in activity with metabolic activation. Simultaneous treatment of the CHO cells with a co-mutagen (e.g., B[a]P) caused significant enhancement of the mutagenic activity.[29]

Cheshier et al.[30] have shown that CHO cells readily phagocytize whole diesel particles, which become closely associated with the nucleus. Under these conditions, 100 µg/ml of diesel particles caused a tenfold increase in mutation frequency above the controls.[30]

DNA DAMAGE ASSAYS

Saccharomyces cerevisiae D3 recombinogenic assay

The diploid yeast S. cerevisiae D3 can be used to measure mitotic recombination by scoring for red pigmented mutant colonies formed in the presence of adenine.[31] The mutants are generated from a recombinational event resulting from DNA breakage and repair after exposure to DNA-damaging chemicals. Initial studies on the diesel and comparative samples reported that no reproducible or dose-related responses were observed.[22] Further studies of these samples in the S. cerevisiae assay showed that two of the three diesel samples assayed, the Nissan and VW Rabbit, did result in reproducible dose-related increases in mitotic recombinants. The Nissan sample caused 62 mitotic recombinants/10^5 surviving cells/µg/ml (r^2=0.79) without activation and 46 (r^2=0.64) with activation. The VW Rabbit caused 24 mitotic recombinants/10^5 surviving cells/µg/ml (r^2=0.4) without activation and 7.2 (r^2=0.2) with activation. The response was greater and more reproducible in the absence of metabolic activation. The Oldsmobile sample was weakly positive in the absence of metabolic activation, and the gasoline Mustang sample did not reproducibly increase the number of mitotic recombinants. Polycyclic aromatic hydrocarbons (e.g., B[a]P) do not induce mitotic recombination in S. cerevisiae,[22] and NO_2-PNAs have not been examined in this assay.

DNA strand breaks in Syrian hamster embryo cells

Damage to cellular DNA, which results in the formation of DNA fragments, can be measured directly by alkaline elution techniques.[32] Casto has shown that chemical induction of DNA damage in primary Syrian hamster embryo (SHE) cells can be detected following centrifugation on alkaline sucrose gradients.[33] The diesel Caterpillar, Nissan, Oldsmobile, and VW Rabbit samples were tested at four concentrations from 31 to 250 µg/ml. None of these samples produced a significant

change in the sedimentation profile of DNA from the treated SHE cells. The
gasoline Mustang sample did produce a significant increase in DNA strand breaks
at the highest concentration tested (250 µg/ml). In comparing several in vitro
tests to detect carcinogens in Syrian hamster cells, Casto suggests that the
DNA strand breakage assay is the least sensitive of the assays evaluated.[28]

Unscheduled DNA repair in liver cells

The liver cell DNA repair assay measures autoradiographic unscheduled DNA
synthesis in freshly isolated hepatocytes. The Oldsmobile diesel sample was
evaluated in the hepatocyte primary culture/DNA (HPC/DNA) repair assay by
Williams according to previously published procedures.[34] Unscheduled DNA repair
was induced from 10 to 100 µg/ml with an average of 36.7 grains/nucleus at
100 µg/ml. The response appeared to be dose-related; however, insufficient
numbers of concentrations were tested in any one experiment to perform a
regression analysis. Combination of the data from four separate experiments
resulted in a slope of 0.325 grains/nucleus/µg/ml (r^2=0.78).

Sister chromatid exchange assay in CHO cells

The sister chromatid exchange (SCE) assay measures the increase in exchanges
between two chromatids of each chromosome in cells grown in the presence of
bromodeoxy-uridine (BrdU) during replication. The increase in SCEs observed
after cells have been treated with chemical mutagens has been related to re-
combinational or post replicative repair of DNA damage.[35] The diesel and gaso-
line emission samples were tested in the SCE assay using the CHO cell system
previously described.[22] This method uses a 21.5-hour sample exposure period;
however, due to the cytotoxic effects of the metabolic activation system, only
a 2-hour exposure period was used when the samples were tested with metabolic
activation. It is not possible, therefore, to compare directly the induction
of SCEs with and without metabolic activation. The slope of the dose-response
regression analysis is shown in Table 4.

All of the diesel and gasoline samples, except the Oldsmobile sample, induced
SCEs in the absence of metabolic activation. In the presence of metabolic
activation, all of the diesel samples induced SCEs except the Caterpillar
sample. The significantly lower activity in the presence of activation is
presumably due in part to the much shorter exposure period. The polycyclic
aromatic hydrocarbon (B[a]P) tested in this assay only induced SCEs when meta-
bolic activation was added. Preliminary studies on 1-nitropyrene (95% pure)
showed that it was weakly active in the absence of the metabolic activation
system.

TABLE 4

SISTER CHROMATID EXCHANGES IN CHO CELLS

Vehicle Description		$-MA^a$	Slope: SCE/cell/µg/ml (r^2)	$+MA^b$	(r^2)
Diesel:	Cat	0.011	(.83)	NEG	
	Nissan	0.30	(.93)	0.071	(.87)
	Olds	NEG		0.017	(.46)
	VW Rabbit	0.075	(.99)	0.030	(.92)
Gasoline, unleaded:					
	Mustang	0.076	(.99)	NT^c	
Benzo(a)pyrene		NEG		1.24	
1-Nitropyrene (95%)		0.066	(.80)	NEG	

^{a}-MA exposure 21.5 h.
^{b}+MA exposure 2 h.
^{c}NT = not tested.

CHROMOSOMAL ABERRATIONS

Chromosomal aberrations in CHO cells

Chromosomal aberrations that can be detected as a result of treatment of
cells in culture include both numerical and structural aberrations. Scoring
of numerical aberrations, however, is not generally recommended for this assay.
Structural aberrations include breaks, deletions, gaps, exchanges, or trans-
locations at chromosomal and/or chromatid levels. These aberrations are
generally observed between 6 to 24 hours after cell treatment. In order to
determine the optimal time after treatment to observe aberrations, CHO cells
treated with the Nissan sample for 6 hours were scored for structural chromo-
somal abnormalities at 12, 15, and 21 hours.[36] A summary of those results is
shown in Table 5. A dose-related positive response was observed at all three
time periods.

Chromosomal aberrations in human lymphocytes

Human lymphocytes freshly isolated from blood samples taken from normal
individuals can be exposed to chemicals in vitro and analyzed for chromosomal
aberrations. The diesel Oldsmobile sample was exposed to lymphocytes from two
individuals at five doses ranging from 0.1 to 100 µg/ml with and without an
S9 metabolic activation system. Chromosome aberrations were scored by McKenzie
according to previously published criteria.[37] In the absence of metabolic

TABLE 5

SUMMARY OF CHROMOSOMAL ABERRATIONS IN CHO CELLS

µg/ml Nissan	Hours after Treatment					
	12		15		21	
	Total Cells	Percent Aberrations[a]	Total Cells	Percent Aberrations	Total Cells	Percent Aberrations
0	515	1.75	276	4.06	147	2.00
20	136	5.07	152	10.53	151	6.80
40	129	6.98	59	28.80	98	10.91
60	115	18.26	79	62.03	122	15.28
80	192	20.83	T[b]	T	88	22.72

[a]Percentage of cells with all types of aberrations.
[b]T = toxic.

activation, treatment of lymphocytes with the diesel Oldsmobile sample resulted in a four- to fivefold increase in the percentage of cells with chromosomal aberrations over the dose range tested. Chromosome and chromatid breaks and aneuploidy were observed at 0.1 to 1.0 µg/ml. Chromosomal fragments, dicentrics, and endoreduplications were observed at doses above 5 µg/ml. Chromosomal and chromatid gaps were only observed at 100 µg/ml. In the presence of metabolic activation, no increase in the total percentage of cells with aberrations was observed, although an increase in chromosomal fragments and dicentrics was observed.

IN VIVO MUTAGENESIS BIOASSAYS

The organics extractable from diesel particle emissions are mutagenic in many microbial and mammalian cell assays, as described above. However, these assays are not readily applicable to testing whole diesel emissions nor can they test for the heritability of mutations. For these reasons, plant (Tradescantia), insect (Drosophila), and mammals (mice and hamsters) have been employed to evaluate the in vivo mutagenic activity of diesel emissions, as summarized in Table 6.

The Tradescantia micronucleus test and stamen hair gene mutation assays both have been shown to detect the mutagenic activity of volatile and gaseous chemicals and environmental emissions. Ma[38] reported that diluted diesel exhaust induced micronuclei (broken pieces of chromosomes) in Tradescantia. Whole diesel emissions were also shown by Schairer[39] to induce gene mutations in the Tradescantia stamen hair assay.

TABLE 6

IN VIVO MUTAGENICITY OF DIESEL EMISSIONS

Bioassay System	Endpoint	Reference	Whole Emissions	Gases[a]	Particles[a]	Extractable[a] Organics
Tradescantia	Micronucleus test	Ma et al.[38]	+			
Tradescantia mutation	Stamen hair gene assay	Schairer[39]	+	+		
Drosophila melanogaster	Sex-linked recessive lethal test	Schuler and Niemeir[40]; Nix[41]	-	-		
Mouse	Micronucleus assay	Pereira[42]	-		-	+
Mouse	Bone marrow SCE assay	Pereira[42]	-		+	+
Mouse specific locus	Point mutation test	Russell et al.[44]	-			
Mouse dominant lethal	Chromosome damage test	Russell et al.[44]	-			
Mouse heritable translocation test	Chromosome damage test	Russell et al.[44]	-			
Chinese hamster	Micronucleus assay	Pereira[42]	-		-	(+)[b]
Syrian hamster	Lung cell SCE assay	Rounds[43]	+		+	+
Syrian hamster	Fetal liver SCE assay	Pereira[42]	-		-	+

[a]Where bioassays have not been conducted, no entry in the table is shown.
[b](+) = weakly positive.

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The fruit fly, Drosophila melanogaster, provides a well-defined genetic test system to measure inherited damage. Two independent investigators[40,41] have evaluated the mutagenicity of whole diesel emissions using the D. melanogaster sex-linked recessive lethal assay. Nix[41] also tested the gaseous emissions from filtered exhaust. Neither the whole nor filtered exhaust was found to induce mutations in this assay.

Whole animal rodent bioassays using mice or hamsters provide the opportunity to measure genetic damage (e.g., induction of micronuclei or induction of SCEs) in somatic cells as well as heritable genetic damage. Both mice and hamsters have been used in studies by Pereira[42] and Rounds[43] to measure induction of micronuclei and SCEs in bone marrow, lung cells, and fetal liver after exposure to whole diesel emissions. In all of these studies except the lung cell SCE assay, the whole emissions were negative. After exposure to collected particles, the SCE assays were positive in both bone marrow and lung cells. All of these genetic damage assays in somatic cells were positive when the animals were treated with the organics extracted from diesel particles. These studies suggest that the organics associated with diesel particles are capable of inducing genetic damage in somatic cells in the lung, bone marrow, and fetal liver. However, under conditions where the animals were exposed to high concentrations of whole diesel exhaust for several months, only induction of SCEs in lung cells was observed. These results suggest that insufficient concentrations of the mutagenic organics would reach the germinal cells to cause heritable mutations.

Heritable mutations in mice after exposure to diesel exhaust were assayed for by Russell et al.[44] using the specific locus, dominant lethal, and heritable translocation assays. The results in all the heritable mutagenesis assays were negative.

The in vivo mutagenesis studies further confirm the mutagenic activity of the organics associated with diesel particles, while showing the lack of transmitted genetic effects after animal exposure to whole diesel exhaust emissions. These findings suggest either that the mutagenic components do not reach the gonads, or that the heritable genetic assays are insensitive to the frameshift mutagens present in diesel emissions. Polycyclic aromatic hydrocarbons and other frameshift mutagens such as the NO_2-PNAs have not been well studied in either the Drosophila or mouse heritable mutagenesis assays.

CARCINOGENESIS BIOASSAYS

Oncogenic transformation assays

Chemically induced carcinogenesis is currently considered to be a multistep process that may involve DNA damage or mutation as an initial step. Oncongenic transformation assays measure the induction of morphological transformations that result in the formation of colonies of cells phenotypically similar to malignant cells (Type III foci). These transformed cells generally cause tumors when injected into a syngeneic host.

Several of the diesel and gasoline samples in Table 1 have been tested in two oncogenic transformation assays. Curren et al.,[26] using mouse embryo cells (BALB/c 3T3) found that all of the diesel and gasoline samples, except the Caterpillar sample, induced some transformed foci. Dose-related responses were not observed, which may be due to the problems discussed above with the BALB/c mutagenesis assay that was conducted simultaneously. Casto et al.,[28] using primary SHE cells, found that none of the diesel or gasoline samples caused transformation in these experiments. Unfortunately, lack of induction of transformation by one of the positive controls and difficulties in obtaining acceptable lots of serum for these assays prevented further testing.

Viral enhancement of transformation

The viral enhancement assay measures the increased sensitivity of cells to virus-induced transformation. Although this assay is listed with the transformation assays, Casto et al.[45] have reported the significance of DNA damage and repair in the enhancement of viral transformation by chemicals. This assay may be a measure, therefore, of DNA damage. The viral enhancement of the transformation assay of Casto[46] was employed in the evaluation of the diesel, gasoline, and several comparative samples.[29] The transformation frequency was determined (number of transformed foci per 10^6 surviving cells) in at least three separate experiments. The dose response curves for selected experiments were reported by Casto et al.[28] The combined data from all experiments have been re-analyzed to determine the slope of the dose response. Concentrations resulting in less than 10% survival were not used in determining the slope of the transformation response, as shown in Table 7.

All of the diesel and gasoline samples, except the Caterpillar sample, increased the viral enhancement of transformation. The Oldsmobile and VW Rabbit samples were very weakly active, and the dose responses had low r^2 values, 0.68 and 0.25, respectively. The variation in response between the three separate

TABLE 7

ENHANCEMENT OF VIRAL TRANSFORMATION IN
SYRIAN HAMSTER EMBRYO CELLS

Vehicle Description		Transformation Frequency/μg/ml	(r^2)
Diesel:	Cat	NEG	
	Nissan	0.328	(0.76)
	Olds	0.021	(0.68)
	VW Rabbit	0.059	(0.25)
Gasoline:	Mustang	0.33	(0.18)
	Benzo(a)pyrene	351.0	(0.85)

experiments was significant, and even the Mustang gasoline sample, which caused a 0.33 transformation frequency/μg/ml, had an unacceptably low r^2 value of 0.18 for the combined slope analysis. The Nissan sample caused a transformation frequency equivalent to the Mustang sample, with an r^2 of 0.76.

Skin tumor initiation

   Mice treated topically with chemical carcinogens produce both benign (papillomas) and malignant (squamous cell carcinomas) tumors. The tumor-initiating activity of a chemical can be determined when mice are treated with a single application of the chemical and subsequently treated with a strong tumor promoter (i.e., 12-0-tetradecanoyl phorbol-13-acetate [TPA]). Tumor-initiating chemicals are thought to induce somatic mutations as a result of covalent binding to DNA and other macromolecules.[47] Nesnow et al.[48] have reported the detailed methods and results of skin tumor initiation studies on these diesel and gasoline extracts in SENCAR mice. The skin tumor-initiating activity to produce papillomas of these samples is shown in Table 8.

   Papillomas were induced with all of the samples except the Caterpillar. Complete analysis of the tumor initiation activity and a discussion of the carcinogenic activity of these samples on mouse skin is reported by Nesnow et al.[49]

TABLE 8

SKIN TUMOROGENESIS IN SENCAR MICE

Vehicle Description		Slope: Papillomas/ Mouse/mg[a]	(r^2)
Diesel:	Cat	NEG	
	Nissan	0.52	(.99)
	Olds	0.14	(.83)
	VW Rabbit	0.30	(.53)
	Mercedes	INC	
Gasoline, unleaded:			
	Mustang	0.085	(.76)
Benzo(a)pyrene		86.2	(.99)
1-Nitropyrene (>99%)		INC	

[a]Average of males and females.

SUMMARY AND DISCUSSION

Comparison of mutagenic and carcinogenic activity of extractable organics
from diesel particle emissions in various bioassays

The organics extractable from diesel particle emissions were found to be muta-
genic in all three types of bioassays: gene mutation assays, DNA damage assays,
and chromosomal aberration assays. The three mutagenesis assays that resulted
in reproducible dose-response data and that have also been used to evaluate at
least four organic emission samples are: S. typhimurium bacterial mutagenesis
assay (Table 2), L5178Y mouse lymphoma mutagenesis assay (Table 3), and the SCE
assay in CHO cells (Table 4). The relative activity of the diesel and
gasoline organic emission samples has been compared between these three
assays and with the two short-term carcinogenesis assays, which resulted in re-
producible dose-response data, enhancement of the viral transformation assay
(Table 7), and mouse skin tumor initiation assay (Table 8).

In order to evaluate whether the relative activity of these samples correlated
between assays, the activity determined from the slope of the dose-response for
each sample in one assay was plotted versus the second assay. Linear regression
analysis and confidence bands were determined as shown in Figure 2. The correla-
tions, as indicated by the r^2 values (Table 9) for the gene mutation assays
when plotted versus all the other assays was very good ($r^2 > 0.90$) in the absence
of metabolic activation, except for the mouse lymphoma and the skin tumorigene-
sis versus SCE in CHO cells. The addition of metabolic activation to the S.

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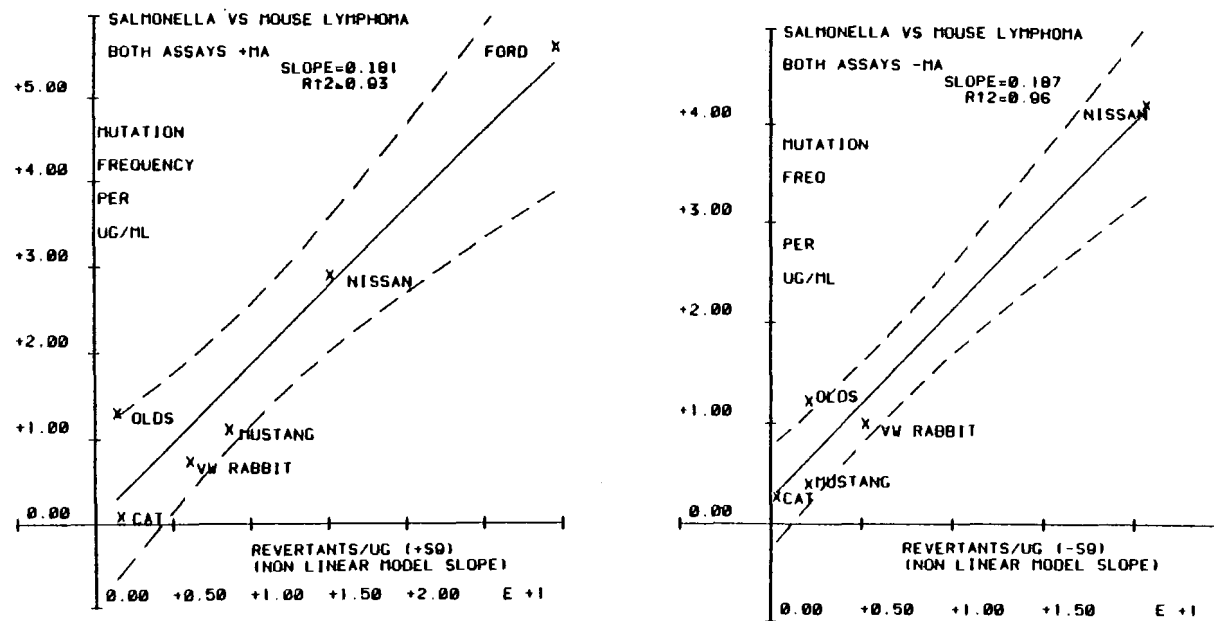
Fig. 2. Linear regression analysis of the mutagenic activity of diesel and gasoline samples in the
S. typhimurium mutagenesis assay versus the L5178Y mouse lymphoma assay. Confidence bands are
shown in dotted lines.

typhimurium assay decreased its correlation with both viral enhancement and skin tumorigenesis. The viral enhancement assay, which is thought to be dependent upon DNA breakage to allow increased frequency of virus insertion, correlated highly ($r^2 > 0.96$) with the mutagenesis assays in the absence of metabolic activation. The mouse lymphoma assay both with and without metabolic activation correlated highly ($r^2 = 0.95$) with the skin tumorigenesis assay.

TABLE 9

CORRELATION OF DOSE-RESPONSE SLOPES
DIESEL AND GASOLINE

Bioassay Comparison	Exogenous Metabolic Activation	r^2
Salmonella versus Mouse Lymphoma	-MA	0.96
Salmonella versus Mouse Lymphoma	+MA	0.93
Salmonella versus SCE in CHO	-MA	0.98
Salmonella versus SCE in CHO	+MA	0.94
Salmonella versus Viral Enhancement	-MA	0.99
Salmonella versus Viral Enhancement	+MA	0.79
Salmonella versus Skin Tumorigenesis	-MA	0.90
Salmonella versus Skin Tumorigenesis	+MA	0.72
Mouse Lymphoma versus SCE in CHO	-MA	0.84
Mouse Lymphoma versus SCE in CHO	+MA	0.87
Mouse Lymphoma versus Viral Enhancement	-MA	0.96
Mouse Lymphoma versus Viral Enhancement	+MA	0.83
Mouse Lymphoma versus Skin Tumorigenesis	-MA	0.95
Mouse Lymphoma versus Skin Tumorigenesis	+MA	0.95
SCE in CHO versus Viral Enhancement	-MA	0.96
SCE in CHO versus Viral Enhancement	+MA	0.93
SCE in CHO versus Skin Tumorigenesis	-MA	0.83
SCE in CHO versus Skin Tumorigenesis	+MA	0.83
Viral Enhancement versus Skin Tumorigenesis	-MA	0.92

[a]Exogenous metabolic activation (S9) added to one or both assays is shown as +MA; when no exogenous metabolic activation system was added to either assay, it is shown as -MA.

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These studies suggest that there is generally good agreement both qualitatively and quantitatively between the short-term mutagenesis and carcinogenesis bio-assays in which a dose-related response is observed for the organics extractable from diesel and gasoline emission particles. Several assays (e.g., DNA strand breaks and oncogenic transformation in SHE cells) do not detect activity in these samples. Other assays (e.g., mutagenesis and oncogenic transformation in BALB/c 3T3 cells) did provide qualitative data to indicate that these organics were active; however, reproducible dose-related responses were not observed. This result may be due to a lack of increasing amounts of chemical reaching the cell as the exposure concentration increased, probably as a result of solubility problems with these complex mixture samples. More solubility problems arose in those in vitro assays where acetone rather than DMSO was used as a solvent.

Conclusions

The studies reviewed here were undertaken to evaluate the mutagenicity of organics associated with diesel particle emissions in a battery of mammalian cell bioassays. These data provide strong evidence that these organics are mutagenic in mammalian cells. Furthermore, the relative activity of a series of emission extract samples, which exhibit approximately one order of magnitude range in activity in the S. typhimurium bacterial mutagenesis assay, exhibits a similar range in activity in mammalian cell assays. These studies suggest that bacterial mutagenesis assays are not overestimating the mutagenicity of these organics compared to mammalian cells, nor are they greatly overestimating the relative tumor-initiating activity in skin carcinogenesis studies.

Since a significant portion of the bacterial mutagenic activity appears to be due to NO_2-PNA compounds, and particularly mono-and di-nitrated pyrene, more studies are needed to evaluate the activity of these compounds in mammalian cells. Preliminary studies reported at this symposium on the activity of 1-nitropyrene (95% pure and contaminated with dinitropyrenes) suggest that these compounds are active in mammalian cells. The concentrations of mono- and di-nitrated pyrenes[50] in the samples tested here, however, can not account for all of the "direct-acting" mutagenic activity observed in mammalian cells treated with diesel particle organics. Furthur research is needed to identify other mutagenic and potentially carcinogenic constituents of diesel emissions.

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GENOTOXICITY OF DIESEL EXHAUST EMISSIONS IN LABORATORY ANIMALS

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INTRODUCTION

The number of diesel powered passenger cars in the United States has
increased. It has been estimated that by 1985 diesel powered passenger cars
will comprise ten to 25 percent of new cars.[1] The exhaust emissions of diesel
powered passenger cars produce 30 to 100 times more particulate matter than a
comparable gasoline engine with catalytic converter. These particulates are
composed of carbonaceous material onto which a complex mixture of organic
chemicals have been adsorbed. Some of the organic chemicals are known
carcinogens and mutagens while many have not been tested for carcinogenic and
mutagenic activity.[2] The assessment of the human health hazard, if any, due to
the increased number of diesel powered cars requires the evaluation of the
genotoxic activity of the complex mixture of organic chemicals adsorbed onto the
particles.

Is this communication, I shall describe the work in progress at the Health
Effects Research Laboratory in Cincinnati, Ohio, to evaluate the genotoxic
activity of diesel exhaust emissions. The complex mixture of organics adsorbed
onto diesel exhaust particles is being tested in laboratory rodents for
mutagenic and clastogenic activity. We are comparing the following three
different types of exposure to these organics, 1) intraperitoneal (i.p.) and
intratracheal administration of methylene chloride extract of particles, 2)
i.p. and intratracheal administration of particles and 3) inhalation of the
exhaust emissions. The methylene chloride extract should be the most genotoxic
of the types of exposure and inhalation of the particles the least. Therefore,
the methylene chloride extract was used to determine, under optimal conditions,
whether the particle contain a significant amounts of genotoxic chemicals to be
detected. The inhalation studies were performed in order to determine the
genotoxic effect under conditions by which humans might be exposed. Exposure to
the particles provided an estimate of the ability of the organics to be released
in vivo from the particles in order to exert a genotoxic activity. The activity
of the particles should be intermediate between the extract and the exhaust.

The genotoxic assays performed include, 1) Ames Salmonella mutagenicity with/without metabolic activation of urine, 2) micronuclei in polychromatic erythrocytes, 3) sister chromatid exchange in bone marrow cells, fetal hamster liver exposed in utero and primary lung cultures from hamster exposed in vivo, 4) metaphase analysis in bone marrow cells and 5) sperm morphology and motility.

MATERIALS AND METHODS

Generation of diesel exhaust[3]

Diesel emission was produced by one of two Nissan CN-6 diesel 6 cylinder engines coupled to a Chrysler torque-flite automatic transmission Model A-727 and mounted on an Eaton-Dynamometer Model 758-DG. The engines were operated by Federal Short Cycle type driving modes. The exhaust was diluted with air 1:9 so that it contained about 12 mg/m^3 particulate matter. In earlier studies the exhaust was diluted 1:18 so that it contained 6-7 mg/m^3.

Preparation of diesel particulate and extract

The particles were collected on teflon coated pallflex T60A20 type filters (Pallflex Products Corp.) and extracted for 24 hr. in a soxhlet extraction apparatus using methylene chloride (Fisher Chemical Co., Pittsburgh, PA) as the eluant. The eluant was filtered through a fluoropore filter (Millipore Corp.) backed by a microfiber glass disc (Millipore Corp.). The mass of the extract was obtained by gravimetric determination of an aliquot of the filtrate after blowing off the methylene chloride with nitrogen. The rest of the filtrate was made up to the desired concentration in dimethyl sulfoxide (DMSO) by solvent exchange using a stream of nitrogen to remove the methylene chloride. All extractions and processing procedures required to obtain the diesel extract in DMSO were performed under yellow lights.

Animals. The animals were maintained in accordance with the standards set forth in the "Guide for the Care and Use of Laboratory Animals" of the Institute of Laboratory Animal Resources, National Research Council. They received water and Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) ad libitum. The animals were exposed to diesel exhaust emissions in chambers 8 hrs/day from 7:00 a.m. to 3:00 p.m. and 7 days/week.

Micronucleus assay[4,5]

The animals were sacrificed by cervical dislocation and the bone marrow cells harvested by the method of Schmidt.[4] Briefly, the intact femurs were removed and cleaned of all muscles. For the mice, both proximal and distal ends were snipped off. Due to the limited yield of bone marrow cells from Chinese hamsters, the ends of the femurs were not snipped off. About 0.2 ml of fetal

bovine serum (FBS) was aspirated into a 1 ml tuberculin syringe fitted with a 23 gauge needle. The needle tip was carefully inserted into the proximal end of the femur, and after the femur was completely submerged in FBS contained in small test tube, the bone marrow was gently aspirated. The needle was removed from the syringe, and FBS containing the bone marrow cells was slowly injected back into the tube. The syringe was then inserted into the distal end and the aspiration repeated. The bone marrow suspension from both femurs was combined and centrifuge at 1000 rpm for 5 minutes. The pellet was gently suspended in a drop of FBS and spread with a coverglass on a slide.

The slides were air-dried and stained within 24 hours. The staining procedure included: 3 minutes in 0.2% (w/v) May Grunwald dissolved in methanol; 2 minutes in 0.2% (w/v) May Grunwald diluted with an equal volume of H_2O; a rinse in H_2O; 10 minutes in Giemsa (1:6, Gurrs Improved R66 Giemsa:H_2O); and a final rinse in H_2O. The slides were blotted dry, cleared in xylene for 5 minutes and immediately covered with a cover glass using Pro-Texx. All slides were coded prior to evaluation. For each animal, the number of micronucleated poly-chromatic erythrocytes in 1000 such cells was determined as recommended by von Ledebur and Schmid.[6]

Sister chromatid exchange (SCE) assay[7,8]

Bone marrow cells. A slight modification of the procedure of Allen et al.[9] for in vivo sister chromatid exchange was used. Briefly, twenty-four hours prior to sacrifice a 60 mg pellet of 5-bromo-2-deoxyuridine (BrdU) was implanted under the skin between the scapulae. Two hours prior to sacrifice the animals were injected intraperitoneally with colchicine (10 mg/Kg bd. wt.). The animals were sacrificed by cervical dislocation and the intact femurs removed. All muscle was removed from the bone and the proximal end of the bone gently snipped off. The bone marrow cells were flushed from the canal with 0.075 M KCl and the contents from both femurs combined.

The cell suspension was incubated in a 37°C water bath, for 30 minutes, followed by centrifugation at 1,000 g for 10 minutes. The supernatant was discarded. The cells were fixed in ice-cold Carnoy's solution (3:1 v/v methanol:glacial acetic acid). After 20 minutes at 4°C the fixative was removed by centrifugation. The fixation process was repeated two more times. The final cell pellet was resuspended in a small volume of the ice-cold fixative and dropped onto cold, wet slides. The slides were air-dried in a dust free atmosphere overnight.

The Hoechst-Giemsa black-light method described by Goto et al.[10] was used to stain the cells. The slides were rinsed in double distilled water, stained with

Hoechst 33158 dye (50 ug/ml) for 15 minutes, rinsed again in water, and blotted dry. Next, the slides were placed on a slide warmer (50°C), covered with a coverglass using MacIlvaines buffer and exposed to black light for 22 minutes. Then the coverglass was removed and the slides allowed to dry for at least 2 hrs. Counterstaining was done in 6% Giemsa for 10 minutes. The coverglass was then remounted onto the microscope slide using Pro-Texx mounting medium.

All slides were coded prior to evaluation. Twenty-five metaphases from each animal were evaluated for the number of SCE. The mitotic index was determined by counting the number of dividing cells per 1,000 cells.

Lung cells

Using the intratracheal instillation procedure of Saffiotti et al.,[11] animals were administered either 1) diesel particles, 2) dichloromethane extract of diesel particles absorbed onto carbon black or 3) benzo(a)pyrene absorbed onto carbon black. The samples were suspended in Hank's balanced salt solution (HBSS) containing 20% (v/v) Emalphor El-620.

The animals were sacrificed by cervical disolocation and the heart and lungs quickly excised. The lung tissue was finely minced with sterile scissors in McCoy's 5A medium suplemented with 10% fetal bovine serum, 100 units of penicillin and 100 ug streptomycin/ml. The minced tissue was applied to a Petri dish and incubated overnight in a 5% CO_2 incubator at 37°C. The unattached cells and tissue fragments were removed, washed in HBSS twice in order to remove erythrocytes and cellular debris, and then distributed onto three additional Petri dishes. The attached cells in the original dish were washed once with HBSS to remove cellular debris and then incubated with complete McCoy's medium supplement with 10% fetal bovine serum.

When the cultures showed colonies containing 50 or more cells, they were treated with BrdU (10 ug/ml) in subdued illumination. The dishes were incubated in the dark for an additional 40 hrs. At that time, the cultures were treated with 0.05 ug/ml colcemid (Grand Island Biological Co.) for four hrs. The cells were then suspended by trypsinization (0.05% trypsin in calcium and magnesium free HBSS) and treated for 30 minutes at 37°C in a hypotonic solution of 0.075 M KCl. Finally the cells were fixed in ice-cold Carnoy's solution and stained by the Hoechst-Giemsa black-light procedure described above.

Fetal liver cells exposed in utero

On day 12 of gestation, pregnant Syrian hamsters were treated with BrdU by implanatation of a pellet under the skin. The diesel particulates, extracts or benzo(a)pyrene were administered two hrs. later by intraperitoneal injection. Eighteen hours after the BrdU was given, the animals were sacrificed by cervical

dislocation and the fetuses quickly removed. Fetal livers from each litter (10-14 fetuses) were pooled in 10 ml of calcium and magnesium free HBSS. After 20 minutes of incubation at 37°C, the livers were gently crushed with a spatula against the side of the flask to release the cells from the connective tissue. The suspension was treated with colcemid for 2 hrs. at 37°C in a CO_2 incubator. The cells were collected by centrifugation and resuspended in 5-6 ml of 0.075 M KCl. After incubation for 10 minutes at 37°C, the cells were collected by centrifugation. The collected cells were fixed with ice-cold Carnoy's solution and stained by the Hoechst-Giemsa black-light method as described above.

RESULTS

Micronucleus assay

The number of micronucleated polychromatic erythrocytes in mice and Chinese hamsters exposed to diesel exhaust emissions (12 mg/m^3 particulate) for one month or administered 640 mg/kg bd. wt. diesel particulate was not different from the corresponding controls of clean air and DMSO controls (Table 1). There appeared to be a slight increase in micronucleated cells in mice but not Chinese hamsters administered an extract of diesel particulate (1000 mg/kg bd. wt.). The clastogenic activity of the organic chemicals present on diesel exhaust particulate would appear to be below the sensitivity of the micronucleus assay.

Sister chromatid exchange in bone marrow cells

Exposure of mice to diesel exhaust emissions (12 mg/m^3 particulate) for one month did not induce SCE in bone marrow cells (Table 2). The administration of either diesel particulates (300 mg/kg bd. wt.) or their extract (800 mg/kg bd. wt.) resulted in an increased incidence of SCE in mice sacrificed two days post-treatment. The increase above DMSO controls in the incidence of SCE was 5.64, 3.68 nd 7.82 SCE/metaphase for diesel particulate, diesel extract and benzo-(a)pyrene (100 mg/kg bd. wt.), respectively. The dose of diesel particulate and extract administered to the mice was only three and eight times the dose of benzo(a)pyrene. It would appear that the activity of benzo(a)pyrene was only 4.2 fold more active than diesel particulate and 17 fold for diesel extract. It should be recognized that these calculations are tenuous and need to be substantiated by comparison of the dose-response relationship for diesel particulate and extract to the dose-response relationship for benzo(a)pyrene.

Sister chromatid exchange in lung cells. While exposure of Syrian hamsters to diesel exhaust emissions (6-7 mg/m^3 particulate) for three months did not increase the incidence of SCE in lung cells, the exposure to 12 mg/m^3 particulates for 3.5 and 8.5 months did induced SCE. The intratracheal

TABLE 1

MICRONUCLEUS ASSAY IN MICE AND CHINESE HAMSTERS[a]

Experiment Treatment	Micronucleated Polychromatic Erythrocytes	
	Mice	Chinese Hamsters
Experiment I		
Diesel Exhaust		
a. 1 month	$0.59 \pm 0.07 (10)$[b]	$0.49 \pm 0.07 (10)$
Clean Air	$0.60 \pm 0.16 (10)$	$0.48 \pm 0.10 (10)$
Water	$0.54 \pm 0.13 (5)$	--
Cyclophosphamide		
a. 1000 mg/kg bd wt	$2.02 \_ 0.43 (5)$	--
Experiment II		
Diesel Particulate		
a. 640 mg/kg bd wt	$0.10 \pm 0.05 (6)$	$0.23 \pm 0.10 (6)$
DMSO	$0.43 \pm 0.07 (6)$	$0.20 \pm 0.04 (6)$
Cyclophosphamide		
100 mg/kg bd wt	$5.77 \pm 0.58 (6)$	$5.38 \pm 0.80 (6)$
Experiment III		
Diesel Extract		
a. 1000 mg/kg bd wt	$0.80 \pm 0.20 (6)$	$0.25 \pm 0.04 (6)$
DMSO	$0.32 \pm 0.06 (6)$	$0.12 \pm 0.03 (6)$
Cyclophosphamide		
a. 100 mg/kg bd wt	$3.83 \pm 0.19 (6)$	$5.58 \pm 0.50 (6)$

[a]Preliminary results from M.A. Pereira (U.S. EPA, HERL–Cincinnati, OH) and
P.S. Sabharwal (Environmental Health Research and Testing, Inc., Cincinnati,
OH). Mice (B6C3F1) were from Harlan, Indianapolis, IN and Chinese hamsters
from Northeastern Univ., Boston, MA.

[b]Percentage of micronucleated polychromatic erythrocytes presented as the
mean $\pm$ SE for groups containing the number of animals in parenthesis.

administration of either diesel particulate or diesel extract resulted in a dose
related increase in SCE. The activity of diesel extract was approximately 10
times greater than the activity of diesel particulate. Since the recovery of
the mass of the particulate in the extract was on the average 23%, it appeared
that at least a third of the genotoxic material adsorbed onto the in-
tratracheally instilled particulate was available. The genotoxic chemicals

adsorbed onto the particles in diesel exhaust emissions were available to exert their activity in lung cells when administered by all three types of exposure studied, i.e. inhalation, particulate and extract.

TABLE 2

INDUCTION OF SISTER CHROMATID EXCHANGE IN BONE MARROW CELLS OF MICE[a]

Experiment Treatment	N	SCE per Metaphase
Experiment I		
Diesel Exhaust a. 6 month	10	$4.57 \pm 0.39 (10)$[b]
Clean Air	6	4.17 ± 0.42
Corn Oil	6	5.87 ± 0.47
Benzo(a)pyrene a. 100 mg/kg bd wt	6	13.16 ± 1.02
Experiment II		
Diesel Particulate a. 300 mg/kg bd wt	6	11.31 ± 0.94
Diesel Extract a. 800 mg/kg bd wt	6	9.35 ± 0.60
DMSO	6	5.67 ± 0.45
Benzo(a)pyrene a. 100 mg/kg bd wt	6	13.49 ± 1.01

[a]Preliminary results from M.A. Pereira (U.S. EPA, HERL-Cincinnati, OH) and P.S. Sabharwal (Environmental Health Research and Testing Inc., Cincinnati, OH). Mice (B6C3F1) were from Harlan, Indianapolis,IN.
[b]Results are means $\pm$ SE.

Sister chromatid exchange in fetal liver exposed in utero. The exposure of pregnant Syrian hamsters from day one of gestation to diesel exhaust emissions (12 mg/m^3) or to diesel particulate at the LD_{50} (300 mg/kg bd. wt.) on day 12 of gestation, did not increase the incidence of SCE in fetal liver when determined on day 13 (Table 4). The administration of diesel extract on day 12 of gestation resulted in a dose-dependent increase in SCE in the fetal liver on day 13. This would indicate that once the organic chemicals absorbed onto the particles in diesel exhaust are eluted, they are capable of crossing the placenta and exerting a genotoxic effect. Metabolic activation, if necessary, of the

TABLE 3

INDUCTION OF SISTER CHROMATID EXCHANGE IN PRIMARY LUNG CULTURES FROM SYRIAN
EXPOSED IN VIVO[a]

Experiment Treatment	SCE per Metaphase
Experiment I	
Diesel Exhaust	
a. 6 mg/m^3; 3 months	11.86 $\pm$ 0.47(5)[b]
Clean Air	
a. 3 months	11.52 $\pm$ 0.58 (8)
Experiment II	
Diesel Exhaust	
a. 12 mg/m^3; 3.5 months	19.41 $\pm$ 1.03(12)
b. 12 mg/m^3; 8.5 months	22.22 $\pm$ 1.02(4)
Clean Air	
a. 3.5 months	10.94 $\pm$ 0.43(12)
b. 8.5 months	9.03 $\pm$ 0.38(5)
Experiment III	
Diesel Particulates	
a. 0	10.74 $\pm$ 0.17(5)
b. 44 mg/kg	12.95 $\pm$ 0.41(5)
c. 87 mg/kg	15.37 $\pm$ 0.21(5)
d. 130 mg/kg	18.18 $\pm$ 0.65(5)
Benzo(a)pyrene	
a. 20 ug/hamster	17.16 $\pm$ 0.50(5)
Experiment IV	
Diesel Extract	
a. 0	10.47 $\pm$ 0.32(5)
b. 3.3 mg/kg	13.90 $\pm$ 0.78(5)
c. 6.6 mg/kg	16.18 $\pm$ 0.21(5)
d. 13.3 mg/kg	19.96 $\pm$ 0.92(5)
Benzo(a)pyrene	
a. 20 ug/hamster	16.34 $\pm$ 0.47(5)

[a]Results from D.E. Rounds (Pasadena Foundation for Medical Research, Pasadena,
CA) Final Report Contract No. 68-03-2945 with U.S. EPA HERL-Cincinnati, OH
(Project Officer: John G. Orthoefer). Syrian hamsters were from Engle Lab,
Farmersburg, IN.

[b]Results are means $\pm$ SE for groups containing the number of animals in paren-
thesis.

genotoxic chemicals could have occurred either maternally prior to crossing the
placenta or in the fetus. It also appeared that sufficient toxic material was
eluted from the administered particles to cross the placenta and decrease the
mitotic index in the fetal liver.

DISCUSSION

The genotoxic activity of diesel exhaust emissions was evaluated in the
micronucleus and sister chromatid exchange assays. The micronucleus assay
measures the ability of a substance to either cause pieces of chromatin to be
severed from chromosomes or to disrupt the spindle apparatus.[5,6,12,13] This
results in pieces and/or whole chromosomes which do not segregate during mitosis
with the rest of chromosomes. Instead, they are excapsulated by a membrane so
that upon examination under a microscope they appear as small as micronuclei.
The sister chromatid exchange assay measures the exchange apparently during
replication of chromatin between two sister chromatids.[8,14,15] This exchange
results in an alteration of the genome. There is a good correlation between the
ability of a substance to increase the incidence of SCE and to cause clastogenic
effects and mutations,[8,16,17] though there are some exceptions.[18,19] By use of
both the micronucleus and SCE assays, it would be possible to detect both the
clastogenic and mutagenic activity of diesel exhaust emissions.

The genotoxic activity of diesel exhaust in lung cells was evaluated for the
three following types of exposure: 1) inhalation, 2) administration of diesel
particulate and 3) administration of an extract of the particulate. The
inhalation of diesel exhaust emissions and the intratracheal instillation of
diesel particulate or diesel extract increased the incidence of SCE. The order
of potency for induction of SCE in lung cells was extract > particulate >
inhalation. The Syrian hamsters were exposed to diesel exhaust emissions
containing 12 mg/m^3 particles for 8 hrs/day, 7 days/week. A Syrian hamster
weighing about 90 gm inhales approximately 0.06 liters/min., so the exposure in
3.5 months would result in the maximum accumulation of 389 mg/kg bd. wt.
particulate. The maximum accumulated dose induced an equivalent number of SCE
compared to an extract equivalent to 58 mg particulate/kg bd. wt. as calculated
with 23% being the recovery of the mass of the particulate in the extract.
Therefore, it would appear that at least 15% of the genotoxic material adsorbed
onto inhaled diesel particles was available to exert its activity in the lung.
The amount of available genotoxic material was probably higher, since not all of
the inhaled particles were deposited in the lungs.

TABLE 4

SUMMARY OF THE EFFECT ON SISTER CHROMATID EXCHANGE AND MITOTIC INDEX IN
HAMSTER FETAL LIVER CELLS EXPOSED IN UTERO[a]

Treatment	SCE per Metaphase	Mitotic Index
Diesel Exhaust	no effect	no effect
Diesel Particulate	no effect	decrease
Diesel Extract	increase	decrease

[a]Results are from M.A. Pereira (U.S. EPA, HERL-Cincinnati, OH) and P.S.
Sabharwal (Environmental Health Research and Testing, Inc., Cincinnati, OH)
and have been submitted for publication

The ability of the genotoxic material in diesel exhaust emissions to be
distributed systemically was evaluated by the micronucleus and SCE assays in
bone marrow cells. The micronucleus assay was not sensitive enough to detect
genotoxic activity by the organics adsorbed onto diesel exhaust particles
administered either by inhalation or by intraperitoneal injection of particles
or extract. In the SCE assay a six month exposure of mice to diesel exhaust
emissions did not increase the incidence of SCE in bone marrow cells, while the
intraperitoneal administration of diesel particulate or diesel extract did
induce SCE. The assumption of a linear dose-response relationship for diesel
particulate to the increased incidence of SCE in bone marrow cells would appear
reasonable, since a linear dose-response relationship was found in lung cells.
This linear extrapolation predicts that an i.p. dose of 75 mg/kg bd. wt. diesel
particulate would have been detected. The six month exposure in mice that did
not increase the incidence of SCE (assuming that a 30 gm mouse inhales 0.02
liters/min.) would have resulted in the maximum accumulation of 667 mg/kg bd.
wt. particulate. This dose of inhaled particles was ten times the predicted
minimum single systemic dose of 75 mg/kg bd. wt. particulate to which the SCE
assay in bone marrow cells would be sensitive. Therefore, a sufficient dose of
genotoxic materials was administered by inhalation to possibly be detected by
the SCE assay in bone marrow cells. However, under the conditions of the
experiment, the SCE assay in bone marrow was unable to demonstrate that the
genotoxic material adsorbed to inhaled particles was available for systemic
distribution. This is in contrast to direct application of the particles to
lung cells by intratracheal instillation and inhalation where most of the
genotoxic material was available to induce SCE in lung cells.

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The induction of heritable mutations, teratogenesis and embryotoxicity by the organic material adsorbed onto diesel particles requires that the material be distributed systemically and when appropriate, cross the blood-testes barrier or the placenta. The SCE assay in bone marrow cells is more sensitive than the specific locus assay[20] used to determine the mutagenic hazard of a chemical. Therefore, one would predict that the genotoxic activity of a substance (inhalation of Diesel Exhaust Emissions) which was below the sensitive of the SCE assay in bone marrow cells, would be too low to be detected in the specific locus assay as a mutagenic hazard. Our evidence would indicate that an insufficient amount of the genotoxic material adsorbed to particles in diesel exhaust emissions (as determined by the SCE assay in bone marrow cells) was available for systemic distribution to the reproductive organs to represent a measurable mutagenic hazard.

When the genotoxic material was administered as an extract, the material did cross the placenta and induce SCE in fetal liver. Intraperitoneal administration of diesel particulate and inhalation of diesel exhaust emissions did not increase the incidence of fetal SCE. We were therefore unable to demonstrate that the genotoxic material when administered adsorbed onto the particles reached the fetus. This distribution to the fetus would be required for the genotoxic material to represent a teratogenic hazard. In conclusion, we were unable to support a possible mutagenic or teratogenic hazard for the exposure in laboratory animals of the particles in diesel exhaust emissions. Therefore, if a genotoxic hazard exists for this exposure it would appear to be limited to the lung where it might cause cancer.

ACKNOWLEDGEMENTS

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HUMAN CELL MUTAGENICITY OF POLYCYCLIC AROMATIC HYDROCARBON COMPONENTS OF DIESEL EMISSIONS

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INTRODUCTION

In an earlier report by Liber et al.[1], it was shown that a methylene chloride
extract of automobile diesel exhaust particulate was significantly mutagenic to
both Salmonella typhimurium and diploid human lymphoblasts in the concentration
range of 50-100 µg/ml when treatment was in conjunction with an Aroclor-induced
rat liver microsome-containing postmitochondrial supernatant (PMS). This die-
sel exhaust particulate extract also contained "direct-acting" mutagens for S.
typhimurium. However, no "direct-acting" mutagenic activity was detected by
the human lymphoblast mutation assay.

Fractionation of the methylene chloride extract was performed and the
seven resultant fractions were tested for their mutation inducing ability in the
S. typhimurium 8-azaguanine resistance forward mutation assay, with and without
rat liver PMS.[1,2] A hexane/toluene fraction, which represents 6.5% by weight of
the total methylene chloride extract, contained the polycylic aromatic hydro-
carbons (PAH) and was found to be the most mutagenic fraction to S. typhimurium
with PMS activation.[1,2] Although others have reported that organic extracts of
diesel exhaust particulate from various sources contain "direct-acting" mutagens
for mammalian cells[3,4], we have placed an emphasis upon determining what PAH may
be responsible for the mutagenicity of our diesel exhaust particulate extract to
human lymphoblasts. In addition, we have initiated studies to determine the
mutagenicity of PAH and their derivatives that are found in other organic ex-
tracts of emissions collected from diesel engines.

MATERIALS AND METHODS

Diesel exhaust particulate sample. The diesel exhaust extract sample utili-
zed in these experiments was a gift of Dr. Morton Beltzer of Exxon Research and
Engineering Company, Linden, New Jersey. The diesel exhaust particulate was

collected from a 1978 Oldsmobile 350 CID engine burning blended commercial No. 2 diesel fuel operated with repetitive hot start Federal Testing Procedures. The exhaust was passed through a dilution chamber and the particulate was collected on a Pallflex type T60A20 filter. The filter was Soxhlet extracted with methylene chloride for 16 hr followed by solvent evaporation on a steam bath with nitrogen purging.

The methylene chloride extract of the diesel soot was then fractionated based on polarity for bacterial mutagenicity testing. Details of the fractionation procedure have been published in a separate paper.[2] The PAH containing hexane/toluene fraction was analyzed by gas chromatography/mass spectrometry (GC/MS) for the identification of individual PAH and derivatives.[2]

Source of PAH. Benz(a)anthracene and phenanthrene were purchased from Eastman Chemical Co., Rochester, NY. Benzo(a)pyrene was purchased from Sigma Chemical Co., St. Louis, MO. Chrysene, triphenylene, 1-methylphenanthrene, 2-methylphenanthrene and 1-methylpyrene were obtained from ICN Life Sciences Group, Planview, NY. Anthracene, 2-methylanthracene, 9-methylanthracene, 9,10-dimethylanthracene, fluoranthene and pyrene were obtained from Aldrich Chemical Co., Milwaukee, WI. The 9-methylphenanthrene was obtained as a gift from M. L. Lee, Bringham Young University. The fluoranthene 2,3-dihydrodiol was provided by W. Rastetter, Massachusetts Institute of Technology.

Cyclopenteno(c,d)pyrene and its derivatives, cyclopentano(c,d)pyrene (CPAP), CPAP-3,4-oxide, CPAP-3,4-trans-diol and CPAP-3,4-cis-diol were synthesized in the laboratory of E. L. Cavalieri. A detailed report of the synthesis of cyclopenteno(c,d)pyrene (CPEP) and its derivatives will be reported elsewhere.

Source of PAH metabolizing element. Liver microsome containing postmitochondrial supernatant (PMS) from Aroclor 1254-induced male Sprague-Dawley rats was prepared by the method of Ames et al.[5] and purchased from Litton Bionetics, Kensington, MD. The PMS had a concentration of 29.3 mg protein/ml as determined by the supplier and was utilized at a final concentration of 5% v/v (1.5 mg protein/ml) in the human lymphoblast mutation assay. The PMS was radiosterilized at $-80°C$[6] and maintained at this temperature until being thawed immediately before use.

Human lymphoblast mutation assay. The diploid human lymphoblast cell line TK6, a presumptive heterozygote at the thymidine kinase locus, was utilized to select mutants resistant to trifluorothymidine (F_3tdR).[7,8] The cells were routinely maintained in suspension culture at 37°C in RPMI 1640 culture medium supplemented to 10% v/v with heat inactivated horse serum, both purchased from Flow Laboratories, Inc., McClean, VA.

A minimum of 2×10^7 exponential phase cells were treated per culture and duplicate cultures were treated per experimental point. Treatment with either the diesel exhaust particulate extract or individual PAH was in the presence of rat liver PMS and the necessary cofactors.[8,9] Following treatment at 37°C for 3 hr, the cells were pelleted by centrifugation, resuspended in fresh medium, and counted to determine the initial cell density. At this time a small aliquot was also taken, diluted and plated to determine the toxicity of the treatment.

Expression of resistance to F_3tdR was allowed to develop for a minimum period of three days before mutant selection occurred. This amount of time is more than sufficient to allow maximum phenotypic expression of the induced F_3tdR resistant mutants.[8]

F_3tdR resistant mutants were selected by diluting the cultures to a cell density of 2×10^5/ml and trifluorothymidine was added to give a final concentration of 2 μg/ml. An aliquot of cells was also diluted to 10 cells/ml and plated to determine cloning efficiency. Four 96 well microtiter plates (Linbro Scientific Inc., Hamden, CT) at 0.2 ml of cell suspension/well were plated to select the F_3tdR resistant mutants and 2 plates were plated at low cell density (2 cells/well) to determine the cloning efficiency of each culture. Plates were incubated at 37°C in a 5% CO_2 in air incubator for 14 days before scoring of clones took place.

Calculation of mutational data. The use of the Poisson distribution and associated calculations to determine the cloning efficiency and F_3tdR resistant mutant fraction for the TK6 human lymphoblast mutation assay has been reported.[8,10] The minimal concentration of a treatment required to induce a significant mutant fraction is determined by interpolation on the concentration vs. mutation frequency curves to the historic upper 99% confidence limit of the spontaneous background mutation frequency. The concentration at this interpolation point is taken as the minimal concentration which would induce a statistically significant mutant fraction. The historic upper 99% confidence limit for the spontaneous background mutation frequency for the TK6 human lymphoblast F_3tdR resistance mutation assay is 5×10^{-6}.

RESULTS

The ability of the total methylene chloride extract of our automobile diesel exhaust particulate sample to induce resistance to F_3tdR in human lymphoblasts is shown in Figure 1. A concentration of 70 μg/ml of the diesel soot extract was required to induce a significant mutant fraction with a survival
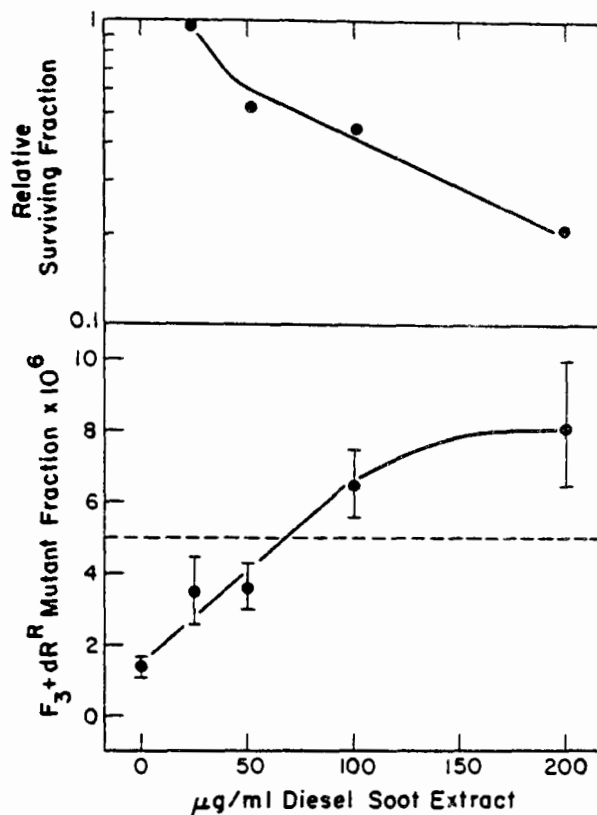
Fig. 1. Toxicity (upper portion) and mutagenicity (low portion) of a methylene chloride extract of diesel soot to human lymphoblasts. The error bars repre- sent the 95% confidence limits.

of 0.45. As reported earlier, this diesel soot extract is not mutagenically active toward human lymphoblasts in the absence of metabolic activation.[1]

Fractionation of the methylene chloride extract yielded a 1:1 hexane/toluene fraction that was most mutagenic in Salmonella typhimurium with metabolic acti- vation[1] and has subsequently been shown to contain the PAH.[2] Gas chromatog- raphy/mass spectrometry analysis of this PAH containing fraction has produced the qualitative and quantitative data summarized in Table 1. A total of 27 individual PAH and alkylated derivatives have been identified. Note that the alkylated phenanthrenes alone represent 46% by weight of the identified PAH, followed by the alkylated fluorenes which represent approximately 15% by weight of the PAH. Fluoranthene, one of the most abundant individual PAH in the die- sel exhaust extract accounts for 6% by weight of the identfied PAH. Others have also found that alkylated anthracenes/phenanthrenes and fluoranthene
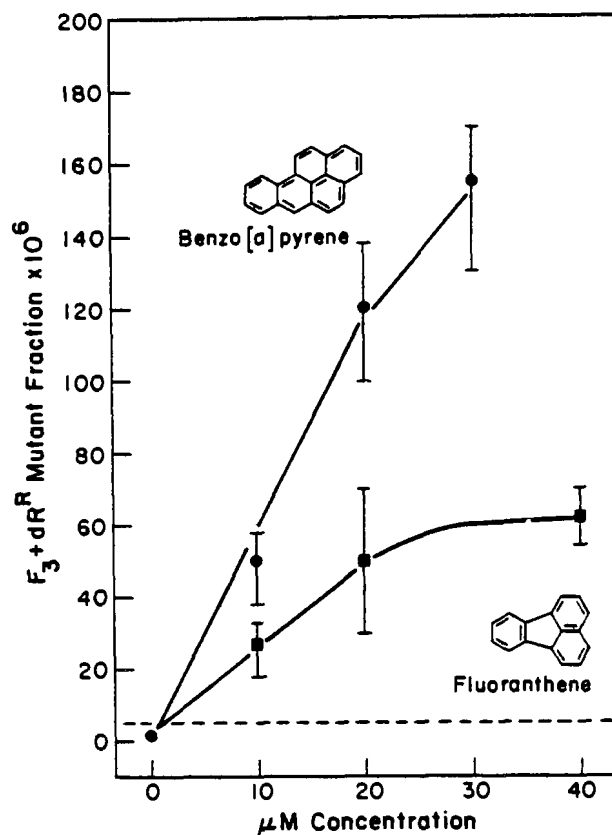
Fig. 2. Mutagenicity of benzo(a)pyrene (●) and fluoranthene (■) to human lymphoblasts. The error bars represent the 95% confidence limits. The dashed line represents the historic upper 99% confidence limit of the spontaneous background mutation frequency.

represent a major proportion of the PAH associated with diesel exhaust particulate.[11-13]

Based on the above chemical analysis of the PAH containing fraction of our diesel exhaust particulate extract, we initiated testing of the available individual PAH to determine which ones play a role in the mutagenicity of the whole extract to human lymphoblasts. Eleven PAH found in the diesel exhaust extract have been tested to determine their mutagenic potency.

Fluoranthene, one of the most abundant PAH found in our diesel exhaust particulate extract, induces a significant mutant fraction at a concentration of 2 μM and has approximately 50% of the mutagenic potency of benzo(a)pyrene (BaP), as shown in Figure 2 and Table 2.

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TABLE 1

POLYCYCLIC AROMATIC HYDROCARBONS AND DERIVATIVES IDENTIFIED IN A METHYLENE CHLORIDE EXTRACT OF DIESEL EXHAUST PARTICULATE AS DETERMINED BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY ANALYSIS

Compound	WT %
methylfluorenes	0.03
dibenzothiophene	0.02
phenanthrene	0.21
C_2-fluorenes	0.17
3-methylphenanthrene	0.15
2-methylphenanthrene	0.21
9- and 4-methylphenanthrene	0.13
1-methylphenanthrene	0.13
C_3-fluorenes	0.11
phenylnaphthalene	0.065
C_2-phenanthrene	0.52
fluoranthene	0.21
C_4-fluorenes	0.21
pyrene	0.21
methylphenylnaphthalenes	0.21
C_3-phenanthrenes	0.30
methyl fluoranthenes & methyl pyrenes	0.17
C_2-phenylnaphthalenes	0.13
C_4-phenanthrenes	0.17
C_3-phenylnaphthalenes	0.03
benzo(ghi)fluoranthene	0.03
benz(a)anthracene	0.007
chrysene/triphenylene	0.01
nitropyrene	0.03
benzofluoranthenes	0.03
benzopyrenes	0.03
perylene	0.002
TOTAL	3.5

Figure 3 presents the results obtained when phenanthrene and three of its monomethyl derivatives were tested for their mutation inducing ability in human lymphoblasts. Phenanthrene was weakly active requiring a concentration of 100 μM to induce a significant mutant fraction. 2-Methylphenanthrene was
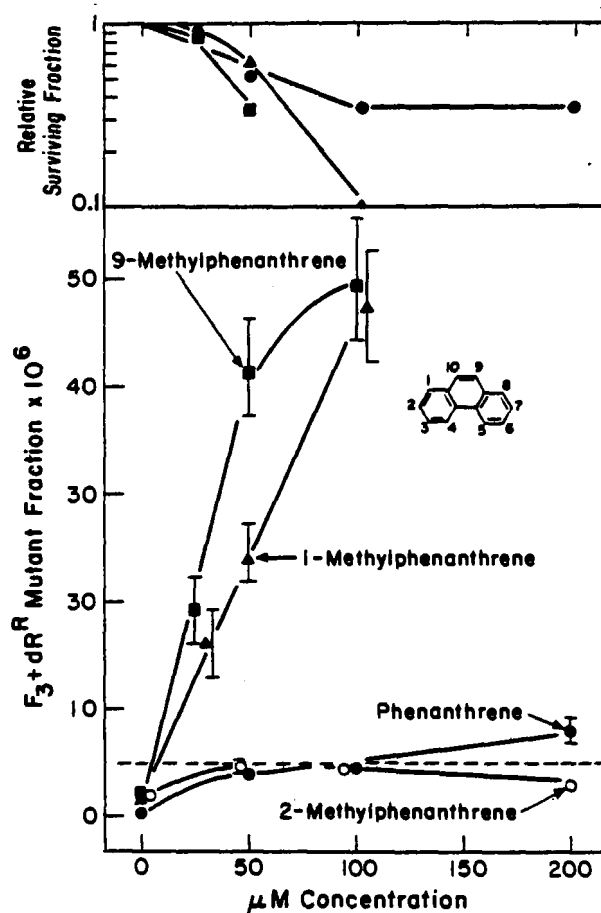
Fig. 3. Toxicity and mutagenicity of phenanthrene and some methylated derivatives to human lymphoblasts. Error bars are as in Fig. 1.

inactive up to a concentration of 200 μM. However, both 1-methylphenathrene and 9-methylphenanthrene were relatively active mutagens to human lymphoblasts inducing significant mutant fractions at the concentrations of 5 μM and 4 μM, respectively (Table 2).

In addition to the carcinogen BaP,[14,15] the known carcinogens benz(a)anthracene (BA), chrysene (CH), and the noncarcinogen triphenylene[14,15] are present in our diesel soot extract and have been tested to determine their mutagenic potency to human lymphoblasts. The results presented in Figure 4 show that BA and CH have similar mutagenic activity for human lymphoblasts, inducing significant mutant fractions at 9 μM and 6 μM, respectively. The noncarcinogen triphenylene was significantly less active, requiring a concentration of 20 μM to induce a significant mutant fraction.
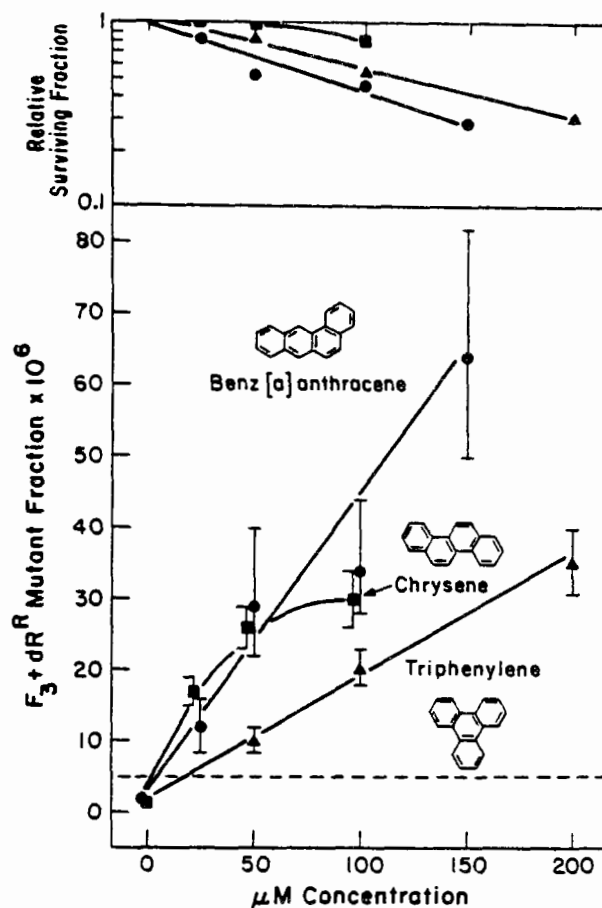
Fig. 4. Toxicity and mutagenicity of benz(a)anthracene (●), chrysene (■) and triphenylene (▲) to human lymphoblasts. Error bars are as in Fig. 1.

Other PAH components of our diesel soot extract that have been tested for their mutagenic potency to human lymphoblasts are pyrene and 1-methylpyrene. These two PAH were inactive up to the tested concentrations of 300 μM and 100 μM respectively. Mutagenicity results for the 11 tested PAH that are present in our diesel exhaust particulate extract are summarized in Table 2.

Many other PAH and derivatives have been found to be associated with particulates produced by the combustion of diesel fuel, some of which are shown in Table 3. Anthracene and its alkylated derivatives have been identified as components of various organic extracts of diesel exhaust emissions,[11-13] which led us to test several of these PAH for their ability to induce mutations in the human lymphoblast assay.

TABLE 2

MUTAGENIC CONTRIBUTION OF INDIVIDUAL POLYCYCLIC AROMATIC HYDROCARBONS TO AN AUTOMOBILE DIESEL EXHAUST
PARTICULATE EXTRACT FOR HUMAN LYMPHOBLASTS

Compound	Induced[a] Mutation	Relative[b] Mutagenicity	Weight Percent	Concentration[c] (µM)	Mutation Contribution[d] Mutant Fraction x 10^6
benz(a)anthracene	+ 9	0.1	0.007	0.04	0
chrysene	+ 6	0.2	0.01	0.06	0
fluoranthene	+ 2	0.5	0.2	0.9	1.3
phenanthrene	+100	0.01	0.2	1.3	0
1-methylphenanthrene	+ 5	0.2	0.1	0.7	0.4
2-methylphenanthrene	-200		0.2	1.0	0
9-methylphenanthrene	+ 4	0.25	0.07	0.3	0.2
pyrene	-300		0.2	1.0	0
1-methylpyrene	-100				0
triphenylene	+ 20	0.04	0.01	0.06	0
benzo(a)pyrene	+ 1	1	0.03	0.01	0.03
Component contribution			1.03	5.4 µM	1.93
Total extract			100	100 µg/ml	4.3

[a](+) indicates the induction of a significant mutant fraction at the indicated µM concentration or (-) indi-
cates the response was negative up to the µM concentration tested.

[b]Indicates the mutagenic potency relative to that of benzo(a)pyrene which induces a significant mutant
fraction at a concentration of 1 µM in the human lymphoblast mutation assay.

[c]The µM concentration of the individual PAH in 100 µg/ml of the methylene chloride extract of our diesel
exhaust particulate extract.

[d]The predicted amount of the mutant fraction contributed by the individual PAH to the mutagenicity of 100
µg/ml of our diesel exhaust particulate extract after correction for spontaneous background mutant fractions.

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TABLE 3

OTHER POLYCYCLIC AROMATIC HYDROCARBONS AND DERIVATIVES IDENTIFIED IN VARIOUS
EXTRACTS OF DIESEL EXHAUST PARTICULATES

Compound	Source
alkylnaphthalenes	a,c
alkylanthracenes	a
cyclopenteno(c,d)pyrene	b,c
methyl benz(a)anthracene, chrysene or triphenylene	c
methylbenzofluoranthenes	c
methyl benzo(a)pyrene, benzo(e)pyrene or perylene	c

[a]Rodriguez, C. F., Fischer, J. B., and Johnson, D. E., Health Effects of Diesel
Engine Emissions, Pepelko, W. E., Danner, R. M., and Clarke, N. A. ed., U.S.
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[b]Stenberg, U., Alsbert, T., Blomberg, L., and Wännman, T., Polynuclear
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Figure 5 shows that anthracene and 9,10-dimethylanthracene were not muta-
genic to human lymphoblasts up to the tested concentrations of 200 μM and 100
μM, respectively. 2-Methylanthracene was only weakly active as a mutagen to the
human lymphoblasts inducing a significant mutant fraction at the concentration
of 60 μM. However, 9-methylanthracene was mutagenic to human lymphoblasts at a
concentration of 9 μM, which is similar to the mutagenic potencies of BA and CH.
A summary of the results for anthracene and its methylated derivatives is pre-
sented in Table 4.

We are not only interested in determining what role PAH play in the muta-
genicity of diesel exhaust emission to human cells, but also in how certain PAH
are metabolized to their ultimate mutagenic forms for human cells. We have car-
ried out such a study with the carcinogen cyclopenteno(c,d)pyrene (CPEP)[16-18],
which is a major component of gasoline exhaust emissions and is associated with
the particulate of diesel exhaust as well.[13,19-21] The parent PAH, CPEP, in-
duced a significant mutant fraction at a concentration of 7 μM, (Figures 6,
Table 4) which is similar to the results in a previous report utilizing a dif-
ferent human lymphoblast cell line.[9] An arene oxide at the 3,4-position of
CPEP (CPAP-3,4-oxide) was highly mutagenic to the human lymphoblasts without
metabolic activation (Figure 6), inducing a significant mutant fraction at a

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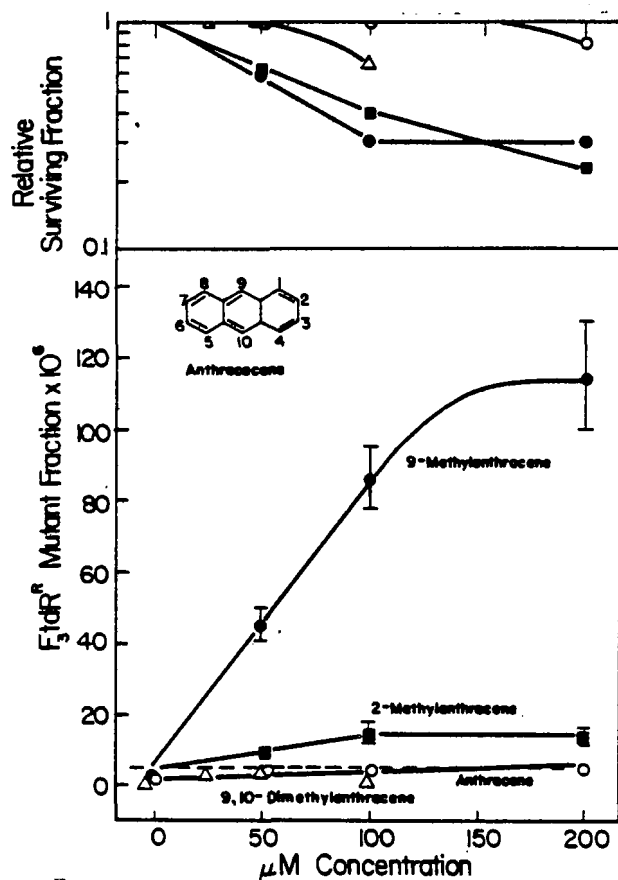
Fig. 5. Toxicity and mutagenicity of anthracene (0), 2-methylanthracene (■), 9-methylanthracene (●) and 9,10-dimethylanthracene (△). Error bars are as in Fig. 1.

concentration of 0.4 μM. These data show that CPAP-3,4-oxide is an ultimate mutagen for human lymphoblasts. However, in the presence of rat liver PMS this arene oxide was mutagenically inactive (Figure 6). Cyclopentano(c,d)-pyrene (CPAP), which lacks the 3,4-double bond of CPEP and therefore cannot form the 3,4 arene oxide, was significantly less mutagenic to human lympho-blasts compared with the activity of CPEP (Figure 6). CPAP was approximately 6-fold less mutagenically active in the human lymphoblast mutation assay rela-tive to CPEP as indicated by the data shown in Table 4.

The mutagenicity of two dihydrodiol derivatives of CPEP, CPAP-3,4-cis-diol and CPAP-3,4-trans-diol was also determined. CPAP-3,4-trans-diol, which is the major rat liver microsome metabolite of CPEP,[22] was not mutagenically active in the human lymphoblast mutation assay up to a concentration of 80 μM (data not
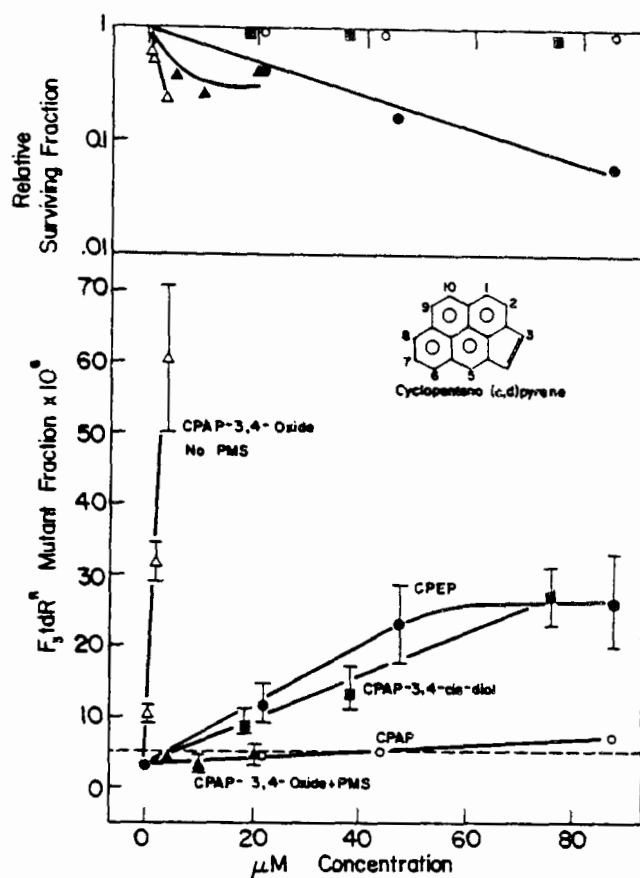
Fig. 6. Toxicity and mutagenicity of cyclopenteno(c,d)pyrene (●), CPAP (○), CPAP-3,4-oxide, no PMS (△), CPAP-3,4-oxide, +PMS (▲) and CPAP-3,4-cis-diol (■). Error bars are as in Fig. 1.

shown). However, the results presented in Figure 6 demonstrate that CPAP-3,4-cis-diol has the same mutagenic activity as the parent, CPEP. This interesting result suggests that CPAP-3,4-cis-diol is a proximate mutagen of CPEP.

In addition to our studies with the possible metabolites of CPEP, we have tested the ability of trans-2,3-dihydrodiolfluoranthene to serve as a proximate mutagen of fluoranthene to human lymphoblasts. In the presence of rat liver PMS, trans-2,3-dihydrodiolfluoranthene was metabolized to a mutagen that was as active as the parent, inducing a significant mutant fraction at a concentration of 2 µM (data not shown).

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TABLE 4

MUTAGENICITY OF PAH FOUND IN OTHER EXTRACTS OF DIESEL EXHAUST TO
HUMAN LYMPHOBLASTS

Compound	Induced[a] Mutation	μM[b] Conc.	Relative[c] Mutagenicity
anthracene	-	200	
2-methylanthracene	+	60	0.017
9-methylanthracene	+	9	0.1
9,10-dimethylanthracene	-	100	
cyclopenteno(c,d)pyrene	+	7	0.14
cyclopentano(c,d)pyrene	+	40	0.025
CPAP-3,4-oxide[d]	+[e]	0.4	2.5
CPAP-3,4-trans-diol	-	80	
CPAP-3,4-cis-diol	+	6	0.17
fluoranthene-2,3-dihydrodiol	+	2	0.5

[a](+) indicates the induction of a significant mutant fraction or (-) indicates no significant mutagenic effect.

[b]The μM concentration that induces a significant mutant fraction or the highest concentration tested in the case of a negative result.

[c]Mutagenic potency of the individual PAH relative to benzo(a)pyrene which induces a significant mutant fraction in the human lymphoblast assay at a concentration of 1 μM.

[d]CPAP is cyclopentano(c,d)pyrene.

[e]Tested without metabolic activation.

DISCUSSION

The results presented in Figure 1 show that a methylene chloride extract of automobile diesel exhaust particulate is mutagenic to human lymphoblasts inducing a significant mutant fraction at a concentration of 70 $\mu g/ml$ when a metabolic activation system was present. These results are similar to those reported by Liber et al.[1] who also found that our diesel soot extract was not mutagenic to human lymphoblasts without metabolic activation. However, McCormick et al.[23] have found that extracts of diesel engine particulate and the whole particles themselves are significantly cytotoxic to normal human fibroblasts and xeroderma pigmentosum (XP) fibroblasts which suggest that "direct-acting" cytotoxic agents for human fibroblasts are associated with diesel exhaust particulates. In addition, they (J. J. McCormick, personal communication) have found that both the organic extracts of diesel combustion particulates and the whole particulates induce resistance to 6-thioguanine in both cell

types. Further, the XP fibroblasts had an approximately 9-fold higher induced mutation frequency relative to the normal human fibroblasts, suggesting that the DNA adducts formed by these "direct-acting" mutagens are excisible.

Since our extract of diesel soot was mutagenic to the human lymphoblasts only in the presence of a metabolic activation system, we have concentrated on determining what promutagens are responsible for the mutagenicity of the diesel soot extract to these human cells. Results of fractionation experiments demonstrated that the hexane/toluene PAH containing fraction of our diesel soot extract was the most mutagenic to S. typhimurium in the presence of rat liver PMS,[1,2] suggesting that this fraction could be responsible for a major proportion of the human cell mutagenicity. Eleven of the 27 identified PAH have been tested for their ability to induce mutations in human lymphoblasts. The results are summarized in Table 2. Based on this mutational data and the chemical analysis presented in Table 1, it was possible to calculate the induced mutant fraction contribution of each tested PAH to the total mutagenicity of 100 μg/ml of the methylene chloride extract of our diesel soot sample (Table 2). The data suggest that as few as three PAH; fluoranthene, 1-methylphenanthrene, and 9-methylphenanthene, may account for up to 44% of the total mutability of the diesel emissions particulate extract to human lymphoblasts. Fluoranthene alone may be responsible for approximately 30% of the total mutagenicity of our diesel soot extract to human cells. Benzo(a)pyrene, which has routinely been used as a standard indicator of PAH levels, is present in too small a concentration to play any significant role in the mutagenicity of our diesel exhaust particulate extract to human lymphoblasts (Table 2).

It is our belief, that once the mutagenic potency to human lymphoblasts of the other PAH present in our diesel soot extract has been determined, the PAH alone will account for the entire mutagenicity of our extract to human lymphoblasts.

We have also carried out studies on the mutagenicity to human lymphoblasts of PAH found in other diesel soot extracts. Methylated anthracenes have been found to be associated with the particulate emitted from several diesel engines.[11-13] Comparison of the data presented in Tables 2 and 4 demonstrates that 9-methylanthracene has a mutagenic potency to human lymphoblasts similar to that of the known carcinogens benz(a)anthracene, chrysene and cyclopenteno(c,d)pyrene.[14-18] Therefore, in light of the role played by alkylated phenanthrenes in determining the mutagenicity of diesel emissions extracts, one should not overlook the role played by simple alkylated PAH in determining the genotoxic effect of complex combustion mixtures.

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The carcinogen cyclopenteno(c,d)pyrene[16-18] (CPEP) is a major PAH component of gasoline exhaust particulate and is known to be associated with diesel exhaust particulate as well.[13,19-21] CPEP appears to be converted to its ultimate mutagenic form via a one step epoxidation across the 3,4 double bond.[22,24] Our results demonstrate (Figure 6) that the 3,4-oxide of CPEP is a potent direct-acting mutagen to human lymphoblasts and are similar to those obtained with the L5178Y mouse lymphoma mutation assay.[25] Therefore, CPAP-3,4-oxide is an ultimate mutagen of CPEP to mammalian cells. However, our results suggest that there is more than one pathway for the activation of CPEP. CPAP, which lacks the 3,4 ethylene bond of CPEP and therefore cannot form the 3,4-oxide, still induced a significant mutant fraction in human lymphoblasts (Figure 6), albeit at a higher concentration relative to CPEP (Table 4). In addition, though CPAP-3,4-trans-diol, the major rat liver microsome metabolite of CPEP[22], was mutagenically inactive, CPAP-3,4-cis-diol had the same activity as CPEP. Recently, Gold and Eisenstadt[22] have reported that a 9,10 "K-region" dihydrodiol of CPEP is a minor rat liver microsome metabolite of the parent PAH. Therefore, it is possible that CPAP-3,4-cis-diol is oxidatively metabolized to CPAP-3,4-cis-diol-9,10-oxide which serves a second ultimate mutagen of CPEP.

We conclude that there are at least three pathways for the metabolic activation of CPEP; 1) a predominate pathway which proceeds via the epoxidation at the 3,4 double bond, 2) a pathway independent of the 3,4-ethylene double bond and, 3) a pathway specific to CPAP-3,4-cis-diol. We caution the reader that this third pathway of activation may not pose a genotoxic threat to man in that the formation of cis-dihydrodiols of PAH are not known to be produced by mammalian cell metabolism.

In our initial studies to determine the metabolic pathway for the activation of fluoranthene to an ultimate mutagen for human cells, we found that trans-2,3-dihydrodiolfluoranthene had the same mutagenic potency as the parent PAH with metabolic activation. This result suggests that trans-2,3-dihydrodiolfluoranthene is a potential proximate mutagen of fluoranthene for human lymphoblasts. Furthermore, our unpublished data demonstrate that trans-2,3-dihydrodiol-1,10B-epoxyfluoranthene is a potent direct-acting mutagen for Salmonella typhimurium indicating that this diol-epoxide of fluoranthene is an ultimate mutagen for bacterial cells.

In Table 5 we present a summary of the PAH and derivatives that have been tested for mutation induction in other human cell mutation assays. Several epoxide derivatives have been tested and with the exception of benz(a)anthracene-5,6-oxide, all were mutagenic to human fibroblasts. This finding suggests that other oxide derivatives of PAH are mutagens for human cells.

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TABLE 5

POLYCYCLIC AROMATIC HYDROCARBONS AND DERIVATIVES TESTED FOR MUTATION INDUCTION
IN OTHER HUMAN CELL SYSTEMS

Compound	Cell Type	Mutation Induction	Genetic Marker[a]
benz(a)anthracene	Epithelial[b]	−	DT^R
benz(a)anthracene-5,6-oxide	Fibroblast[c]	−	$8AG^R$
benzo(a)pyrene	Epithelial/ Fibroblast[d]	+/+	$DT^R/6TG^R$
benzo(a)pyrene-4,5-oxide	Fibroblast	+	$8AG^R$
benzo(a)pyrene-7,8-diol-9,10-oxide	Fibroblast[e]	+	$8AG^R$
dibenz(a,c)anthracene	Epithelial	+	DT^R
dibenz(a,h)anthracene	Epithelial	−	DT^R
dibenz(a,h)anthracene-5,6-oxide	Fibroblast	+	$8AG^R$
7,12-dimethylbenz(a)anthracene-5,6-oxide	Fibroblast	+	$8AG^R$
anthracene	Epithelial	−	DT^R
chrysene	Epithelial	−	DT^R

[a]DT^R - diphtheria toxin resistance; $8AG^R$ - 8-azaguanine resistance; $6TG^R$ - 6-thioguanine resistance.

[b]Rocchi, P., Ferreri, A.M., Borgia, R., and Prodi, G., Carcinogenesis 1, 765-767 (1980).

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Rocchi et al.[26] found that benz(a)anthracene and chrysene were inactive as mutagens in their human epithelial cell mutation assay (Table 5) which is contrary to our results with human lymphoblasts. However, Rocchi et al.[26] used a concentration of only 1 µM, which is most likely too low a concentration to induce a significant mutagenic response.

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CYTOTOXICITY, MUTAGENICITY AND COMUTAGENICITY IN DIESEL EXHAUST PARTICLE EXTRACTS ON CHINESE HAMSTER OVARY CELLS IN VITRO

by

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Diesel exhaust particle extracts were found cytotoxic to Chinese hamster ovary (CHO) cells. Extracts from cars of different manufacturers had different cytotoxicity. The emission rates of cytotoxic chemicals were calculated for the different cars using the cytotoxicity of the extracts, the percentage of extractable chemicals on the exhaust particles, and particulate emission rates. The ranking of emission rates of cytoxic chemicals for the different cars were found to be the reverse of the ranking of the cytotoxicity of the extracts (1). Our data indicate the need to include emission data other than the activities of the extracts, when the emission of noxious agents from different vehicles are compared.

The cytotoxicity of diesel exhaust particle extracts is antagonized by serum, lung and liver cytosols, and sulfhydryl agents in vitro (2) [Figures 1, 2]. The detoxifying effects of the cytosols is enhanced further by the addition of cofactors (NADP and glucose-t-phosphate); therefore, suggesting enzymatic detoxification in addition to protein binding. Our data suggest that similar detoxification of the toxic chemicals associated diesel exhaust particles may occur in vivo.

All diesel exhaust particle extracts had low mutagenicity towards CHO cells. This low activity was observed using different endpoints including sister-chromatid-exchange and mutation at the hypoxanthine-guanine phosphoribosyl (HGPRT) gene locus (3). The mutagenicity was slightly enhanced by the addition of exogenous aroclor 1254-induced liver S9. Although the extracts had only low mutagenicity, they were found to have definite co-mutagenic activities (4). Treatment of CHO cells with a combination of a mutagen (N-methyl, N'-nitro, N-nitrosoguanidine or benzo(a)pyrene) and diesel exhaust extract yielded a 2-3 fold higher mutant frequency than that calculated by the mutagenicity of the mutagen and the diesel exhaust extract alone [Table 1]. This co-mutagenicity was observed for all extracts tested, using three different endpoints: mutation at the HGPRT gene locus, mutation at the Na^+-Ka^+-ATPase gene locus, and sister-chromatid-exchange. We have shown that diesel exhaust particles are associated with chemicals with cytotoxic, mutagenic, and co-mutagenic properties. Engineering variables, biological detoxifying molecules, and other environment mutagens/carcinogens, all could possibly modify the health-effect of the diesel exhaust emission.

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    hancement of chemical-induced mutagenesis in cultured Chinese hamster
    ovary cells: Possible interaction of diesel exhaust with environmental
    carcinogens. Mutat. Res. In press.

Table 1. Co-mutagenicity of Diesel Exhaust Particle Extracts
in the Presence of Exogenous Activation System[a]
(Li and Royer, 1981)

Treatment	Mutant Frequency			
	-B(a)P	+B(a)P (o.5 μg/ml) Expected[b]	Observed	Enhancement[c]
Solvent (DMSO) Control	7 (A)	-	56 (B)	-
Exhaust Extracts (60 μg/ml)				
Car A	28	77	218	2.8
Car B	6	56	170	3.0
Car C	11	60	194	3.2
Car D	9	58	184	3.2
Car E	25	74	229	3.1

[a] An Aroclor 1254 - induced rat liver cytosol/cofactors mixture was used
for exogenous activation.

[b] Expected mutant frequency = mutant frequency (B(a)P alone) (B) + mutant
frequency (diesel exhaust particle extract alone) - mutant frequency
(DMSO alone) (A).

[c] Enhancement = observed mutant frequency ÷ expected mutation frequency.
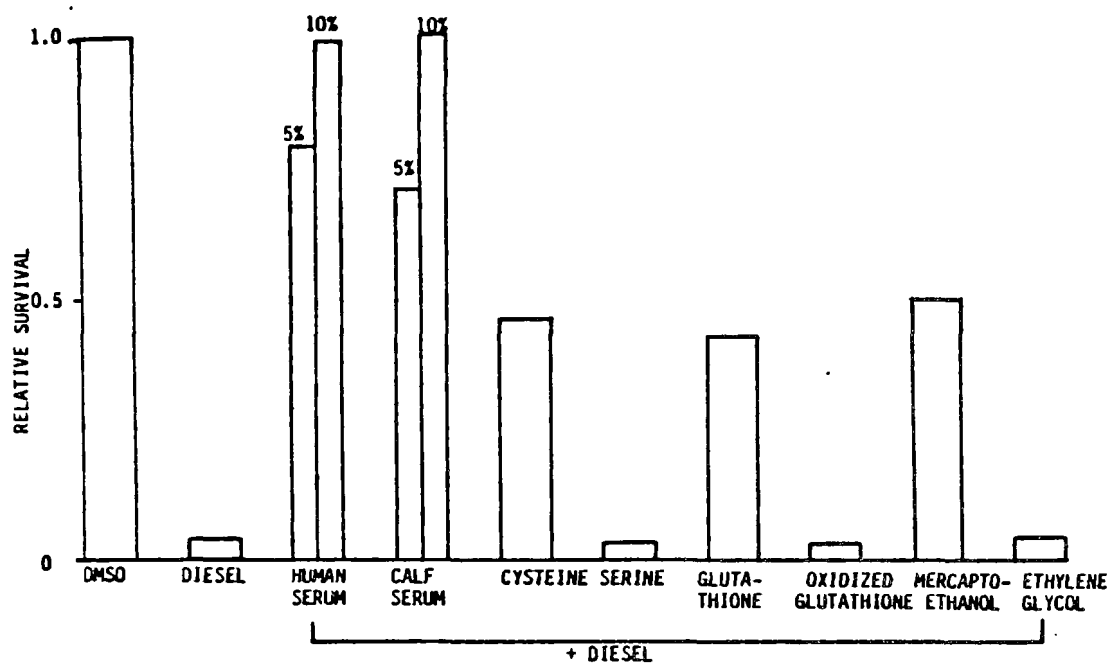
Figure 1 (Li, 1981). Effects of animal sera, sulfhydryl compounds and their non-sulfhydryl analogs, on the cytotoxicity of diesel exhaust extract.
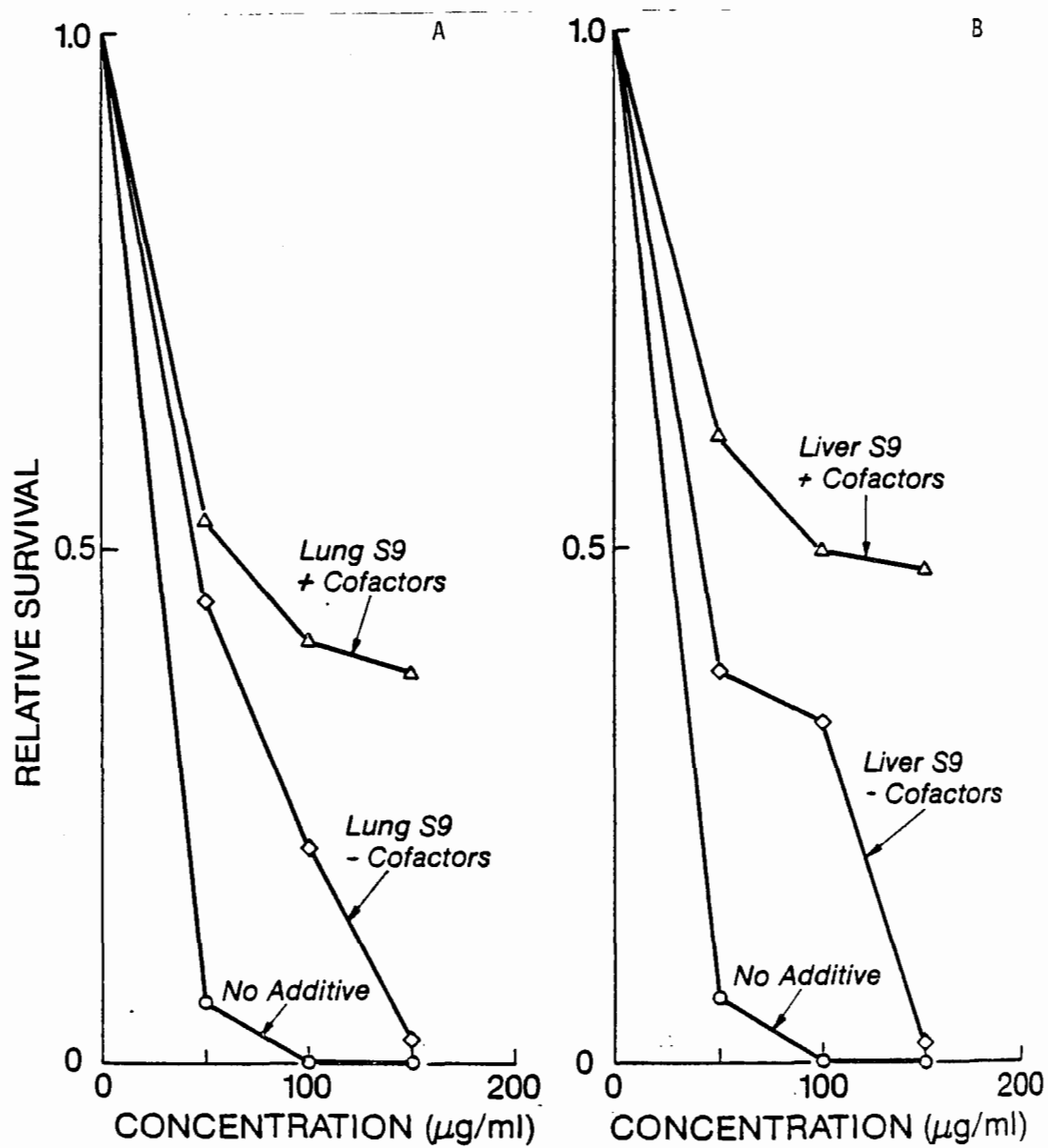
Figure 2 (Li, 1981). Effects of lung (A) and liver (B) cytosols on the cytotoxicity of diesel exhaust extract.

MUTAGENIC ACTIVITY OF DIESEL PARTICLES IN ALVEOLAR MACROPHAGES FROM RATS EXPOSED TO DIESEL ENGINE EXHAUST

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Diesel engine exhaust contains submicron size carbonaceous particles. Dichloromethane extracts of these particles collected by filtration or electrostatic precipitation elicited mutagenic responses in bacterial mutagenicity assay. Currently, there are no data on the mutagenic properties of inhaled diesel particles that are deposited directly in the lung. The purpose of this experiment was to determine the mutagenicity of the inhaled diesel particles and the interaction between the particles and alveolar macrophages.

Adult male Fischer 344 rats were exposed to 6 mg/m^3 of diesel particles for 3 days (20 hrs/day). Alveolar macrophages were obtained by bronchopulmonary lavage immediately after exposure and at 1, 4, and 7 days thereafter. Macrophages from forty animals were pooled for each data point, sized and counted. The mass of diesel particles phagocytized in alveolar macrophages was determined by a spectrophotometric method (Rudd and Strom, J. Appl. Tox., 1(2):83-87, 1981). The alveolar macrophages were concentrated by filtration on pre-washed fiberglass filters and dried at room temperature to constant weight. The filters were extracted with dichloromethane in a Soxhlet apparatus for 4 hours (20-25 solvent cycles). The resulting extracts were oily, indicating cellular lipids and surfactant were extracted from the macrophages. The *Salmonella typhimurium* strain TA98 was used for mutagenicity assay. For thin layer chromatography, Whatman LK6 plates were used and the developing solvent was toluene:hexane (5:1).

Table 1 shows the diesel particle mass recovered in alveolar macrophages from exposed rats. The mass of diesel particle recovered from the lavage accounted for 45-50% of the particles deposited in rat lungs. The extracts of diesel particles (DPE) collected from the exposure chamber by filtration were used as reference for the thin layer chromatographic and mutagenic analysis of the macrophage extracts. The TLC fluorescence banding pattern of the samples from macrophages obtained immediately or one day post-exposure were similar to that of chamber DPE. However, the extracts of macrophages recovered on the fourth and seventh day post-exposure lost their fluorescence patterns. Figure 1 shows the results of the mutagenicity assay. The data indicate that the cellular lipids extracted from macrophages mitigated the mutagenic response of the airborne DPE, but a positive result was still detectable in the extracts of the macrophages obtained immediately, and one day after the exposure. In contrast, the mutagenic activity of extracts from macrophages obtained on the fourth and seventh

day after the exposure was undetectable. The data indicate that: 1) inhaled diesel particles contain extractable mutagenic compounds - whether they are the same as those found in the particles collected by other means has yet to be resolved; 2) alveolar macrophages have the ability to release or transform the fluorescent and mutagenic extractable hydrocarbons from phagocytized diesel particles over a period of several days and thus may significantly influence their biological activity in the respiratory system.

Table 1

DIESEL PARTICLE MASS RECOVERY
IN ALVEOLAR MACROPHAGES FROM EXPOSED RATS

GROUPS	DP ug/mL Lavage Fluid	DP ug/10^6 Macrophages	Total Recovery (mg)
IMMEDIATELY	5.0	41.3	8.4
1 DAY-POST	5.9	34.3	10.3
4 DAY-POST	6.3	25.5	10.6
7 DAY-POST	6.6	25.8	11.1

Mutagenic activity of airborne diesel particle extract and macrophage extracts.

————●———— Airborne diesel particle extract.

— —●— — Airborne diesel particle extract + 800 µg control macrophage extract.

————○———— Macrophage extract from exposed rats immediately after exposure.

————◉———— Macrophage extract from exposed rats 7 days after exposure.
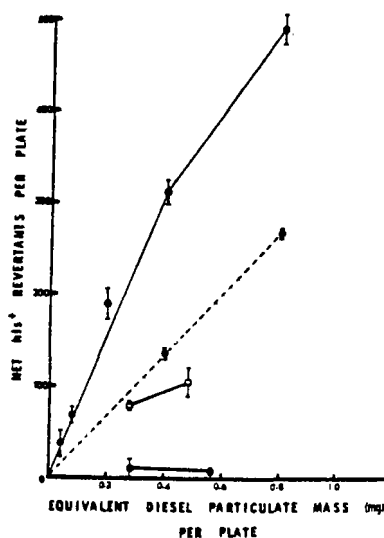
SECTION 6

CARCINOGENESIS

SKIN CARCINOGENESIS STUDIES OF EMISSION EXTRACTS

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INTRODUCTION

The incomplete combustion of fossil fuels results in the emission of particulate and organic vapor-phase components to the atmosphere. The particulate phase of these emissions contains organic materials adsorbed onto the particulate matrix. These organic materials have been subjected to intense chemical analysis, fractionation and characterization.[1-15] The characterization of the biological activities of organic materials emitted in the environment has been reported for samples collected as whole condensates and as particles. Emissions from gasoline engines, collected as condensates, were tumorigenic when applied dermally to mice.[16-22] Extracts from particles collected from a gasoline engine were also tumorigenic on mouse skin.[23]

Kotin et al.[24] reported that extracts of particles collected from diesel engines were active in producing tumors on strain A mice, while Mittler and Nicholson[22] reported little such activity from diesel exhaust condensates.

We have previously reported that extracts from particulate emissions from coke oven, roofing tar, and several diesel and gasoline engines produced papillomas when applied to SENCAR mice.[25,26] These tumor initiation experiments indicated that the tumorigenic activities of the diesel engines were dependent upon the particular engineering qualities of each engine and that these emission samples produced a wide range of activities.

The SENCAR mouse has been shown to be highly sensitive to chemical carcinogens and useful in mechanistic studies of carcinogenesis.[27-31] This paper describes the results of a systematic study of the ability of extracts of particulate emissions to induce benign and malignant tumors in SENCAR mice and their abilities to act as tumor initiators, tumor promoters, or complete carcinogens.

MATERIALS AND METHODS

Sample Generation and Isolation

The details of sample generation and isolation have been reported elsewhere.[2]
Particulate emissions were collected from a 1973 preproduction Nissan Datsun, a
1978 Oldsmobile, a prototype 1976 VW Turbo Rabbit, a 1977 Mercedes 300D and a
1977 Mustang vehicle (Table 1), each which was mounted on a chassis dynamometer
with a repeated highway fuel economy (HWFET) cycle of 10.24 mi, an average speed
of 48 mph, and a running time of 12.75 min. Another sample was collected from a
1972 heavy-duty Caterpillar 3304 engine mounted on an engine dynamometer at 2200
rpm steady state with an 85-lb load. The residential furnace sample was
collected from a Day and Night Air Conditioning Model 125-OU-AC-A furnace,
100,000 BTU, 0.9 gal/min firing rate, operating at 20% lean and using a cyclic
mode of 10 min on, 20 min off. The Caterpillar, Nissan, Mercedes, VW and
Oldsmobile engines were fueled with the same batch of No. 2 diesel fuel. Parti-
cle samples were collected with a dilution tunnel in which the hot exhaust was
diluted, cooled and filtered through Pallflex Teflon-coated fiberglass filters.
Topside coke oven samples were collected from the top of a coke oven battery at
Republic Steel, Gadston, AL, by use of a Massive Air Volume Sampler. Because of
the topside ambient location and local wind conditions, an unknown portion of this
emission sample contains particles from the local urban environment. The coke
oven main sample was collected from a separator located between the gas collector

TABLE 1

DIESEL AND GASOLINE SAMPLES

Source	Fuel	Driving Cycle
1972 Caterpillar 3304	Diesel No. 2[a]	Mode II[b]
1973 Preproduction Nissan Datsun 220C	Diesel No. 2	HWFET[c]
1978 Oldsmobile 350	Diesel No. 2	HWFET
~1976 Prototype VW Turbo Rabbit	Diesel No. 2	HWFET
1977 Mercedes 300D	Diesel No. 2	HWFET
Residential Furnace	Diesel No. 2	10 min on/ 20 min off
1977 Mustang II-302, V-8 catalyst and EGR	Unleaded gasoline	HWFET

[a]All diesel fuel samples were from the same lot.
[b]Mode II cycle was conducted at 2200 rpm steady state with an 85-lb load.
[c]Highway fuel economy cycle (HWFET) was a 10.24-mi cycle averaging 48 mph and taking 12.75 min.

and the primary coolers within the coke oven battery. The roofing tar emission sample was collected from a conventional tar pot with external propane burner. Pitch-based tar was heated to 182° to 193°C and emissions were collected with a 1.8-m stack extension and Teflon socks in a baghouse. It should be noted that only one vehicle or source was used for each sample, therefore each sample may not be representative of a particular technology.

All samples were Soxhlet extracted with dichloromethane, which was then removed by evaporation under dry nitrogen gas.

Animals

SENCAR mice, selected for their increased sensitivity to carcinogens,[32] were used in this study. These mice were derived by breeding Charles River CD-1 mice with male skin-tumor sensitive (STS) mice that were originally derived from Rockland mice. Mice were selected for sensitivity to the 7,12-dimethylbenz(a)anthracene-12-0-tetradecanoylphorbol-13-acetate (TPA) two-stage system of tumorigenesis for eight generations. These mice were obtained initially from Dr. R. Boutwell (McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI) and are now raised at the Oak Ridge National Laboratory, Oak Ridge, TN.

Chemicals

TPA was obtained from Dr. P. Borchert (University of Minnesota, Minneapolis, MN) and benzo(a)pyrene B(a)P from Aldrich Chemical Co. (Milwaukee, WI). All agents were prepared under yellow light immediately before use and were applied topically in 0.2 ml of spectral-quality acetone.

Tumor Experiments

Studies involved 80 7- to 9-week-old mice per treatment group (40 of each sex). Animals were housed in plastic cages (10 per cage) under yellow light with hardwood chip bedding, fed Purina chow and water ad libitum, and maintained at 22° to 23°C with 10 changes of air per hour. All mice were shaved with surgical clippers two days before the initial treatment and only those mice in the resting phase of the hair cycle were used. Under the tumor initiation protocol, all samples at all doses were applied as a single topical treatment, except for the 10 mg dose, which was administered in five daily doses of 2 mg. One week after treatment, 2.0 µg of the tumor promoter TPA was administered topically twice weekly. Under the complete carcinogenesis protocol, samples were administered weekly (or twice weekly for the highest dose level) for 50

to 52 weeks. Under the tumor promotion protocol, mice were first initiated with 50.5 µg of B(a)P and then treated weekly (or twice weekly at the highest dose level) for 34 weeks with the sample. Skin tumor formation was recorded weekly, and papillomas greater than 2 mm in diameter and carcinomas were included in the cumulative total if they persisted for one week or longer. The number of mice with tumors, the number of mice surviving, and total number of tumors were determined and recorded weekly. At six months the numbers of papillomas per surviving animal were recorded for statistical purposes. Histological verification of tumors as well as histopathological identification of nondermal tumors will be reported elsewhere.

Data Collection, Validation and Storage

A unique identifier was assigned to each treatment group. Treatment information for each group and raw data were coded onto forms for data processing. Data entered on the form were punched onto cards which underwent 100% verification against the forms. Weekly group scores (numbers of surviving mice, carcinomas, papillomas and mice with tumors) and periodic individual animal scorings for each group were validated by a computer program, corrected, and updated on a cumulative file maintained on a Univac 1110.

The main data base was sampled periodically by use of Dodge-Romig sample inspection tables and verified against the data entry forms.[33] An error rate of less than 2% was maintained. Following each update of new data, reports listing treatment protocol, weekly tumor scores and individual papilloma and carcinoma scores by animal group identifier were generated. The program which generates these reports also builds subset card image data files from the main data base, which are input to various statistical analysis routines.

Statistical Analysis Methods

Analyses were carried out on tumor scorings performed 24 to 26 weeks after initiation. Two types of statistical analyses were performed. For tumor incidence data, a probit model was fitted, taking into account the numbers of spontaneous tumors occuring in the TPA control groups. The probit formula used is

$$P = \beta_o + (1-\beta_o) \ \Phi \ (\beta_1 + \beta_2 \ \ln x)$$

where P is the probit proportion, x is the dose applied and Φ is the standard normal cumulative distribution function.[34] The model parameters β_o, β_1, and β_2 were estimated from the raw data by maximum likelihood methods. The dose which produces tumors in 50% of the surviving mice over the TPA-treated controls was

then estimated as a function of the estimated parameters. The 95% confidence
limits were estimated using the asymptotic variance-covariance matrix estimated
during the model-fitting process. Chi-square goodness-of-fit and likelihood
ratio tests were also computed to examine the appropriateness of the model and the
strength of the dose effect.

The tumor multiplicity data were analyzed by a nonlinear Poisson model:[35]

$$\lambda_i = \beta_o + e^{\beta_1 + \beta_2 \ln (x_i)}$$

where λ_i is the number of papillomas per mouse, x_i the dose and β_o, β_1 and β_2
the model parameters. Using maximum likelihood methods, the model parameters
were estimated from the raw data and used to calculate the number of papillomas
per mouse for a dose of 1 mg. Asymptotic 95% confidence intervals for these
activities were obtained. Tests for the Poisson assumption, adequacy of the
model, and strength of the dose response were also calculated.[35]

RESULTS

The mouse skin bioassay system can be used to evaluate agents as tumor
initiators, tumor promoters, cocarcinogens and complete carcinogens. The two
protocols that can be employed to detect chemical carcinogens in the mouse
skin tumorigenesis assay are complete carcinogenesis and tumor initiation, as
illustrated in Figure 1. Multiple application of the test agent for up to 60
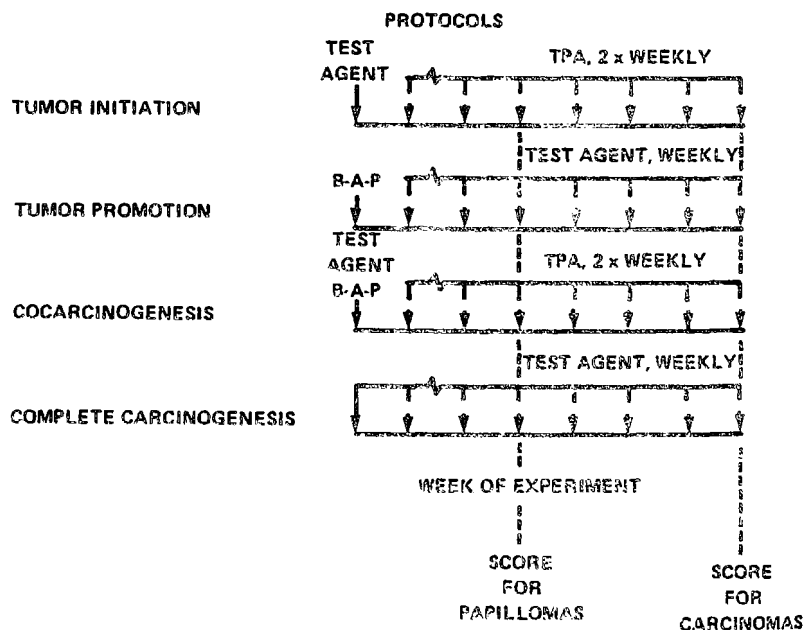
Fig. 1. Schematic diagram of tumor initiation, tumor promotion, cocarcinogenesis
and complete carcinogenesis bioassay protocols.

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weeks will give rise primarily to malignant carcinomas of the skin. This protocol for complete carcinogens is a test for agents exhibiting both tumor-initiating and tumor-promoting activities. The bioassay protocol for tumor initiators is a single application of test agent followed one week later by multiple applications of the potent tumor promoter TPA. The bioassay protocol for tumor promoters is initiation with a strong tumor initiator, B(a)P, followed by weekly applications of the test agent.

Tumor Initiation

Tumor formation after application of B(a)P or Nissan extract began after a 7- to 8-week latency period and reached a plateau (Figure 2) for both tumor multiplicity and tumor incidence.

The results of the tumor initiation experiments on SENCAR mice for B(a)P and for topside coke oven, coke oven main, Nissan, roofing tar, Oldsmobile, VW Rabbit,
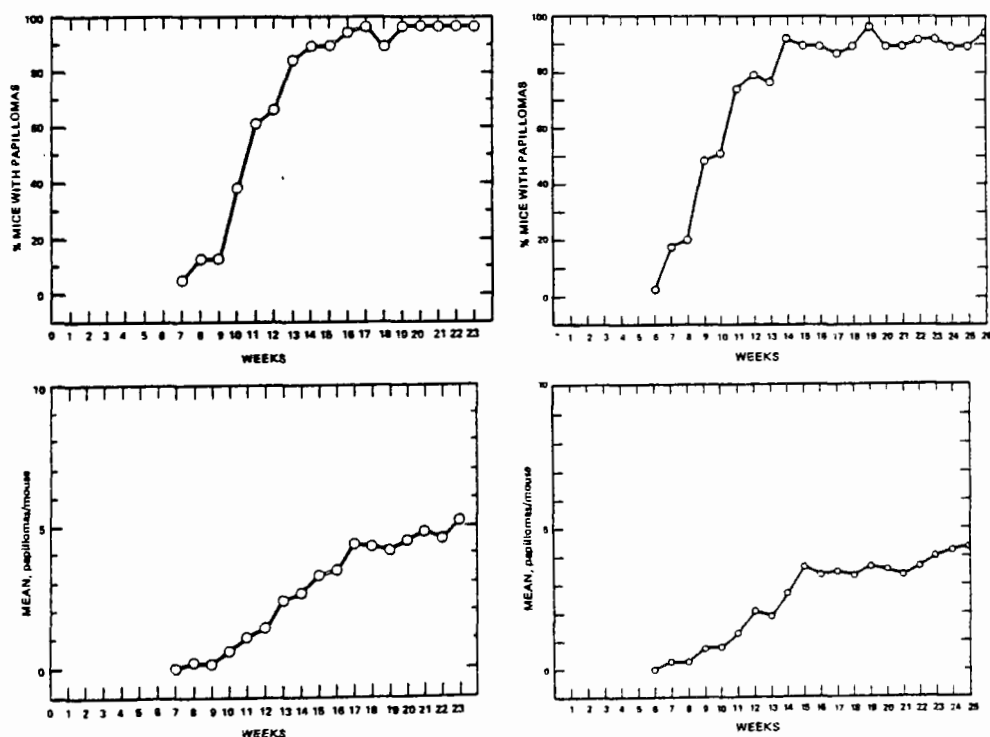
Fig. 2. SENCAR mouse skin tumor initiation. Male SENCAR mice were initiated with either a single dose of B(a)P (50.5 µg) or five daily treatments of Nissan extract (2 mg). Animals were then treated biweekly with TPA (2 µg). Left, B(a)P; right, Nissan extract.

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Mercedes, Caterpillar, residential furnace, and Mustang extracts are shown in Tables 2 to 12, respectively. Animals were scored at six months for papilloma formation and at one year for carcinomas. The carcinoma data represent the cumulative number of carcinomas found in each treatment group and therefore include tumors on both living and dead animals. The B(a)P, topside coke oven, coke oven main, Nissan, and roofing tar samples produced an 89% or greater tumor incidence at the highest dose level applied. Tumor multiplicity ranged from 5 to 6 papillomas per mouse in the roofing tar and Nissan samples to greater than 7 in the B(a)P, topside coke oven and coke oven main samples. These groups of animals also produced a significant number of squamous cell carcinomas, ranging from 13 to 65% of the mice bearing carcinomas at the highest dose evaluated. In general, samples which produced a papilloma response of greater than five papillomas per mouse at six months produced a carcinoma response of 0.15 to 0.65 carcinomas per animal, with 13 to 65% of the animals bearing at least one tumor per year.

The Oldsmobile sample (Table 7) produced a biphasic response in both male and female animals; the highest activity occurred at 2 mg/mouse with 0.35 to 0.40 papillomas per mouse and 20 to 40% of the mice bearing tumors. Some carcinomas were observed after one year, but their numbers were not appreciably above those observed in the TPA control animals (Table 2).

The VW Rabbit sample (Table 8) produced dose-related increases in papillomas in both male and female mice, with the maximum activity for each sex at 10 mg. At this dose there were 0.34 to 0.47 papillomas per mouse, with 24 to 42% of the animals bearing tumors. Few carcinomas were scored at one year.

The Mercedes sample (Table 9) was also a weak tumor initiator on SENCAR mouse skin, producing a response in male mice at 10 mg of 0.47 papillomas per mouse and a similar response in female mice but at 1.00 mg. As in the Oldsmobile sample, the response in female mice was biphasic. Animals scored at one year produced no more carcinomas than the TPA controls.

The Caterpillar sample (Table 10) did not elicit a dose response in either sex of SENCAR mice and was marginally higher than the background TPA control. Carcinoma formation was minimal.

The residential furnace sample (Table 11) produced a maximal response at the highest dose applied (10 mg/mouse) of 0.29 to 0.38 papillomas per mouse with 21 to 25% of the animals bearing tumors. Carcinoma formation was minimal.

The Mustang sample (Table 12) was tested at doses from 0.1 to 3 mg/mouse due to sample limitations. The response was maximal in the female animals at 3 mg/ mouse and activity plateaued at 2 to 3 mg/mouse in the male animals. Twenty percent of the female mice produced carcinomas at the highest dose tested.

TABLE 2

SENCAR MOUSE SKIN TUMORIGENESIS
BENZO(a)PYRENE - TUMOR INITIATION

Dose (μg/mouse)		No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
0	(M)	37	8	0.08	5	0.05
0	(F)	39	5	0.05	0	0
2.52	(M)	40	45	0.50	5	0.07
2.52	(F)	39	31	0.44	5	0.05
12.6	(M)	40	73	1.8	20	0.20
12.6	(F)	37	57	1.1	23	0.23
50.5	(M)	39	100	5.8	25	0.25
50.5	(F)	40	75	2.8	20	0.20
101	(M)	38	95	10.2	30	0.33
101	(F)	38	97	7.9	25	0.25

[a]Scored at 6 months.
[b]Cumulative score after one year.

TABLE 3

SENCAR MOUSE SKIN TUMORIGENESIS
TOPSIDE COKE OVEN - TUMOR INITIATION

Dose (μg/mouse)		No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100	(M)	40	13	0.13	0	0
100	(F)	40	10	0.20	8	0.08
500	(M)	40	73	1.6	5	0.05
500	(F)	40	70	1.8	15	0.15
1000	(M)	37	95	2.6	15	0.15
1000	(F)	39	72	2.0	3	0.03
2000	(M)	39	95	4.0	13	0.13
2000	(F)	38	90	3.5	10	0.10
10,000	(M)	39	100	7.1	13	0.15
10,000	(F)	40	100	7.7	20	0.23

[a]Scored at 6 months.
[b]Cumulative score after one year.

TABLE 4

SENCAR MOUSE SKIN TUMORIGENESIS
COKE OVEN MAIN - TUMOR INITIATION

Dose (µg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	38	50	0.63	10	0.10
100 (F)	39	31	0.38	25	0.25
500 (M)	39	90	3.7	54	0.59
500 (F)	39	82	2.2	54	0.54
1000 (M)	39	87	3.3	53	0.53
1000 (F)	39	90	3.1	48	0.48
2000 (M)	40	78	3.1	48	0.48
2000 (F)	40	100	5.3	45	0.45
10,000 (M)	38	100	8.9	55	0.55
10,000 (F)	37	100	8.1	65	0.65

[a]Scored at 6 months.
[b]Cumulative score after one year.

TABLE 5

SENCAR MOUSE SKIN TUMORIGENESIS
NISSAN - TUMOR INITIATION

Dose (µg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	37	0	0	0	0
100 (F)	39	3	0.03	5	0.05
500 (M)	38	26	0.34	13	0.13
500 (F)	39	23	0.39	10	0.10
1000 (M)	40	33	0.38	20	0.20
1000 (F)	38	39	0.53	13	0.13
2000 (M)	35	66	1.1	13	0.13
2000 (F)	40	58	1.6	15	0.15
10,000 (M)	38	89	5.5	36	0.36
10,000 (F)	38	97	5.7	31	0.31

[a]Scored at 6 months.
[b]Cumulative score after one year.

TABLE 6

SENCAR MOUSE SKIN TUMORIGENESIS
ROOFING TAR - TUMOR INITIATION

Dose (μg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	40	10	0.13	5	0.05
100 (F)	39	15	0.21	10	0.10
500 (M)	40	28	0.35	10	0.10
500 (F)	39	13	0.15	18	0.18
1000 (M)	39	38	0.41	5	0.05
1000 (F)	40	45	0.80	15	0.15
2000 (M)	39	36	0.62	13	0.13
2000 (F)	38	37	0.45	15	0.15
10,000 (M)	39	100	6.4	23	0.25
10,000 (F)	40	95	5.7	48	0.48

[a]Scored at 6 months.
[b]Cumulative score after one year.

TABLE 7

SENCAR MOUSE SKIN TUMORIGENESIS
OLDSMOBILE - TUMOR INITIATION

Dose (μg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	40	13	0.13	8	0.10
100 (F)	40	15	0.18	8	0.10
1000 (M)	39	26	0.36	8	0.08
1000 (F)	40	18	0.25	8	0.08
2000 (M)	40	20	0.35	3	0.03
2000 (F)	40	40	0.40	5	0.05
10,000 (M)	39	21	0.21	8	0.08
10,000 (F)	39	10	0.13	13	0.13

[a]Scored at 6 months.
[b]Cumulative score after one year.

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TABLE 8

SENCAR MOUSE SKIN TUMORIGENESIS
VW-RABBIT - TUMOR INITIATION

Dose (μg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	40	18	0.18	0	0
100 (F)	37	14	0.14	0	0
500 (M)	37	14	0.14	0	0
500 (F)	40	5	0.05	0	0
1000 (M)	38	21	0.21	3	0.03
1000 (F)	39	18	0.26	3	0.03
2000 (M)	38	21	0.24	5	0.05
2000 (F)	35	14	0.17	6	0.06
10,000 (M)	38	24	0.34	5	0.05
10,000 (F)	38	42	0.47	10	0.10

[a]Scored at 6 months.
[b]Cumulative score after one year.

TABLE 9

SENCAR MOUSE SKIN TUMORIGENESIS
MERCEDES - TUMOR INITIATION

Dose (μg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	39	13	0.13	0	0
100 (F)	38	5	0.05	0	0
500 (M)	29	21	0.21	5	0.05
500 (F)	39	3	0.03	0	0
1000 (M)	40	23	0.25	5	0.05
1000 (F)	39	21	0.48	0	0
2000 (M)	40	5	0.05	0	0
2000 (F)	38	11	0.13	0	0
10,000 (M)	38	37	0.47	5	0.05
10,000 (F)	40	15	0.18	0	0

[a]Scored at 6 months.
[b]Cumulative score after one year.

TABLE 10

SENCAR MOUSE SKIN TUMORIGENESIS
CATERPILLAR - TUMOR INITIATION

Dose (µg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	39	10	0.15	5	0.05
100 (F)	38	0	0	0	0
500 (M)	39	10	0.13	0	0
500 (F)	38	8	0.08	0	0
1000 (M)	39	15	0.15	3	0.03
1000 (F)	40	5	0.05	3	0.03
2000 (M)	38	11	0.11	3	0.03
2000 (F)	39	5	0.05	0	0
10,000 (M)	40	10	0.10	0	0
10,000 (F)	39	5	0.05	0	0

[a]Scored at 6 months.
[b]Cumulative score after one year.

TABLE 11

SENCAR MOUSE SKIN TUMORIGENESIS
RESIDENTIAL FURNACE - TUMOR INITIATION

Dose (µg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	40	0	0	8	0.08
100 (F)	40	0	0	3	0.03
500 (M)	39	5	0.05	3	0.03
500 (F)	40	10	0.10	8	0.08
1000 (M)	40	18	0.20	3	0.03
1000 (F)	40	3	0.03	0	0
2000 (M)	39	5	0.05	3	0.03
2000 (F)	40	3	0.03	3	0.03
10,000 (M)	38	21	0.29	5	0.05
10,000 (F)	40	25	0.38	8	0.08

[a]Scored at 6 months.
[b]Cumulative score after one year.

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TABLE 12

SENCAR MOUSE SKIN TUMORIGENESIS
MUSTANG - TUMOR INITIATION

Dose (μg/mouse)	No. Mice Surviving	Mice with Papillomas[a] (%)	Papillomas per Mouse[a]	Mice with Carcinomas[b] (%)	Carcinomas per Mouse[b]
100 (M)	39	5	0.05	5	0.05
100 (F)	39	13	0.23	13	0.13
500 (M)	39	13	0.15	0	0
500 (F)	38	18	0.24	10	0.10
1000 (M)	40	18	0.18	5	0.05
1000 (F)	40	10	0.13	10	0.10
2000 (M)	37	22	0.24	15	0.15
2000 (F)	39	21	0.23	13	0.13
3,000 (M)	34	18	0.24	5	0.05
3,000 (F)	40	23	0.28	20	0.20

[a]Scored at 6 months.
[b]Cumulative score after one year.

The lack of a monotonic dose response across the complete dose range tested in the Oldsmobile, VW Rabbit, Mercedes, Caterpillar and Mustang samples may indicate a toxic response to these samples. Damage to the skin epidermal cells will result in lower expression of the tumorigenic response by these complex mixtures. This is particularly clear with the Oldsmobile and Mustang samples, where a 5-fold increase in dose (to 10 mg/mouse) results in equal or lower tumor response. In these examples the highest dose (10 mg) was administered in five daily increments of 2 mg, which should have lowered the toxic responses when compared to a single administration.

Complete Carcinogenesis

Six agents were examined for their ability to act as complete carcinogens in the SENCAR mouse skin system: benzo(a)pyrene, coke oven main, roofing tar, Nissan, Oldsmobile, and Caterpillar extracts. Benzo(a)pyrene (Table 13), when applied once per week produced greater than 93% carcinoma incidence at 50.5 μg/week, with almost one carcinoma per mouse. Higher doses did not increase the tumor multiplicity.

Coke oven main also produced a strong complete carcinogen response in both male and female mice (Table 14). Male mice seemed to be more sensitive, as 98% of the mice bore approximately one carcinoma, while only 75% of the female mice responded. The roofing tar sample produced a significant response only at the highest dose applied (4 mg per mouse per week) with 25 to 28% of the mice bearing tumors (Table 14).

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The diesel samples (Nissan, Oldsmobile and Caterpillar) were essentially in-active as complete carcinogens at the doses applied on SENCAR mouse skin (Table 15). No tumors were observed in the negative control animals (Table 13).

TABLE 13

SENCAR MOUSE SKIN TUMORIGENESIS
BENZO(a)PYRENE - COMPLETE CARCINOGENESIS

Dose µg/mouse/week	Mice with carcinomas[a] (%)	Carcinomas per mouse[a]
12.6 (M)	10	0.10
12.6 (F)	8	0.08
25.2 (M)	63	0.63
25.2 (F)	43	0.43
50.5 (M)	93	0.93
50.5 (F)	98	0.98
101 (M)	80	0.83
101 (F)	90	0.98
202 (M)	80	0.80
202 (F)	93	0.98
0 (M)	0	0
0 (F)	0	0

[a]Cumulative score after one year.

TABLE 14

SENCAR MOUSE SKIN TUMORIGENESIS
COKE OVEN MAIN AND ROOFING TAR - COMPLETE CARCINOGENESIS

Dose (µg/mouse/week)	Mice with carcinomas[a] (%)		Carcinomas per mouse[a]	
	Coke Oven Main	Roofing Tar	Coke Oven Main	Roofing Tar
100 (M)	5	0	0.05	0
100 (F)	5	0	0.05	0
500 (M)	36	0	0.36	0
500 (F)	30	0	0.30	0
1000 (M)	48	3	0.55	0.03
1000 (F)	60	0	0.60	0
2000 (M)	82	3	1.00	0.03
2000 (F)	78	8	0.78	0.08
4000 (M)	98	25	0.98	0.28
4000 (F)	75	28	0.85	0.28

[a]Cumulative score after one year.

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TABLE 15

SENCAR MOUSE SKIN TUMORIGENESIS
NISSAN OLDSMOBILE AND CATERPILLAR - COMPLETE CARCINOGENESIS

Dose (μg/mouse/week)	Mice with carcinomas[a] (%)			Carcinomas per mouse[a]		
	Nissan	Oldsmobile	Caterpillar	Nissan	Oldsmobile	Caterpillar
100 (M)	0	0	0	0	0	0
100 (F)	0	0	3	0	0	0.03
500 (M)	0	3	0	0	0.03	0
500 (F)	0	0	0	0	0	0
1000 (M)	0	0	0	0	0	0
1000 (F)	0	0	0	0	0	0
2000 (M)	0	0	0	0	0	0
2000 (F)	0	0	0	0	0	0
4000 (M)	3	0	0	0.03	0	0
4000 (F)	5	0	0	0.05	0	0

[a]Cumulative score after one year.

Quantitative Analysis

The mouse skin data were stored in computer files as described in the
Materials and Methods section. These data were subjected to computer modeling
and statistical procedures specifically designed for analysis of tumor multi-
plicity and tumor incidence data using an interactive computer terminal graphics
system. Tumor incidence data were applied to a probit model with background
correction. From the model the dose which elicits tumors in 50% of the animals
over the TPA control rate (TID_{50}) was estimated. An example of the data and
probit analysis for the Nissan sample is shown in Figure 3a. The plot of mice
with papillomas vs dose applied shows the data points and the probit curve calcu-
lated from the data. The TID_{50} and associated 95% confidence intervals calculated
from the fitted parameters are shown as well as the raw data. Tumor multiplicity
data were analyzed by a nonlinear Poisson model with a background correction term.
The data were fitted to the model and the model parameters were estimated; from
these values the number of papillomas per mouse at 1 mg and the associated 95%
confidence intervals were estimated. An example of the graphics display is shown
in Figure 3b.

TEST AGENT CODE: 0051   PROTOCOL: TI   STRAIN: S SEX: M WEEK: 26
TEST AGENT NAME: NISSAN DCM 1979   START DATE: 072879

DOSE	#MICE	%PAPS	MEAN	S.D.
.000	37	8.108	.001	.277
100.000	37	.000	.000	.000
500.000	38	26.316	.342	.627
1000.000	40	32.500	.375	.586
2000.000	35	65.714	1.143	1.167
10000.000	38	89.474	5.474	3.269

PROBIT MODEL WITH BACKGROUND ESTIMATES

OBS & EXP US DOSE

BETA	INITIAL	FINAL	ASYM VAR
0	.0811	.0440	.0005
1	-2.5203	-5.6797	.7488
2	.3071	.7671	.0133

TEST	CHI-SQ	DF	P
G-O-F	5.75	3	.1243
DOSE	102.92	2	.0000

	ESTIMATE	LOWER 95% UPPER
ED50	1522.95	1100.93 2106.75
TD50	1642.18	1289.64 2229.17

(a)

MICROGRAMS
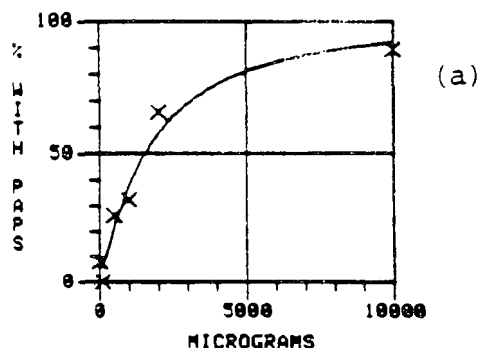
TEST AGENT CODE: 0051   PROTOCOL: TI   STRAIN: S SEX: F WEEK: 26
TEST AGENT NAME: NISSAN DCM 1979   START DATE: 072879

DOSE	#MICE	%PAPS	MEAN	S.D.
.000	118	5.085	.059	.271
100.000	39	2.564	.026	.160
500.000	39	23.077	.385	.847
1000.000	38	39.474	.526	.762
2000.000	40	57.500	1.600	1.892
10000.000	38	97.368	5.658	3.656

NONLIN POISSON MODEL WITH BACKGROUND ESTIMATES

OBS & EXP US DOSE

BETA	INITIAL	FINAL	ASYM VAR
0	.0593	.0479	.0003
1	-1.7472	-7.1132	.1688
2	.3138	.9624	.0022

TEST	CHI-SQ	DF	P
POISS	326.69	306	.1990
ADQCY	9.01	3	.0291
DOSE	674.83	2	.0000

PAPS/M @ 1 MG	LOWER	95% UPPER
SPEC	.676	.573 .798
EXCS	.628	.521 .757

(b)

MICROGRAMS
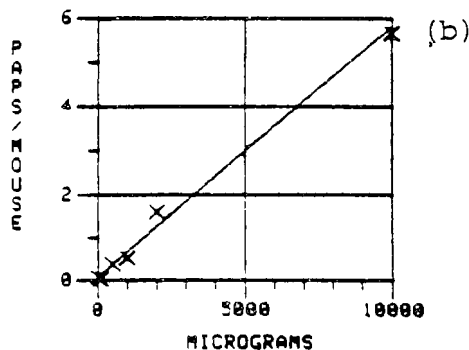
Fig. 3.   Samples of computer-generated analyses of tumor data: (a) tumor
          incidence data and probit analysis for the Nissan sample, (b) nonlinear
          Poisson model analysis of tumor multiplicity data for the Nissan sample.

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Tumor Promotion

The coke oven main and roofing tar samples were applied weekly to mice previously initiated with a single dose of 50.5 µg of benzo(a)pyrene. Coke oven main produced a response equal to the positive control TPA but at 1/500 the dose applied (Table 16). The roofing tar sample was also active as a tumor promoter (Table 16) and produced a dose-related effect up to the highest dose applied. Mice treated with only a single dose of B(a)P produced no tumors.

TABLE 16

SENCAR MOUSE SKIN TUMORIGENESIS
COKE OVEN MAIN AND ROOFING TAR - TUMOR PROMOTION

Dose (µg/mouse/	Mice with Papillomas[a] (%)		Papillomas per mouse[a]	
	Coke Oven Main	Roofing Tar	Coke Oven Main	Roofing Tar
0 (M)[b]	0	0	0	0
0 (F)	0	0	0	0
100 (M)[c]	3	0	0.02	0
100 (F)	10	0	0.10	0
500 (M)	26	5	0.44	0.05
500 (F)	38	0	0.83	0
1000 (M)	53	20	1.2	0.27
1000 (F)	68	16	1.2	0.36
2000 (M)	84	23	2.5	0.32
2000 (F)	85	13	3.1	0.15
4000 (M)[d]	100	55	8.2	1.2
4000 (F)	100	30	8.8	0.6
TPA, 4 µg (M)[e]	86	100	3.1	5.2
TPA, 4 µg (F)	97	100	5.9	7.2

[a]Scored at 34 weeks.
[b]Mice initiated with B(a)P (50.5 µg) and subsequently treated weekly with acetone.
[c]Mice initiated with B(a)P (50.5 µg) and subsequently treated weekly with Coke Oven Main or Roofing Tar.
[d]Mice initiated with B(a)P (50.5 µg) and subsequently treated twice weekly with 2 mg Coke Oven Main or Roofing Tar.
[e]Mice initiated with B(a)P (50.5 µg) and subsequently treated twice weekly with 2 µg. TPA.

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DISCUSSION

The SENCAR mouse, specifically bred for increased sensitivity towards two-stage (initiation-promotion) carcinogenesis, has demonstrated its ability to respond to carcinogens.[29,30] Of three mouse strains and stocks examined, the SENCAR mouse was the most sensitive to the initiating effects of B(a)P (Table 17) with the C57 Black strain completely inactive. The exact nature of the inability of C57 Black mice to respond to B(a)P is unknown, but certain lines of evidence indicate that there is a lack of promotion response in their skin epithelial cells.[36]

This study of the effects of ten complex mixtures and B(a)P on SENCAR mouse skin is the most extensive to date and the results confirm the applicability of this mouse strain to the analysis of complex mixtures. The qualitative results from these studies as summarized in Table 18 are based on decisions from the following empirical rules: (1) a tumor initiation-promotion assay is considered positive for papilloma formation if there is evidence of a dose response and if at least two doses yield a papilloma-per-mouse value equal to three times the background value, and (2) a tumor initiation or complete carcinogenesis assay is considered positive for carcinoma formation if at least one dose produces a tumor incidence of at least 20%.

TABLE 17

COMPARISON OF THE TUMOR INITIATING ACTIVITY OF
BENZO(a)PYRENE IN THREE MOUSE STRAINS AND STOCKS[a]

Strain (Stock)	B(a)P (µg)	Papillomas per Mouse[b]	Mice with Papillomas[b] (%)
SENCAR	50.4	8.2	100
	25.2	3.8	80
	12.6	1.6	60
	2.5	0.9	42
CD 1	50.4	3.8	72
	25.2	1.8	58
	12.6	0.7	40
	2.5	0.1	10
C57 Black	404	0	0
	202	0	0
	101	0	0
	50.4	0	0
	25.2	0	0
	12.6	0	0

[a]Data taken from DiGiovanni et al., (29), and Slaga and Nesnow (unpublished).
[b]Scored at six months.

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TABLE 18

SUMMARY

Sample	Tumor Initiation		Complete Carcinogenesis	Tumor Promotion
	Papillomas[a]	Carcinomas[b]	Carcinomas[b]	Papillomas[a]
Benzo(a)pyrene	+/+[c]	+/+	+/+	+/+
Topside Coke Oven	+/+	-/+	ND[d]	ND
Coke Oven Main	+/+	+/+	+/+	+/+
Roofing Tar	+/+	+/+	+/+	+/+
Nissan	+/+	+/+	-/-	ND
Oldsmobile	+/+	-/-	-/-	ND
VW Rabbit	+/+	-/-	I[e]	ND
Mercedes	+/-	-/-	ND	ND
Caterpillar	-/-	-/-	-/-	ND
Residential Furnace	-/-	-/-	ND	ND
Mustang	+/+	-/+	ND	ND

[a] Scored at 6 months.
[b] Cumulative score at 1 year.
[c] Male/Female.
[d] ND = Not Determined.
[e] I = Incomplete.

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Benzo(a)pyrene, coke oven main, and roofing tar samples were positive in both sexes as tumor initiators (papillomas and carcinomas), tumor promoters and complete carcinogens. In general, those agents which produced a strong tumor-initiation papilloma response also produced carcinomas in the same animals when scored at one year. Four diesel samples were positive as tumor initiators (Nissan, Oldsmobile, VW Rabbit and Mercedes) as was the gasoline engine sample (Mustang). Of all the strong tumor initiators, Nissan extract was the only sample which was not a complete carcinogen at the doses tested and presumably had no tumor promoting activity on mouse skin. In fact, none of the diesel samples evaluated was found to be a complete carcinogen in the dose ranges tested. Benzo(a)pyrene, coke oven main, and roofing tar, all which were complete carcinogens, possessed tumor-promoting activity. The lack of tumor-promoting activity in the Nissan sample is probably a function of the composition of the Nissan mixture. The skin tumorigenesis results indicate that the coke oven main was a stronger tumor promoter than the roofing tar sample. Chemical fractionation and mutagenesis studies show that both the chemical composition and genetically active components of diesel, roofing tar and coke oven main samples are significantly different (J. Lewtas, personal communication).

Tumor initiators on mouse skin may also possess complete carcinogenic activity when administered by other routes to mice and rats. A review of the literature indicates that urethane[37] and triethylenemelamine[38] are both probably pure mouse skin tumor initiators: repeated applications of these agents on mouse skin do not yield tumors. However, urethane administered intraperitoneally, subcutaneously or orally to mice produced a variety of lesions, including lung, liver and lymphoid tumors. Urethane administered orally to rats also produces multiple tumors. Triethylenemelamine produces lung tumors in mice after intraperitoneal injection and muscle tumors in rats after subcutaneous injection.

It is compelling to postulate that the B(a)P in these complex mixtures could account for their tumorigenic activity, since mouse skin is exquisitely sensitive to this agent. The results presented here reveal that a single application of less than 5 µg of B(a)P will yield a 50% tumor incidence as a tumor initiator. However, the relationship between B(a)P content in each mixture, and papilloma response for each mixture, is not linear (Figure 4). Probably none of the activity of the coke oven sample can be explained by B(a)P content, as the B(a)P-induced tumor response at the B(a)P level in the coke oven sample is quite small. Even the B(a)P level in the Nissan sample (11 µg/10mg extract) can only account for 20 to 30% of the papilloma response elicited
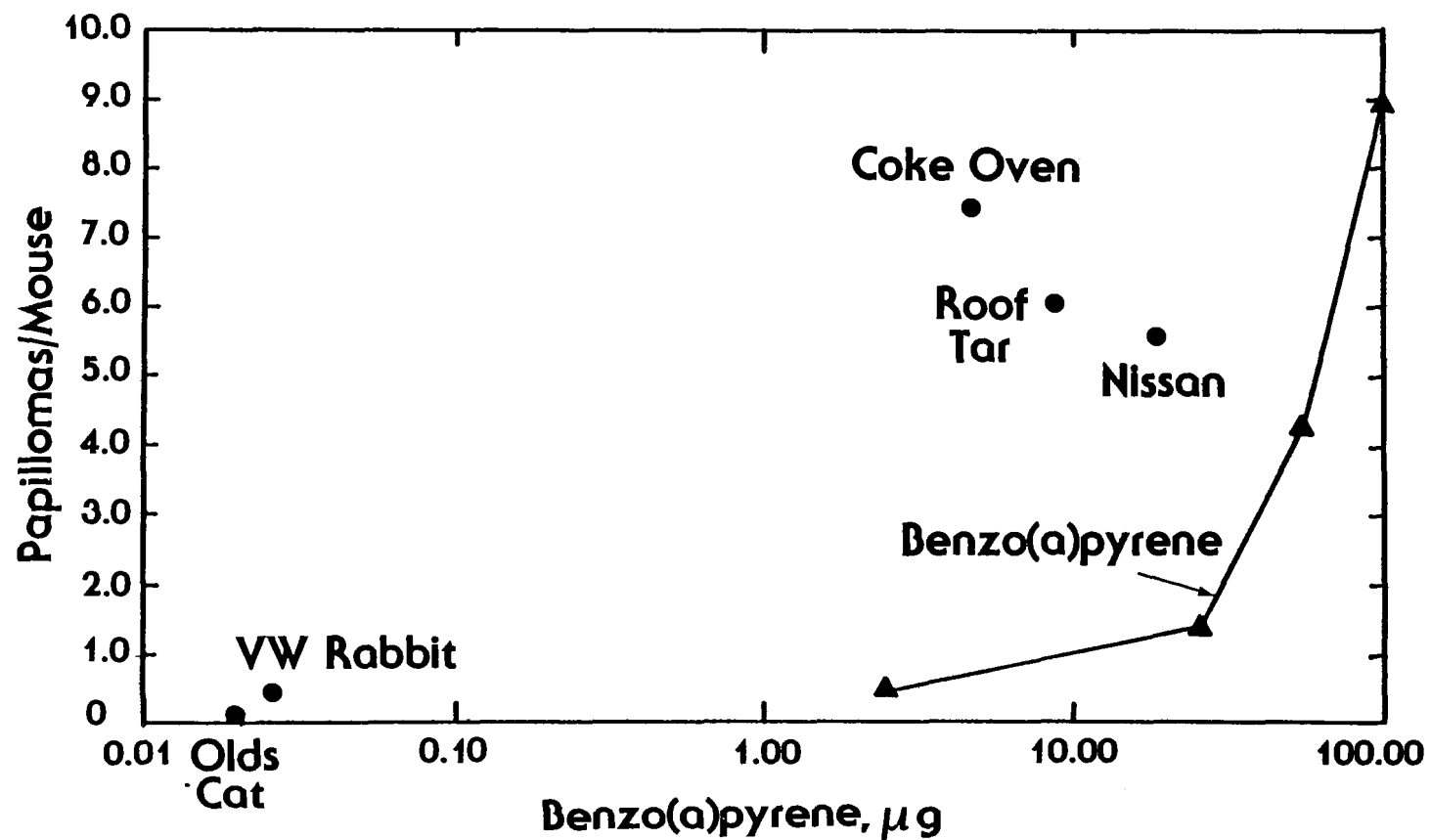
Fig. 4. Relationship of skin tumor initiating activities of complex mixtures
to their benzo(a)pyrene levels: comparison to skin tumor initiating
activity of pure benzo(a)pyrene.

by the Nissan sample. Other components of the mixtures may play an important role in their tumorigenic activities.

Quantitative methods for the analysis of tumor data are many and employ tumor incidence, tumor multiplicity, and tumor latency data. Statistical methods have been employed using Poisson and other distribution assumptions, as well as both uni- and multivariate analytical approaches.[20,21,39-41] We have chosen to apply a nonlinear Poisson model to the papilloma incidence data. This model assumes a Poisson distribution of tumors, that tumor multiplicity is related to dose, that the response may be nonlinear, and that there is a background response. Estimates from the models are presented only if they are in the range from which the data were obtained and if the observed data adequately fit the calculated model (Table 19).

Results from the nonlinear Poisson model suggest the following ranking: topside coke oven > Nissan ≥ roofing tar ≥ VW Rabbit = Mustang. The values calcu- lated are only estimates and in some cases all the assumptions made to derive the estimates are only partially fulfilled.

A probit model has been chosen to evaluate the tumor incidence data. The probit model examines animals with tumors (regardless of multiplicity) and animals without tumors. Results from the probit analysis suggest the ranking: B(a)P > coke oven main ≥ topside coke oven > Nissan = roofing tar. These are not the only models which can be applied to these data, and although they appear effective in this case, more effort is being placed in improving statistical and modeling techniques.

In addition to the tumorigenesis studies described above, detailed gross and histopathological analyses of selected animals have been undertaken. Further results from these detailed pathological studies on the formation of internal tumors and the appearance of tumors with longer latency periods will be presented at a later date.

TABLE 19

SENCAR MOUSE SKIN TUMOR INITIATION
ESTIMATES FROM TWO MODELS BASED ON PAPILLOMA DATA AT 6 MONTHS[a]

		Nonlinear Poisson		Probit	
		Papillomas/Mouse at 1 mg	95% Confidence Intervals	Dose for 50% Papilloma Incidence (TID_{50}), mg	95% Confidence Intervals
Benzo(a)pyrene	M	[b]		0.0036	0.0021 - 0.0062
	F			0.0091	0.0057 - 0.015
Coke Oven Main	M	ND[c]		0.079	0.027 - 0.23
	F			0.19	0.14 - 0.28
Topside Coke Oven	M	2.2[d]	2.00 - 2.40	0.30	0.22 - 0.40
	F	2.0[d]	1.90 - 2.20	0.42	0.31 - 0.58
Nissan	M	0.49[d]	0.38 - 0.63	1.60	1.2 - 2.2
	F	0.68[d]	0.57 - 0.79	1.50	1.1 - 1.9
Roofing Tar	M	0.38[d]	0.30 - 0.49	1.8	1.2 - 2.7
	F	0.44[d]	0.35 - 0.55	2.1	1.5 - 2.8
VW Rabbit	M	0.21	0.14 - 0.30	[e]	
	F	0.17	0.11 - 0.25		
Mustang	M	0.17	0.12 - 0.24		

[a]Estimates calculated from models according to Materials and Methods section.
[b]Not calculated since data were obtained at a lower dose range.
[c]ND = not determined.
[d]The distribution of tumors at all dose levels was not Poisson as the variances exceeded the means.
[e]Not calculated since tumor incidence did not equal 50%.

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DERMAL CARCINOGENESIS BIOASSAYS OF DIESEL PARTICULATES AND DICHLOROMETHANE EXTRACT OF DIESEL PARTICULATES IN C3H MICE

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ABSTRACT

   Diesel particulates (DP) and dichloromethane (DCM) extract of DP were tested to assess their potential as complete carcinogens and as initiators or promoters of carcinogenesis. The test agents were applied as suspensions in acetone to the dorsal skin of 40 male C3H mice per group at various concentrations to obtain information on dose-response relationships. Dosing was performed 3 times weekly in the initiation and complete carcinogenesis studies and 5 times per week in the promotion studies. Positive control groups received repeated applications of benzo[a]pyrene (BaP) for complete carcinogenesis, or a single application of BaP followed by repeated applications of phorbol myristate acetate (PMA) for the initiation and promotion studies. The test agents were applied in place of BaP in the complete carcinogenesis and initiation studies and in place of PMA in the promotion studies. One tumor-bearing animal has been observed at the highest dosage of DCM extract in the complete carcinogenesis study. In the promotion study, 1 and 2 tumor-bearing mice, respectively, have been observed in the 2 highest dosage groups of DCM extract. In the initiation study, 3, 3, 2 and 1 tumor-bearing mice have been observed in the groups that received DP, DCM extract and 2 negative control groups respectively. The results of the initiation and promotion studies suggest that the test agents did not significantly increase tumor incidence compared to the controls. The results of the complete carcinogenesis study are equivocal because of the single tumor observed, and the absence of tumors in the concurrent and historical controls. Since the studies were not completed at the time of manuscript preparation, final conclusions have been deferred until all the data are available.

INTRODUCTION

   In recent years, the need for improved fuel economy has led to an increase in the production and use of fuel-efficient diesel vehicles. Because of the considerably higher particulate emissions of diesel engines compared with

gasoline engines[1] it has become important to determine the toxicological impact of increased exposure of the general population to diesel particulates. Since chemical analysis of organic extracts of diesel particulates has demonstrated the presence of polycyclic aromatic hydrocarbons, including benzo[a]-pyrene[2,3], the possible oncogenicity of diesel emissions is an important public health consideration. In fact, previous studies have shown that extracts of diesel particulates are mutagenic in Salmonella assays[4,5] and oncogenic for mouse skin.[6,7]

With respect to oncogenicity, Kotin et al[6] reported the induction of skin tumors in C57 and A strain mice painted with diesel exhaust extract. More recently, Slaga et al[7] reported the initiation of papillomas in SENCAR mice treated with dichloromethane extract of particulates from Nissan and Olds diesel engines, although the latter extract had extremely low activity. Caterpillar diesel extracts were negative in those studies.

The present studies were designed to assess more definitively the potential of diesel emission extracts as complete carcinogens and as initiators or promoters of carcinogenesis.

MATERIALS AND METHODS

Test Substances. Samples of diesel particulates (DP) and dichloromethane (DCM) extract of DP were supplied by General Motors Research Laboratories to the Bushy Run Research Center on a regular basis throughout the study. The samples were collected from a GM Oldsmobile 350D engine with a road load condition of 65 kilometers/hour. The particulates were collected in a bag house filter at a temperature of $100 \pm 10°C$. The DCM extracts were prepared by a Soxhlet continuous extraction procedure. The samples were shipped on dry ice and stored in a freezer (-12°C) except during preparation of dilutions.

Phorbol 12-myristate 13-acetate (PMA) from PL Biochemicals, Milwaukee, WI were used as the promoting agent in the initiation studies. Benzo[a]pyrene (BaP) from Eastman Kodak, Rochester, N.Y. was used as the initiating agent in the promotion studies and as the positive control substance for the complete carcinogenesis studies. Acetone, spectrophotometric grade, from Fisher Scientific Co., Pittsburgh, PA was used as the diluent for preparation of dosing dilutions and as the negative control substance.

Animals and Husbandry. C3H/HeJ mice from Jackson Laboratories, Bar Harbor, Maine were used in these studies because of their low spontaneous skin tumor incidence and our experience with chemical induction of skin tumors in this strain. The mice were housed 5 per cage in stainless steel suspended cages

located in Airo-Neg Safety Enclosures (Airo Clean Engineering Inc., Broomall, PA). The mice received Zeigler Block feed (Zeigler Brothers Inc., Gardners, PA) and water from an automatic watering system, both ad libitum.

Experimental Design and Procedures. The mice were randomized into 18 groups of 40 mice each such that the means and variances of the body weights were statistically equivalent before treatment began. In the complete carcinogenesis studies, DP was applied as either a 10% or 5% suspension in acetone. DCM extract of DP was applied as suspensions of 50%, 25%, 10% or 5%. A positive control group received 0.2% BaP, and a negative control group received acetone only.

In the promotion studies, a single initiating dose of 1.5% BaP was applied followed after one week by repeated applications of one of the following: a) 10% DP; b) 50% DCM extract; c) 25% DCM extract; d) acetone only; e) 0.0001% PMA (positive control for initiation and promotion studies). An additional group was untreated after the initiating dose of BaP.

In the initiation studies, a single initiating dose of 10% DP, 50% DCM extract, acetone or PMA was followed after one week by repeated applications of 0.0001% PMA. The concentration of PMA was changed for the initiation and promotion studies after 8 months of treatment to 0.01%.

The test substances were applied with an Eppendorf automatic pipet set to deliver 25 microliters. Animals were treated 3 times per week in the complete carcinogenesis and initiation studies and 5 times per week in the promotion study. All test substances were applied to the skin of the back from which the fur was clipped once each week. All suspensions were prepared on a weight/weight basis.

Mice were observed frequently for clinical signs and the appearance of tumor-like growths. Formal observation of each mouse for tumors was performed monthly. The studies were designed to last until the death of all animals. Dosing was stopped only if all the mice in a group had malignant skin tumors by gross observation.

Necropsies were performed on dead mice as soon as possible after death. Mice were sacrificed when found moribund. All body cavities were examined and suspect internal tumors were fixed in 10% neutral buffered formalin for histopathologic examination. The dorsal skin of all mice with or without neoplastic skin lesions, was also fixed for histopathologic examination.

Data Analysis. The mortality and tumor incidence among the various dosage groups were compared using the Breslow[8] and Mantel-Cox[9] tests.

RESULTS

Complete Carcinogenesis Studies. The results of the complete carcinogenesis studies are presented in Table 1. The number of animals alive, as shown in this and the other Tables, was accurate as of the time of manuscript preparation. The dosages were calculated based on measurements of the volume administered, the density of the solution or suspension, and the concentration of test substance.

One tumor-bearing animal was observed at the highest dosage of DCM extract after 714 days of treatment. The tumor was in the treatment area and was diagnosed as a squamous cell carcinoma. Thirty eight tumor-bearing animals were observed in the positive control (BaP) group for an effective tumor incidence of 100%, since the two animals which did not have tumors died early in the study. No skin tumors were observed in the negative controls or in any other dosage group. Survival was not affected by treatment except for the positive controls which died early with skin tumors.

Promotion Studies. One mouse in the 50% DCM extract group and one in the 25% DCM extract group have been diagnosed with squamous cell carcinomas with pulmonary metastases (Table 2). A second animal in the 25% DCM extract group was alive with a grossly diagnosed papilloma. In the positive controls, 19 tumor-bearing animals (11 papillomas, 8 carcinomas) were observed, representing a statistically significant increase over the negative controls. Survival was also significantly reduced in this group. No tumors have been seen in any other dosage group.

Initiation Studies. Three tumor-bearing mice have been observed in each of the groups initiated with DP or DCM extract (Table 3). These include 2 papillomas and 1 carcinoma in the DP group, and 2 papillomas plus 1 fibrosarcoma in the DCM extract group. In the acetone-initiated group, 1 papilloma-bearing mouse was observed. In the PMA-initiated group, 1 carcinoma- and 1 papilloma-bearing mouse were recorded. Statistical analysis of the tumor and survival data revealed no significant differences.

DISCUSSION

The results to date suggest that DP and DCM extract of DP have little, if any, tumor-initiating or tumor-promoting activity under the conditions of these bioassays. This conclusion is based on the absence of a statistically signifi-

cant increase in tumor incidence (or reduction in time to tumor) in any treatment group. The statistically significant tumor response in the 2 positive control groups clearly established the susceptibility of the animals to the induction of skin tumors.

The above conclusion must be qualified because of the observation of a carcinoma in the high dosage DCM extract group of the complete carcinogenesis study. Although the presence of a single tumor is clearly not statistically significant, its importance must be considered in the light of extensive historical control data. The C3H/HeJ strain has been found to have an extremely low spontaneous skin tumor incidence in this laboratory. Of 474 acetone-treated controls, only a single mouse with a squamous cell carcinoma of the eyelid has been observed. No tumors have been observed in the treatment area. Thus, the tumor in the treatment area of a DCM extract-treated mouse may have toxicological importance. Interpretation of this finding is further complicated by the absence of a definite increase in tumor incidence in the 50% DCM extract group of the promotion study in which the animals received a larger total dose than that in the complete carcinogenesis study, following an initiating dose of BaP.

Although the final results and conclusions of these studies are not yet available, the results to date are not consistent with the highly significant tumor yield reported by Kotin et al[6]. The difference in results may be attributed to differences in the composition of the diesel emissions, which itself is a function of engine speed, load and maintenance[6]. The differences in mouse strain and dosage (not clearly defined) may also be important.

The recent preliminary report[7] of positive tumor-initiating activity by DCM extracts from an Olds diesel engine is not definitive because of the "extremely low" activity observed. In addition, the differences between those studies and ours include the source of test and control substances, mouse strain, sex, treatment regimen and specific response parameters. More definitive conclusions from both studies will be possible when the complete data are available.

TABLE 1

RESULTS OF COMPLETE CARCINOGENESIS STUDIES

	DP		DCM EXTRACT				B(a)P	ACETONE
Concentration (%)	10	5	50	25	10	5	0.2	100
Dosage (mg/day)	2.0	1.0	12.0	5.1	2.2	1.0	0.038	17.1
Tumor-Bearing Animals	0	0	1	0	0	0	38[c]	0
Number Alive	3	1	5	4	6	3	0	4
Time To First Tumor (days)	-	-	714	-	-	-	175	-
Median Time To Tumor (days)	-	-	714	-		-	252	
Mean Survival (days)	477	545	551	541	541	541	311[c]	508

All mice received 3 days/week doses of the test agents
DP=Diesel Particulates
DCM=Dichloromethane
BaP=Benzo[a]pyrene
[c] $p < 0.001$

TABLE 2

RESULTS OF PROMOTION STUDIES

	DP	DCM EXTRACT		ACETONE CONTROL	UNTREATED CONTROL	POSITIVE CONTROL (PMA)
Concentration (%)	10	50	25	100	-	(0.01)
Dosage (mg/day)	2.0	12.0	5.1	17.1	-	1.5×10^{-3}
Tumor-Bearing Animals	0	1	2	0	0	19[c]
Number Alive	7	4	10	8	6	0
Time To First Tumor (days)	-	361	452	-	-	354
Median Time To Tumor (days)		361	567	-	-	465
Mean Survival (days)	523	478	586	552	562	452[b]

All mice received 1 dose of benzopyrene (0.23 mg) followed by 5 days/week doses
of test agent.
DP=Diesel Particulates
DCM=Dichloromethane
[b] $p < 0.01$ [c] $p < 0.001$
PMA=Phorbol Myristate Acetate

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TABLE 3

RESULTS OF INITIATION STUDIES

	DP	DCM EXTRACT	ACETONE CONTROL	PMA CONTROL
Concentration (%)	10	50	100	0.01
Dosage (mg/day)	2.0	12.0	17.1	1.5×10^{-3}
Tumor-Bearing Animals	3	3	1	2
Number Alive	0	0	0	1
Time To First Tumor (days)	319	395	532	452
Median Time To Tumor (days)	528	508	562	612
Mean Survival (days)	516	476	516	468

All mice received PMA 1.5 µg/day 3 times/week after one initiating dose of test agent.
DP=Diesel Particulates
DCM=Dichloromethane
PMA=Phorbol Myristate Acetate

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RESPIRATORY CARCINOGENICITY OF DIESEL FUEL EMISSIONS
INTERIM RESULTS

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INTRODUCTION

An experiment is in progress in which diesel engine emission particles (DP), organic solvent extracts of diesel particles (DE), extracts of roofing tar volatiles (RT) and coke oven mains (CO), and cigarette smoke condensate (CS) are being evaluated for their carcinogenic potential when administered by intratracheal instillation to hamsters. Appropriate control animal groups-- untreated colony controls (CC), solvent (SV), solvent plus ferric oxide (SF), benzo(a)pyrene (BP) as a positive control, and gel-saline plus ferric oxide (GS)--are included in the study. Because of the number of hamsters being treated, the experiment was conducted in two replicates identical in design except for a gel-saline control included in the second replicate (Table 1). At the time of this interim report, hamsters in Replicate 1 had been on test for 61 weeks and those in Replicate 2 for 44 weeks. Histopathologic findings are reported for a subset of the animals from Replicate 1 that were sacrificed at 12 months of age after being on test for approximately 9 months.

MATERIALS AND METHODS
Test Materials

Test materials were supplied through the courtesy of EPA and sample generation and collection has been previously described.[1]

Whole Particle Diesel Exhaust Suspensions. The whole particle diesel exhaust material was received as a dry powder that had been scraped from collection filter substrates. Microscopical examination of the powder as received showed that the individual submicrometer carbonaceous exhaust particles had agglomerated and aggregated during generation and after capture on the collection filter to form large diameter hollow carbonaceous spheres (up to 70 μm), and large thin flakes (up to 150 μm). To produce particle suspensions suitable for intratracheal instillation, the whole particle diesel exhaust had

TABLE 1.

EXPERIMENTAL DESIGN FOR EACH REPLICATE OF THE CHRONIC STUDY[a]

Treatment Group	Dose[b] (mg/wk)	Sex	Number of Animals	12 Month Sacrifice Number of Animals	Total
Diesel Particle	5.0	M	50	10	60
	2.5	M	50	10	60
	1.25	M	50	10	60
Diesel Particle	5.0	M	50	10	60
+ Fe_2O_3	2.5	M	50	10	60
	1.25	M	50	10	60
Diesel Extract	5.0	M	50	10	60
+ Fe_2O_3	2.5	M	50	10	60
	1.25	M	50	10	60
Coke Oven	5.0	M	50	10	60
+ Fe_2O_3	2.5	M	50	10	60
	1.25	M	50	10	60
Cigarette Smoke	5.0	M	50	10	60
+ Fe_2O_3	2.5	M	50	10	60
	1.25	M	50	10	60
Roofing Tar	5.0	M	50	10	60
+ Fe_2O_3	2.5	M	50	10	60
	1.25	M	50	10	60
Benzo(a)pyrene + Fe_2O_3	2.0	M	50	10	60
Solvent		M	50	10	60
Solvent + Fe_2O_3	5.0	M	125	25	150
Gelatin Saline Control[c]		M	50	10	60
Colony Control		M	100	20	120
Total Replicate 1			1,225	245	1,470
Total Replicate 2			1,275	255	1,530

[a] The numbers shown are the number of animals in each group for each replicate of the study.
[b] Dose refers to the quantity of test material or Fe_2O_3 when only one component was present, and to the quantity of each component when test materials were admixed with Fe_2O_3.
[c] Gelatin Saline Control was included in only the second replicate.

to be reduced in size so that 90 percent by mass of the material was below 10 μm in size and suspended in physiological saline.

Certain physical properties of the whole particle diesel exhaust such as its low density, wide particle size range, and high electrostatic charging tendency prevented application of conventional dry grinding techniques. The hydrophobic nature of the powder and the requirement for sterility in the final suspension made it necessary to achieve particle size reduction and suspension simultaneously. Two different size reduction-suspension preparation methods were developed over the course of the instillation phases of the bioassay experiments. Before any size reduction or particle suspension techniques could be developed, however, a nontoxic, saline-miscible wetting agent for the diesel exhaust particles had to be selected. Reagent grade propylene glycol was found to wet the diesel exhaust particles adequately without dissolving (at room temperature) the extractable organics adsorbed on the carbonaceous diesel soot particles. The reported low toxicity of propylene glycol,[2] its high viscosity, and its miscibility with saline rendered it a desirable material for use in suspension preparation. Gelatin was dissolved in the saline to improve suspension stability and was found to be soluble in the propylene glycol as well.

For preliminary dose-response bioassay experiments, a simple ball milling technique was selected to simultaneously reduce the diesel exhaust particles in size and to suspend them in the gelatin saline suspending fluid. The ball-milling technique has been described in detail elsewhere.[3] Briefly, the technique involves wetting the particles with propylene glycol before adding them to a sterilized wide-mouth jar containing 3 to 5 mm glass beads and the gelatin-saline suspending fluid; the filled milling jar is placed on a roller for a 10 day milling period.

The ball-milling method was adequate for the small-scale, short term acute toxicity experiments, but was inadequate for the greater materials requirements of the long term chronic toxicity and carcinogenic potential evaluation bioassays. Therefore, a mechanical mixer that simultaneously generates both mechanical shearing and ultrasonic energies was investigated to determine if it could perform both the particle grinding and the particle suspension operations required. The mixer—a Polytron® (Brinkmann Instruments) fitted with the special purpose PT-35/4 probe generator—was tested and found to grind the diesel particle aggregates to the desired sizes and suspend them in the gelatin-saline vehicle. Simple probe generator designs such as the standard saw tooth (ST) probes did not produce adequate mechanical shear energy to reduce the

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particle size sufficiently. Propylene glycol was still required as a wetting agent. The concentration of propylene glycol used in these suspensions was slightly higher than that in the ball-milled suspensions. Gum arabic was added to the suspending fluid (Table 2), only to make the suspending fluid for the diesel particles suspensions and the diesel exhaust emulsions identical in composition.

TABLE 2.
COMPOSITION OF WHOLE PARTICLE DIESEL EXHAUST SUSPENSION

	Ball-Milled	Polytron® Mixed
Dose Range	5, 3, and 1 mg/0.2 ml	5, 2.5, and 1.25 mg/0.2 ml
Carrier Liquid	Saline with 0.5 percent w/v gelatin	Saline with 0.5 percent w/v gelatin and 0.5 percent w/v gum arabic
Wetting Agent	Propylene glycol—7 percent by volume	Propylene glycol—10 percent by volume
Carrier Dust	Fe_2O_3—5, 3, and 1 mg/0.2 ml	Fe_2O_3—5, 2.5, and 1.25 mg/0.2 ml

The method of preparing the Polytron® suspension was straightforward. The diesel particles were placed in sterile pharmaceutical graduate cylinders and the propylene glycol was added, with hand-swirling of the cylinder, to wet the particles. The sterile saline, with gum arabic and gelatin dissolved in it, was then added. The suspensions were mixed for 2 min with the PT-35/4 at a moderately high speed (7 on a scale of 10) to complete the grinding-suspension process. For the iron oxide containing suspensions, the iron oxide was added after the grinding-suspension was completed and was mixed into the suspensions with the standard PT-10ST generator since no additional particle size reduction was desired at this point.

   Diesel Exhaust Extract Suspensions (Emulsions). Solvent extracts of whole particle diesel exhaust were submitted for the bioassay experiments as solutions of the extracted materials in dichloromethane. Before any emulsion preparation could be prepared, the solvent had to be removed under a slow stream of pure, dry nitrogen. Upon removal of the solvent, the diesel exhaust extract was found to be composed of a light amber oily phase and a semi-solid, dark brown tarry phase, neither of which was appreciably soluble in, or miscible with, saline. Thus, in order to produce emulsions of the extract in the saline instillation

carrier fluid, a wetting agent was required, which would be miscible with the oily phase and saline and which would dissolve the tarry phase. Propylene glycol was again found to fulfill the requirements of a nontoxic wetting agent.

Two different methods for diesel extract emulsion preparation were also developed over the course of the program. The initial preparation method, described in detail elsewhere,[3] was a very tedious hand-mixing method, and is briefly described here. The extract solutions were pipetted into glass tissue grinders and the solvent was blown-off under nitrogen. Propylene glycol was added to the solvent-free extract, and the mixture was heated to 50-65°C to aid solvation of the tarry phase. SPAN-80®, a surface active agent, was then added and mixing with the glass pestle began. Once the components had been thoroughly emulsified, the gum-arabic-gelatin-saline fluid (also heated to 50°C) was slowly poured in with continuous mixing with the pestle. Emulsification required 10-15 min of vigorous action of the pestle in the tissue grinder. Iron oxide was added once emulsification was completed.

A more mechanized method of emulsion preparation was obviously required for the larger-scale chronic studies. The Polytron® mixer, equipped with a standard PT-10ST probe generator was tested and found to produce an excellent suspension. Emulsions were prepared directly in the pharmaceutical graduate cylinders in which the solvent removal step was conducted. Warm propylene glycol was then added and mixed with the Polytron® operating at moderate speed for 30-60 sec. The preliminary emulsification step did not require the addition of a surfactant. With the mixer running, the warmed gum-arabic-gelatin-saline mixture was slowly added. Once all the saline was added (1 min), mixing at moderately high speed was continued for another minute. Iron oxide was mixed in at moderate speed for 30 sec after emulsification was completed. The Polytron® preparation technique simplified the preparation procedure, as well as the composition of the final product (Table 3).

Other Extracts. The extracts of coke oven mains and roofing tar volatiles were also received as solutions in dichloromethane. Upon removal of the solvent, the coke oven extract was found to be a very viscous, dark brown-black, sticky, tar-like material, while the roofing tar extract was a light green, waxy material. Both substances were highly odorous.

The preparation of stable emulsions of these extracts in gelatin-saline followed the same general procedures described for the diesel engine exhaust extract. The order of addition of components and mixing steps (with the glass tissue grinder emulsifying apparatus as well as the Polytron®), were somewhat different for these materials, however. For the coke oven mains, after gently

TABLE 3.

COMPOSITION OF DIESEL EXHAUST EXTRACT EMULSIONS

	Tissue Grinder--Hand Emulsified	Polytron® Emulsified
Dose Range	5, 3, and 1 mg/0.2 ml	5, 2.5, and 1.25 mg/0.2ml
Carrier Liquid	Saline with 0.5 percent w/v gelatin and 0.25 percent w/v gum arabic	Saline with 0.5 percent w/v gelatin and 0.5 percent w/v gum arabic
Wetting Agent	Propylene glycol--10 percent by volume and sorbitan monooleate (SPAN-80®) 0.1 percent by volume	Propylene glycol-10 percent by volume
Carrier Dust	Fe_2O_3 - 5, 3, and 1 mg/0.2 ml	Fe_2O_3 - 5, 2.5, and 1.25 mg/0.2 ml

warming the extract plus propylene glycol until the very viscous tar essentially melted, one-third of the required saline heated to 60°C was added to the mixing container before emulsification was begun. This initial mixing with the Polytron® required 45-60 sec before the remainder of the heated saline was slowly added. Final emulsification required 2-3 min of mixing with the Polytron® operating at maximum speed. For the roofing tar extract, after addition of the propylene glycol followed by gentle heating, the initial emulsification was conducted for 1-2 min with the Polytron® operating at top speed. The iron oxide was then added and emulsified for 3 sec, and finally, room temperature saline (plus the gelatin and gum arabic in solution) was added with the Polytron® running at moderately high speed. The final emulsification required mixing at top speed for 1-2 min.

The cigarette smoke condensate was received as a solution in acetone. Removal of the solvent left a viscous, dark brown, somewhat tarry residue. Emulsification was conducted following steps identical to those used for the diesel extract.

   Benzo(a)pyrene-Ferric Oxide Mixture. The positive control material, a 1:1 by mass mixture of benzo(a)pyrene and ferric oxide was prepared by precipitating the benzo(a)pyrene onto the iron oxide. The iron oxide was suspended in 20 volumes of distilled water and stirred constantly with a magnetic stirrer. The benzo(a)pyrene, dissolved in one volume of acetone, was added to the iron oxide suspension by slowly pouring the solution into the vortex. The benzo(a)pyrene immediately precipitated from the acetone solution upon impact in the water,

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thereby capturing iron oxide particles within the crystals formed. The
benzo(a)pyrene-iron oxide particles thus formed were filtered from the water
suspension and dried under a nitrogen stream.

Animals

Male Syrian Golden hamsters (LAK:LVG(SYR)) were obtained from Charles River
Breeding Laboratories, Wilmington, MA, at 6-8 weeks of age and were held in
quarantine until one week before they were placed on test at 12-13 weeks of age.
The animals were inspected for health status upon arrival and periodically
during quarantine. For each replicate, 10-15 hamsters were randomly selected,
killed, and examined for pathogenic bacteria, mycoplasma, yeast, fungi,
endoparasites, and ectoparasites. There were no problems with the health status
of the animals during quarantine other than the death of a small number of
animals, as is consistent with shipping stress.

The hamsters were maintained in plastic, solid-bottom cages on a bedding of
hardwood chips (Ab-sorb-dri®). In the first replicate, three hamsters were
housed per cage initially. By Week 27 of the study, however, the hamsters had
to be rehoused, two per cage, separated by a stainless steel divider because of
fighting. Hamsters in the second replicate were housed two per cage at the
start of the study; dividers were added by Week 4. Food (Wayne Blox, Locke
Erikson Labs, Melrose Park, Ill.) and tap water were available for *ad libitum*
consumption.

During the week preceding the first treatment, the hamsters were randomly
allocated to treatment groups, as shown in Table 1. Groups that received
different test materials were housed in rooms separated from other test
groups. Animals in the solvent and solvent plus ferric oxide control groups
were together with animals for which they were the control. The 125 solvent
plus ferric oxide control animals in each replicate were distributed among the
appropriate five test rooms. Colony control animals were housed separately from
all other animals. In the first replicate the positive control group
(benzo(a)pyrene:ferric oxide) was housed separately, whereas in the second
replicate, it was in a room with the gelatin saline controls. Animal rooms were
maintained on a 12 hour light:12 hour dark cycle at a room temperature of 76°
±2°F, and humidity was controlled to avoid extreme excursions outside the range
of 40-70 percent RH.

Treatment

   Test and control materials were administered by the intratracheal instillation method described by Saffiotti and co-workers.[4,5] Before each intratracheal instillation the hamster was anesthetized with halothane dispensed from an Airco Veterinary Anesthesia Machine, Heedbrink, Model 960.[6] When the righting reflex was lost the animal was placed on a slanted board, its back on the board and its mouth kept open by hanging the lower incisor teeth on a wire hook, while the upper incisors were retained by a tight rubber band (Figure 1).

Figure 1. Administration of diesel particles to hamsters by intratracheal instillation.

A volume of 0.2 ml of the test material was delivered via a 0.25 ml tuberculin syringe fitted with a blunt 19 ga needle about 3 in. long and bent at a 135° angle at 45 mm from the tip. The tongue was pulled outward with forceps and the rhythmic opening and closing of the vocal cords observed. The blunt end of the needle was inserted into the tracheal lumen past the open vocal cords and pushed almost to the bottom of the trachea. The suspension was gently injected and the hamster was retained on the board for approximately 1 min to make certain no suspension was regurgitated. Treatment, initiated at 12-13 weeks of age, was performed once weekly for 15 weeks.

Observations

The hamsters were weighed weekly during the 15-week treatment period and biweekly thereafter. Physical examinations including palpation for tumors, were performed at the time of weight determinations. In addition, the hamsters were observed daily for overt physical or behavioral signs of toxicity or disease; a second check was performed each afternoon to allow identification of dead or moribund hamsters.

Extensive necropsy examination was performed for all animals at the time of death. Moribund animals were sacrificed for immediate necropsy examination. At 12 months of age, a subset of the test and control hamsters (Table 1) were randomly selected for interim sacrifice and necropsy. Surviving animals will be held up to 2 years of age, at which time they will be killed and necropsy examinations will be performed. The necropsy procedure involves a thorough examination of all external surfaces, body cavities, and orifices, with approximately 35 tissues being examined and collected. For animals killed at the interim sacrifices, the brain, heart, liver, spleen, kidneys, and testes were weighed at the time of necropsy. The eyes were fixed in gluteraldehyde and the testes in Bouin's solution for 24 hr and were then transferred to alcohol for preservation. The remaining tissues were fixed and stored in 10 percent neutral buffered formalin. Tissues were blocked in paraffin and 5-6 micron sections were cut and stained with hematoxylin and eosin.

All tissues collected were examined under a light microscope for all hamsters killed for the interim sacrifices and will similarly be examined for all control hamsters and hamsters in the high-dosage test groups whether death is spontaneous or by moribund or terminal sacrifice. For the remaining animals, the respiratory tract will be examined microscopically and the other tissues will be saved for examination in the event treatment-related lesions are identified in the high-dosage test groups. In addition to light microscopic examination, the lung and thoracic lymph nodes of hamsters sacrificed at 12 months were examined with a polarized light microscope.

Data Handling

Data from each replicate of the study were summarized separately. Body weight data were summarized by test or control group. Single factor analysis of variance tests were performed to determine whether there were significant differences among the dosage levels of a specified test article and the solvent or solvent plus ferric oxide hamsters housed within the same room. If a significant F ratio was obtained, the test groups at different dosage levels

were individually compared to the solvent control or appropriate subset of the solvent plus ferric oxide control animals. Organ weight data collected at the interim sacrifices were similarily analyzed. Survival data were summarized as the percent animals surviving at the end of the various test weeks. Clinical observations are continuously summarized based upon calculations and statistical comparisons of the median time for each group between the first observance of a specific sign and the death of the animal. Necropsy observations were tabulated as incidence of specific lesions within groups. Histopathologic data were summarized as the incidence and average severity of lesions for the different test or control groups.

RESULTS

Test Material Characterization

The Polytron® prepared diesel exhaust particle suspensions proved to be more stable than the ball-milled suspensions and contained smaller sized particles (Table 4). The Polytron® preparation method had the additional advantage of eliminating of the glass fragments fractured from the milling beads and vessels.

The final suspensions were evaluated microscopically for particle morphology (Figures 2 and 3) and particle size. Samples were assayed by filtering measured aliquots (diesel particles only) and by ashing measured aliquots (suspension containing diesel particles and iron oxide).

TABLE 4.

PARTICLE SIZE ANALYSES OF WHOLE PARTICLE DIESEL EXHAUST AND PREPARED SUSPENSIONS

Diameter, μm (Linear Dimension)	Cumulative Mass Percent Greater Than Stated Size		
	As-Received	Ball-Milled	Polytron® Milled
0.0	100.0	100.0	100.0
1.0	99.2	99.6	98.5
3.0	97.2	95.7	80.3
5.5	82.7	76.1	24.0
8.0	63.0	48.1	5.8
10.5	43.6	25.3	0.9
13.0	26.0	12.4	0.9
15.5	14.4	4.7	0.9
18.0	7.3	1.5	0.0
20.5	2.5	0.0	0.0
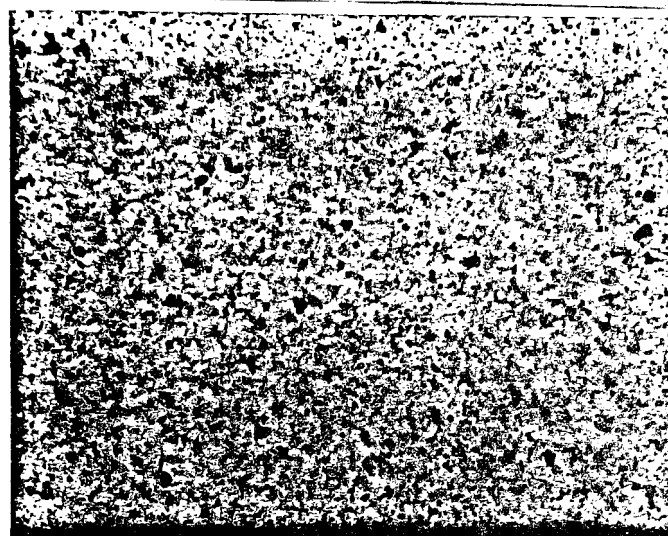
Figure 2. Optical micrograph of whole particle diesel exhaust suspension after dispersion with the Polytron®; 502X. The largest particle visible is approximately 5 μm in diameter.
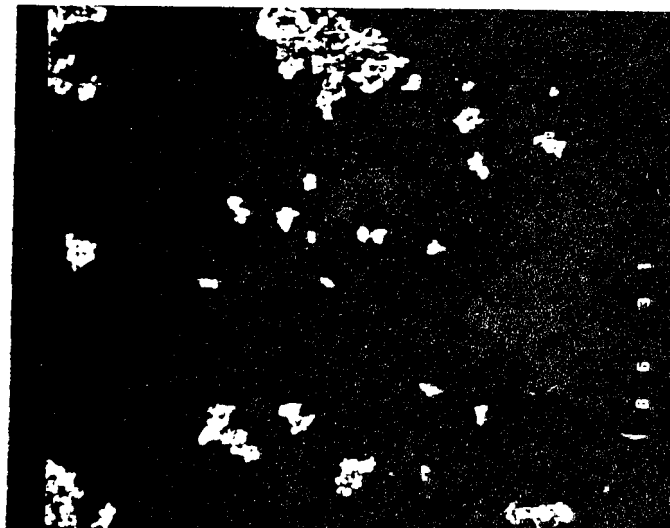
Figure 3. Electron micrograph of whole particle diesel exhaust suspension after dispersion with the Polytron®; 5000X.

The Polytron® prepared diesel extract emulsions also proved to be more stable than the hand-mixed emulsions (Figure 4). The primary improvement of the emulsions introduced by the Polytron® preparation method was the elimination of the surfactant.

No adequate assay method for determining the amount of diesel extract present in the emulsions could be developed because of the complex nature of the extracts. The prepared emulsions were viewed microscopically to determine if droplet sizes of both the tarry and oily phases were comparable (Figure 5).

Clinical Signs

Almost all of the clinical signs exhibited by first replicate hamsters during the observation period preceding to the scheduled sacrifice were the direct result of fighting among cage mates. One hundred percent of the colony control animals developed lumbrosacral skin lesions secondary to fighting. The rest of the test groups exhibited an incidence rate that ranged 94-100 percent. The seriousness of the lesions varied widely among the affected animals. Crusts, necrotic tissue, and one mass that was subsequently identified as an abscess were observed. All of the above lesions resolved after the cage density was reduced to two hamsters per cage and a stainless steel partition was placed in the center of each cage, physically isolating each hamster from his cage mate.

Keratitis was another clinical problem that was observed secondary to fighting. The lesions were reported in the colony controls (1/20), benzo-(a)pyrene (1/10), diesel particle (6/30), diesel particle plus ferric oxide (1/30), diesel extract plus ferric oxide (3/30), cigarette smoke condensate (3/30), and solvent (1/10) treatment groups. No cases of keratitis were reported for the coke oven extract plus ferric oxide group (0/30). The physical isolation of each hamster effectively eliminated this problem from the study.

Survival data to date have indicated no treatment-related differences. In general, the percentage of hamsters surviving at any given time was greater in the second replicate than in the first. This is undoubtedly due to a reduction of deaths from causes related to fight wounds in the second replicate by earlier separation of the hamsters.

Body Weights

Hamsters treated with the highest dose (5.0 mg/wk) of diesel particles had significantly lower body weights than solvent control hamsters in both parts of the study (Tables 5 and 6). A significant effect was first observed in Test Week 6 of the first replicate and was sustained through Test Week 15, the last
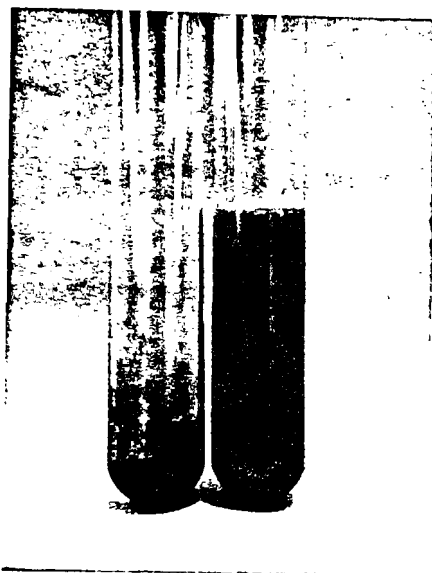
Figure 4. Diesel Exhaust Extract: Left is the extract itself after solvent
removal; Right is the saline based emulsion prepared at a concentration of
2.5 mg/0.2 ml.

Figure 5. Optical micrograph of diesel exhaust extract emulsion. (Dark field
illumination; 520X.) The droplets are all components of the diesel exhaust
extract. The largest droplet is approximately 10 μm in diameter.

TABLE 5.

PATTERN OF SIGNIFICANT DIFFERENCES[a] FOR TREATED VS. CONTROL COMPARISONS OF BODY WEIGHTS BY TEST WEEK FOR REPLICATE 1

Test Week	Test Groups[b]					
	DP	DP+ Fe_2O_3	DE+ Fe_2O_3	CS+ Fe_2O_3	CO+ Fe_2O_3	RT+ Fe_2O_3
0	-	-	-	-	-	-
1	-	-	-	-	-	-
2	F	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	H	HM	-	-	-
6	H	H	HM	-	-	-
7	H	H	-	-	F	-
8	H	H	-	-	F	-
9	-	H	-	-	F	-
10	-	H	-	-	F	-
11	H	H	-	-	F	-
12	H	H	-	-	-	-
13	H	H	-	-	-	-
14	H	H	-	-	-	-
15	H	H	-	-	-	-
17	-	-	-	-	-	-
19	-	-	-	-	-	-
21	-	-	-	-	-	-
23	-	-	-	-	-	-
25	-	-	-	-	-	-
27	-	-	-	-	-	-
29	-	-	-	-	-	-
31	-	-	-	-	-	-
33	-	-	-	-	-	-
35	-	-	-	-	-	-
37	-	-	-	-	-	-
39	-	-	-	-	-	-
41	-	-	-	-	-	-
43	-	-	-	-	H	-
45	-	-	-	-	HM	-
47	-	-	-	-	HM	-
49	-	-	-	-	HM	-
51	-	-	-	-	HM	-
53	-	-	-	-	HM	-
55	-	-	-	-	HM	-
57	-	-	-	-	HM	-
59	-	-	-	-	HM	-
61	-	-	-	-	-	-

[a] A significance level of p 0.05 was used. F indicates that a significant F ratio was obtained in the analysis of variance but was due to difference among the High, Middle, and Low Dosages groups. H, M, or L indicate that the High, Middle, or Low dosage group was significantly lower in body weight than the appropriate control group.

[b] DP = Diesel Emission Particles, DE = Diesel Emission Particle Extract, CS = Cigarette Smoke Condensate, CO = Coke Oven Emission Extract, and RT = Roofing Tar Extract.

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TABLE 6.

PATTERN OF SIGNIFICANT DIFFERENCES[a] FOR TREATED VS. CONTROL COMPARISONS OF
BODY WEIGHTS BY TEST WEEK FOR REPLICATE 2

Test Week	Test Groups[b]					
	DP	DP+ Fe_2O_3	DE+ Fe_2O_3	CS+ Fe_2O_3	CO+ Fe_2O_3	RT+ Fe_2O_3
0	-	-	-	-	-	-
1	H	-	-	-	-	-
2	H	-	-	-	-	F
3	H	-	-	-	-	F
4	HML	-	HM	-	-	F
5	H	-	HML	-	-	F
6	H	-	HM	-	-	F
7	H	-	HM	-	-	F
8	H	c	c	c	c	c
9	H	c	c	c	c	c
10	H	-	HM	-	-	F
11	H	-	HML	-	-	F
12	H	-	HML	F	-	F
13	H	-	HML	-	-	M
14	H	-	HML	-	-	M
15	H	-	HML	-	-	M
16	H	-	HML	-	-	-
18	-	-	HML	-	-	-
20	-	-	HML	-	-	-
22	H	-	HML	-	-	-
24	H	-	HML	-	-	-
26	H	-	-	-	-	-
28	H	-	-	-	-	-
30	H	-	-	F	-	-
32	H	-	-	-	-	-
34	H	-	-	-	-	-
36	-	-	-	-	-	-
38	-	-	-	-	-	-
40	-	-	-	-	-	-
42	-	-	-	-	-	-
44	-	-	-	-	-	-

[a] A significance level of p 0.05 was used. F indicates that a significant F
ratio was obtained in the analysis of variance but was due to differences
among the High, Middle, and Low dosage groups. H, M, or L indicate that the
High, Middle, or Low dosage group was significantly lower in body weight than
the appropriate control group.
[b] DP = Diesel Emission Particles, DE = Diesel Emission Particle Extract,
CS = Cigarette Smoke Condensate, CO = Coke Oven Emission Extract, and
RT = Roofing Tar Extract.
[c] Body weights were not determined at the scheduled times during these weeks.

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week of treatment. In the second replicate, the effects of the highest dose of diesel particles on body weight were apparent earlier, sustained longer, and involved larger differences. In Replicate 2, with the exception of Test Weeks 18 and 20, body weights of the hamsters treated with 5.0 mg/wk diesel particles were significantly lower than control weights for all weeks between Test Weeks 1 and 34. The maximum effect in Replicate 2 was seen during the latter part of treatment and early posttreatment weeks when differences from the control weights averaged 15-20 g.

Body weights of hamsters treated with the highest dose of diesel particles plus ferric oxide were lower than those of their solvent plus ferric oxide control during the treatment period of Replicate 1 (Table 5). Note that the duration of effects closely approximates that seen in the high dosage diesel particles group of Replicate 1. In Replicate 2, body weights of hamsters treated with the high dose of diesel particles plus ferric oxide were somewhat lower than those of their controls during the latter part of treatment and following the treatment period. This difference, however, which ranged 8-12 g between Weeks 12 and 20, failed to achieve statistical significance.

During the latter weeks of treatment in the first replicate and up to Test Week 21, body weight means for hamsters treated with diesel extract or their solvent plus ferric oxide control were distributed in a dose-related fashion: the control mean was the greatest, and the low, middle, and high dosage means were consecutively lower. However, statistically significant differences from control means were limited to Test Weeks 5 and 6 and involved only the high and middle dosage groups (Table 5). In the second replicate, body weight means for all groups of hamsters treated with diesel extract were significantly lower than control values during the latter part of the treatment period and up to Test Week 24. Effects in the high and middle dosage groups were observed as early as Test Week 4 and in the low dosage group in Test Week 5. During the period when significant effects were obtained in all three dosage groups, the means for hamsters treated with the high dose were slightly (4-5 g) but consistently lower than those of the low and middle dosage groups. The means for the latter two groups, however, were often numerically (within 1 g) as well as statistically, equivalent.

To date, no significant reduction in body weight gains has been observed for hamsters treated with cigarette smoke condensate. During the first replicate, hamsters treated with the high dose of this test material appeared to show reduced body weight gains. This was not seen in the second replicate, where the body weight curve for the high dosage group was virtually superimposable on the control curve for much of the period in question.

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For hamsters treated with coke oven emission in the first replicate, no significant differences between body weight means for treated and control groups were observed before Test Week 43. Beginning that week for the high dosage group and in Test Week 45 for the middle dosage group, body weights for these groups were significantly lower than those of the solvent plus ferric oxide controls for all determinations through Test Week 59. At the time of the last determination (Test Week 61), body weights of the high and middle dosage groups still averaged approximately 15 g less than those of the control group, but this difference is not statistically significant. There have been no indications of reductions in body weight gains by coke oven emissions in the second replicate.

These differences in body weight between the high and middle dosage groups and their controls cannot be attributed to body weight loss in these test groups. Rather the mean body weight of their solvent plus ferric oxide controls showed a large increase beginning at about Week 35. This increase in mean body weight coincided with an increase in deaths of animals in the control group. The removal from the experiment of lower weight animals that had died could have resulted in higher calculated mean body weights of the surviving control animals.

Through Test Week 61, there were no indications in Replicate 1 of treatment related effects on body weights of hamsters treated with roofing tar extract. In the second replicate, body weights of hamsters in the low dosage group tended to be greatest and those in the middle dosage group lowest; the high dose and solvent plus ferric oxide control values intermediate between the two. This distribution of body weight means resulted in significant F values for the analysis of variance during Test Weeks 2-15. Through Week 12, however, the significant values were attributable to differences between the low and middle dosage groups. Thereafter, in Weeks 13-15, the body weight means of the middle dosage group were lower than those of both the low dosage group and the control group. The lack of a meaningful dose-response relationship in these results and the absence of similar findings in the first replicate suggest that these results cannot be attributed to the treatment with roofing tar extract.

A gel-saline plus ferric oxide control was included in the second replicate as the appropriate control for the benzo(a)pyrene positive control. In this replicate mean body weights of the benzo(a)pyrene group are lower than their respective control during Weeks 20-40, though body weights of animals from both groups are virtually identical during the treatment period.

Gross Necropsy Observations for Animals Sacrificed at 12 Months of Age
(First Replicate)

The most common gross findings among the sacrificed animals were related to the respiratory system. Mottled black lungs were frequently observed in hamsters treated with diesel particles or diesel particles plus ferric oxide. This occurred slightly more frequently in the high and middle dosage groups in which 60% of the hamsters examined showed this effect. Mottled black or mottled brown lungs were also characteristic of hamsters treated with the high (6/10) and middle (6/9) doses of coke oven extract, but this was not observed at the low dosage for coke oven, and only rarely for hamsters at any dose level for diesel extract, cigarette smoke condensate, or roofing tar extract. Of the 25 solvent plus ferric oxide control animals examined at the interim sacrifice, 3 had black or brown mottled lungs. Gray mottled lungs were observed in a few hamsters treated with diesel particles plus ferric oxide, coke oven extract, and cigarette smoke condensate, as well as in two of the solvent plus ferric oxide control animals. Red mottled lungs was even more common but the distribution of this finding, including its presence in 25% of the colony control hamsters, suggested that it was not a treatment related effect.

Black material was present in the trachea of some hamsters at all dosage levels for diesel particles and diesel particles plus ferric oxide. All diesel particle groups and the middle dosage group for diesel particles plus ferric oxide included hamsters with black material present at necropsy in the respiratory lymph nodes and this was observed occasionally but not consistently for all other test materials except roofing tar extract.

No grossly observable masses were detected in any of the sacrificed animals, however, two of ten hamsters in the diesel particle plus ferric oxide low dosage group had a nodule in the lung. A diverse variety of other gross lesions was sporadically observed in the liver, kidney, adrenal gland, spleen, intestines, and other organs. Considering the low frequency and/or distribution of these across treatment groups, none of these could be attributed to effects of the test articles.

Group mean organ weights of brain, heart, liver, spleen, kidneys, and testes of test hamsters from the first replicate interim sacrifice showed no significant effects of test materials as compared with their solvent controls.

In summary, the gross lesions observed at the interim sacrifice for the first replicate indicate no effects of the test articles other than those which are consistent with the presence of residual test materials in the respiratory and lymphoid systems.

Lung Pathology

Lung lesions of significance among colony control animals consisted of adenomatous hyperplasia of the respiratory epithelium lining terminal bronchioles and/or respiratory bronchioles. The hyperplastic lesion occurred independent of or in close association with small aggregates of mononuclear macrophages located principally in alveolar spaces surrounding the terminal airway structures (terminal bronchioles, respiratory bronchioles, alveolar ducts). The macrophages had a foamy cytoplasm or contained a brown, granular pigment. The above lesions affected approximately one half of the colony control animals and they were primarily focal and minimal in severity. Similar lesions were also seen among animals in the solvent control groups of the respective test materials; however, the incidence and relative severity were slightly higher in most instances. With respect to these animals, small amounts of test materials were also present in alveolar macrophages (except for the DP Solvent group) and associated with a minimal to mild, focal subacute alveolitis in some animals at the site of the macrophage response. The inflammatory response (alveolitis) appeared in lung sections from solvent control animals that contained particles of test material.

Among the various test groups, lesions of adenomatous hyperplasia associated with an alveolar macrophage response, phagocytosis of the test material nad subacute alveolitis were more prevalent and severe among animals in DP and DP:Fe$_2$O$_3$ groups with a dose-response relationship. These lesions were most severe in the DP:Fe$_2$O$_3$ group. Lesions of intermediate severity occurred among animals of the CO:Fe$_2$O$_3$ group. Similar lesions of lesser severity occurred among animals in the DE:Fe$_2$O$_3$, CS:Fe$_2$O$_3$, RT:Fe$_2$O$_3$, and BP:Fe$_2$O$_3$ groups. Squamous metaplasia of the terminal bronchiolar epithelium and/or epithelial cells at the site of the adenomatous hyperplasia was seen among animals at the high and mid dose levels of the RT:Fe$_2$O$_3$ group and all levels of the CO:Fe$_2$O$_3$ group. These lesions were focal and minimal to moderate in severity.

Phagocytosis of test material by alveolar macrophages appeared to be fairly complete among all groups except the DP and DP:Fe$_2$O$_3$ groups. Small amounts of extracellular test material were present in the lung sections for some of these animals of various exposure levels . Extrusion of the test materials via the trachea was evident in most of the animals of all test groups and manifested by the presence of particle-laden macrophages within the lumen of the terminal airway structures.

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Focal lesions of chronic pleuritis, accompanied by particles of test material at the reaction site, occurred among a few test animals of the various treatment groups except the CS:Fe$_2$O$_3$ group. Similar lesions were observed among a few animals in some of the solvent control groups when particles of test material were also present within the lung. These lesions were absent among the colony control animals. However, focal lesions of subacute pleuritis were present among a few animals in the colony control and some of the solvent control and test groups. These lesions were focal and of minimal severity. In view of the above, this lesion appeared to be naturally occurring and unrelated to the test materials.

Other pulmonary lesions of lesser significance among all groups included congestion, recent hemorrhage and peribronchial, peribronchiolar or perivascular lymphoid infiltrates. These lesions did not appear to be compound related and were ascribed to the method of sacrifice and spontaneous disease.

Neoplasms of the respiratory tract, classified as adenomas, were observed among single high-dose animals of the DP and DE:Fe$_2$O$_3$ groups. Advanced lesions of adenomatous hyperplasia graded as moderate to marked (Grade III to IV) were usually multifocal and rather extensive in their development. However, they were regarded as proliferative lesions in response to chronic irritation rather than neoplastic. The exact pathogenesis of this lesion was not established but, it could be characterized as an extension of the terminal bronchiolar epithelium into the region of the respiratory bronchioles, alveolar ducts, and alveoli. However, a metaplastic origin of this lesion could not be excluded. The epithelial cells were cuboidal with cilia on their apical border and they appeared to undergo both hypertrophy and hyperplasia. These cells also assumed an adenomatous to papillary pattern depending upon the relative severity of the lesion. The presence of moderate to large aggregates of particle-laden macrophages was a relatively constant feature of the lesion. Plugs of mucinous secretory product were also present at the site of some of these lesions.

Treatment-related lesions were also observed in tissues other than the lung and consisted of the following.

Trachea and Larynx

Particles of test material were present in the submucosa of the trachea and/or larynx of some animals of all treatment groups with a dose response relationship with regard to the severity of the lesion. There was no tissue reaction to their presence. This lesion was also seen in some solvent control animals where particles of test material also appeared in the lung.

Thoracic Lymph Node

Particles of test material, within macrophages, were observed in the thoracic lymph node of animals in all test groups and was dose-related in severity.

Nontreatment-Related Lesions

A number of spontaneous and age-related non-neoplastic lesions were observed among animals in the colony control, solvent control, and all test groups. The lesions most commonly observed consisted of thyroid cysts, vacuolation of the pituitary, subcapsular cortical cell hyperplasia of the adrenal gland, mononuclear cell infiltrates in the liver with nuclear hypertrophy and/or nuclear inclusions, and degenerative changes in the kidney (nephropathy) accompanied by mineralized foci.

Tumors in various organ sites were also found upon microscopic examination of tissue sections of interim sacrifice hamsters from the first replicate. These included adenomas of the thyroid, kidney and adrenal, melanomas of the eye, and spleen hemangiomas. These tumors did not appear to be present as a response to treatment but rather as background common to hamsters of this age range.

Conclusions

Only limited conclusions can be drawn prior to the completion of this lifetime toxicity/carcinogenicity evaluation of diesel engine emission particles and the other materials on test. Treatment effects on body weight gain were most pronounced in the case of hamsters treated with diesel exhaust particles and with diesel exhaust extract. Reductions in body weight gain were generally dose related and diesel particle treated hamsters in the first replicate gained weight rapidly following cessation of treatment. Effects of treatment in the second replicate were longer lasting and greater in degree. Admixture of ferric oxide with the diesel exhaust particles did not increase the effect on body weight gain. Treatment with diesel exhaust particle extract produced substantial and prolonged decreased weight gain in the second replicate and lesser but significant effects in the first replicate. Decreases in body weight gain in other test groups were not as severe and recovery was rapid following the end of treatment. Thus it appears that toxicity as measured by decreased body weight gains was test material and dose-related and reversible in nature in that recovery generally occurred rapidly once treatment ended.

At the time of the 12-month interim sacrifice a six month period had elapsed since the last intratracheal instillation of test material. Even after this considerable period of time phagocytosis of diesel particles by alveolar

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macrophages was not complete. Some of the hamsters in these test groups still showed the presence of free particles in extracellular spaces.

All particles tested induced marked responses of alveolar macrophages and extensive phagocytosis. Particle laden macrophages were found within the lumen of the terminal airway structures and in the thoracic lymph nodes of animals from all particle test groups. The severity of the response was both dose and test material related. Adenomatous hyperplasia was most severe in diesel particle and diesel particle plus ferric oxide test groups, intermediate in response in the coke oven group, and least severe in the diesel particle extract, cigarette smoke condensate and roofing tar groups. Two lung adenomas were found on microscopic examination; one in a high dose DP hamster and the other in a high dose DE animal.

Thus good correlation was observed between the severity of test material effects on body weight gain and the response of lung tissue to the administration of specific test substances. It cannot be determined at this time whether the hyperplasia, metaplasia and other pathologic findings induced by test material exposure are recoverable in nature or whether they are indicative of future deleterious processes.

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CARCINOGENICITY OF EXTRACTS OF DIESEL AND RELATED
ENVIRONMENTAL EMISSIONS UPON LUNG TUMOR INDUCTION IN
STRAIN 'A' MICE

by

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INTRODUCTION

The predicted increase in the use of diesel engines has resulted in a regulatory need for data assessing the relative carcinogenicity of diesel exhaust. Since cigarette smoke, roofing tar and coke oven emissions have been shown to be carcinogenic, a matrix of experiments was designed to compare the relative potency of these pollutants with diesel exhaust particulate. The present study is one of several, including skin painting of Sencar mice, intratracheal instillation in hamsters and in vitro testing, designed to provide such a comparison.

METHODS

Compounds

Nissan diesel particulate matter was collected with a high volume sampler using Pallflex T60A20 (teflon coated) filters. The samples were collected from a large mixing chamber containing exhaust diluted with about 9 parts clean air to produce a particulate matter concentration of 12 mg/m^3. Exhaust was produced with a 6 cylinder, 90 cubic inch displacement Nissan diesel engine run on the Federal Short Cycle. For details see Hinners et al (1979). The Oldsmobile sample differed from the Nissan sample in that it was generated at a steady state (40 mph). Both samples were Soxhlet extracted with dichloromethane. Cigarette smoke condensate (CSC) was supplied by the Chemical Repository and Tobacco Smoke Chemistry Division of the Tobacco and Health Research Institute, University of

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Kentucky. The CSC was produced from Kentucky Reference 2RI Cigarettes. For details of collection and generation see Patel (1977). The coke oven emissions were collected using massive volume samplers from the top of a coke oven battery at Republic Steel in Godsden, Alabama. The roofing tar emissions were collected using a baghouse filter fitted with special nonreacting filter bags. Details of collection procedures for both coke oven and roofing tar have been described in detail by Huisingh et al (1979). Most samples were dissolved in DMSO: for the Nissan particulate matter 5% of the DMSO solution was EL620.

Animals Used and Experimental Design

Strain A/Jax mice approximately 8 weeks of age, were randomly assigned to 9 groups: 1) uninjected controls, 2) vehicle controls, 3) positive controls, 4) Nissan generated particulate matter, 5) Nissan particulate extract, 6) Oldsmobile particulate extract, 7) cigarette smoke condensate, 8) coke oven emissions, and 9) roofing tar. The doses for each group are listed in Table 1. Because of limited availability of Oldsmobile extract only males were injected. Due to high mortality rates among mice injected with Nissan particulate the dose level was halved in the second experiment. The experiment was carried out in 2 parts because of the limited availability of animals and manpower. The mice were injected 3 times weekly for 8 weeks with the test substances. The injection volume was 50 microliters, delivered by Hamilton syringes fitted with 26 G needles. The urethane positive controls received only one injection as the start of the experiment.

Collection and Analysis of Data

The mice were sacrificed at 9 months of age with an overdose of nembutal. The lungs were removed and placed in buffered formalin. After 2 weeks the lobes were detached from the bronchi and the number of adenomas visible on the surface counted. Questionable areas were examined micro-scopically. Analysis of variance compared the number of tumors per mouse among groups; Chi Square analysis compared the frequency of mice with tumors.

RESULTS

From the data presented in Table 1 it is clear that a significant increase in number of lung adenomas per mouse and percent of mice with tumors occurred in the positive controls (urethane injected). In experiment 1 a significant increase in number of tumors per mouse was noted in males injected with Nissan diesel extract compared with controls or those injected with Oldsmobile extract. In experiment 2, a significant increase in lung tumor rates was detected in females injected with coke oven emissions. After combining the data from both experiments, no statistically significant differences were noted, except for the positive controls.

DISCUSSION

There were no consistent, statistically significant differences between the exposed and control groups with respect to either number of adenomas per mouse or number of mice with adenomas. The response to urethane was equivalent to the numbers predicted from numerous previous studies indicating that the animals were sensitive to tumor induction (Shimkin and Stoner, 1975). It was concluded that either the carcinogens present in the test substances were very weak or that an insufficient concentration reached the lungs to produce a positive result.

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Table 1 - Strain A Mouse Data for Induction of
Adenomas by Environmental Mixtures

Group	Sex	Percent of Mice with Tumors	Av. Number of Tumors per Mouse	Percent Surviving	Dose
Uninjected	M	40	0.6 ± 0.2	75	-
Controls	F	47	0.6 ± 0.2	85	-
DMSO +	M	40	0.9 ± 0.5	50	0.05 ml/injection
5% EL620	F	47	0.7 ± 0.2	75	
Urethane	M	100[A]	22.5 ± 1.9[A]	75	20 mg/mouse
	F	100[A]	21.8 ± 1.5[A]	85	
Nissan Diesel	M	40	0.4 ± 0.1	33	4 mg/injection
Particulate	F	20	0.5 ± 0.1	33	
Nissan Diesel	M	73	1.4 ± 0.3[B]	50	1 mg/injection
Extract	F	55	1.0 ± 0.3	67	
Olds. Diesel	M	37	0.4 ± 0.1	63	1 mg/injection
Extract	F	-	-	-	
Cigarette Smoke	M	59	0.8 ± 0.3	73	0.20 mg/injection
Condensate	F	54	1.1 ± 0.2	80	
Coke Oven	M	54	1.2 ± 0.3	87	0.02 mg/injection
	F	31	0.5 ± 0.2	87	
Roofing	M	41	0.7 ± 0.2	73	0.02 mg/injection
Tar	F	44	0.7 ± 0.3	47	

Experiment 1

Group	Sex	Percent of Mice with Tumors	Av. Number of Tumors per Mouse	Percent Surviving	Dose
Injected	M	13	0.2 ± 0.1	100	-
Controls	F	26	0.4 ± 0.2	100	-
DMSO +	M	44	0.5 ± 0.2	80	0.05 ml/injection
5% EL620	F	26	0.3 ± 0.1	95	
Urethane	M	95[A]	7.3 ± 0.7[A]	100	10 mg/mouse
	F	100[A]	11.3 ± 0.9[A]	96	
Nissan Diesel	M	27	0.3 ± 0.1	68	2 mg/injection
Particulate	F	32	0.3 ± 0.1	42	
Nissan Diesel	M	33	0.4 ± 0.1	60	1 mg/injection
Extract	F	50	0.7 ± 0.1	71	
Olds. Diesel	M	33	0.4 ± 0.1	69	1 mg/injection
Extract	F	-	-	-	
Cigarette Smoke	M	30	0.4 ± 0.1	77	0.20 mg/injection
Condensate	F	21	0.2 ± 0.1	80	
Coke Oven	M	31	0.4 ± 0.1	83	0.02 mg/injection
	F	65	0.9 ± 0.2[A]	89	
Roofing	M	44	0.7 ± 0.2	91	0.02 mg/injection
Tar	F	22	0.3 ± 0.1	91	

Experiment 2

[A] Significantly different from uninjected and injected controls ($p < 0.05$).

[B] Significantly different from uninjected controls and Olds. diesel extract ($p < 0.05$).

THE INFLUENCE OF INHALED DIESEL ENGINE EMISSIONS UPON LUNG TUMOR INDUCTION IN STRAIN 'A' MICE

by

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The Strain 'A' mouse was one of several animal models chosen by the Environmental Protection Agency to assess the carcinogenic risk of exposure to diesel engine emissions. The Strain 'A' mouse was selected because: it has been one of the most extensively used models for assessment of lung tumor induction; the test is well validated; exposure times are fairly short for a cancer assay; the test is relatively straight forward to perform and evaluate and finally; it is one of the most sensitive lung tumor bioassays available (1). The results presented are from experiments in which mice were exposed to exhaust with a particulate concentration of 12 mg/m^3. These studies are a continuation of earlier work in which exposure levels were about one half those used in the present experiments (2).

Details of the exposure conditions and experimental procedures have been published previously (2, 3). Briefly, Strain 'A' mice obtained from Jackson or Strong Laboratories were exposed 8 hrs/day, 7 days/week from 6 weeks to either 9 or 12 months of age. The mice were housed in wire cages and exposed in 100 cubic feet stainless steel chambers. Exhaust was produced by a 6 cylinder, 90 cu inch Nissan diesel engine. In order to simulate city driving conditions, the engine load and speed were varied cyclically using the Federal Short cycle. After completion of exposure, the animals were sacrificed and the lungs fixed and observed for the presence of pulmonary adenomas.

Three experiments were carried out. In the first, 360 animals, 180 of each sex, were exposed to clean air or diesel exhaust. One half of each group received a single intraperitoneal injection of 5 mg urethane prior to the start of exhaust exposure. In the second study, 115 males and 143 females were exposed to diesel exhaust, while 108 males and 142 females were

exposed to clean air. Conditions differed from that of the first study in that the exposure occurred during the dark portion of the daily cycle when the animals were presumably awake, active and respiring at a greater level. In the final study in which only males were used, the mice were sacrificed at 12 months instead of 9 months of age. The number of mice with tumors were compared using Chi Square testing. Results are shown in Table 1. Exposure of mice to diesel exhaust until 9 months of age resulted in a significant decrease in the mean tumor incidence in females, and in males and females combined (P <.05). Decreases were also noted in males, but differences were not significant. In comparing groups treated with an initiating dose of 5 mg urethane prior to exposure, a decreased tumor incidence was again noted in the exhaust exposed mice, (P < .10) for males, (P < .001) for females, and (P < .001) for males and females combined. Exposure to diesel exhaust during the dark portion of the daily cycle also resulted in a decreased tumor incidence compared with clean air controls (P < .01) for males, (P< .001) for females, and (P< .001) for males and females combined. Finally, exposure of males to diesel exhaust until 12 months of age again resulted in a decrease in lung tumor incidence (P < .01).

Overall, there was no indication that exposure of Strain 'A' mice to diesel engine emissions resulted in an increase in lung tumor incidence. On the contrary, the studies consistently showed that tumor rates were decreased in exhaust exposed mice. Such a decrease following exposure to a potentially carcinogenic pollutant is rare, but is not unknown. Nettesheim et al., (4) reported that inhalation of a combination of ozonized gasoline and ferric oxide particles inhibited the tumorigenic effects of injected diethylnitrosamine on the respiratory tract. Kotin and Folk (5) also showed that exposure of C57BL mice for a lifetime to an atmosphere of ozonized gasoline resulted in a significantly lower incidence of malignant lymphomas and hepatomas compared to mice breathing clean air. Finally, Pereira (6) found fewer gamma glutamyl transpeptidase positive liver islands in rats following exposure to diesel exhaust than in clean air controls.

An explanation of the present results must await further study. Possibly, diesel exhaust inhalation inhibits the induction of enzymes responsible for converting procarcinogens to their active forms. The immunocompetence of the animals could also have been altered as a result of the inflammatory reaction to deposited exhaust particulate. The results cannot be explained by increased mortality of mice susceptible to tumor induction since survival rates were not significantly altered by the exposure positions.

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Table 1.   Effects of Inhaled Diesel Engine Emissions Upon Lung Tumor
Incidence in Strain 'A' Mice

Treatment	Age at Sacrifice (Months)	Illumination During Exposure	Sex	Number of Mice with Tumors / Number of Surviving Mice	P Values
Clean Air	9	Light	M	10/44	
			F	11/43	
			M+F	21/87	
Diesel Exhaust	9	Light	M	5/37	NS
			F	4/43	< .05
			M+F	9/80	< .05
Clean Air + 5 mg Urethane	9	Light	M	32/38	
			F	34/37	
			M+F	66/75	
Diesel Exhaust + 5 mg Urethane	9	Light	M	26/39	< .10
			F	16/36	< .001
			M+F	42/75	< .001
Clean Air	12	Light	M	22/38	
Diesel Exhaust	12	Light	M	11/44	< .01
Clean Air	9	Dark	M	28/97	
			F	31/140	
			M+F	59/237	
Diesel Exhaust	9	Dark	M	13/111	< .01
			F	9/139	< .001
			M+F	22/250	< .001

OBJECTIVES AND EXPERIMENTAL CONDITIONS OF A VW/AUDI DIESEL ENGINE EXHAUST INHALATION STUDY

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INTRODUCTION AND METHODS

It has been known for some time that exhaust emissions from passenger car engines contain polycyclic aromatic hydrocarbons (PAH) a number of which has been shown to be carcinogenic in various animal experiments. However, it has not been possible to date to adequately explore the extent to which inhalation of automobile exhaust may increase the risk of cancer in human beings. This situation presents a particular problem in so far as automobile exhaust, in comparison to many other emissions from combustion processes, contains only a relatively small amount of PAH but is closer to the man in the street. So the actual impact on human health is difficult to evaluate.

In the absence of well defined exposure groups and control populations,it cannot be expected that epidemiological studies will provide reliable conclusions as to whether a causal relationship or, even more remote, a dose-effect relationship does indeed exist between automobile exhaust and lung cancer. On the other hand, long-term experimental exposure of human beings would be impossible for purely ethical reasons.

However, i t seems to be possible to devise an adequate experimental inhalation study on laboratory animals where precisely defined exposure conditions may be maintained over a period of years so that pathological reactions of the test animals can be correlated with exposure time and exhaust concentration level. The extrapolation of the results of such animal experiments to human beings, may be limited, and conclusions may be confined to qualitative statements, but, to a limited degree an assessment may be possible provided the experiment is closely tuned to the problem to be investigated. It is easy to realize that negative findings in a

carcinogenicity study of exhaust emissions on a limited number of
rats, hamsters and mice cannot be definitely conclusive for a
population of human beings, numbering in the millions, that are
exposed to exhaust emissions. This drawback applies even to
studies involving several hundred experimental animals, because
the amount of carcinogens inhaled is relatively small and the
available latency period of about 2 years is relatively short. The
use of higher concentrations of exhaust in an animal experiment is
only to a limited degree capable of compensating for the
substantially higher total exposure time and latency period in
human beings, because there are certain limits to an increase of
exposure concentrations. For instance, the concentrations in
animal experiments must remain sufficiently low, in order to
avoid acute toxic effects that might substantially reduce the
life expectancy of the test animals. In addition, the very complex
ambient environment to which man is exposed may modify the effect
of inhaled automobile exhaust, and this is not taken into account
in animal inhalation experiments that simply make use of diluted
exhaust emissions.

The purpose of this inhalation study is to test the emissions
of a VW Diesel engine in an experiment which is designed to provid
more detailed results than in case of a straightforward in-
vestigation merely on the inhalation of dilute exhaust. In this
study, which is financed by the Volkswagen Company, the emissions
are inhaled by laboratory animals which have been pretreated with
various carcinogens in order to induce an elevated basic tumor
incidence rate in the respiratory tract. The probability of
observing a statistically significant syncarcinogenic effect by
inhalationexposure to Diesel exhaust appears to be substantially
greater, if the change in tumor incidence rate occurs within the
steep slope of the sigmoidal dose response curve. This is to be
achieved by establishing an enhanced basic tumor incidence rate in
the laboratory animals. Along the flat and linear initial section
of the curve of the dose-response relationship, it is obviously
necessary to bring about a relatively large change in dosage in
order to obtain a statistically significant additional effect.
However, in the steep slope of this curve a substantially smaller
increase in dosage would be sufficient. This may possibly be the

case for the range of concentrations feasible in our experimental
studies of exhaust emissions. Initial results obtained by using
this experimental animal model are described elsewhere in these
proceedings.

The aim of the VW project is to extend the existing results of
the new animal model beyond the scope of a pilot study and provide
a firm data base. In addition, it is intended to test the
reproducibility of the data.

FACILITIES

Inhalation laboratories and exposure chamber

The inhalation study on the exhaust emissions of the VW Diesel
engine is presently in progress at our new facilities for
environmental hygiene and inhalation toxicology, which is part of
the Fraunhofer Institute of Toxicology and Aerosol Research in
Hannover, West Germany. Special laboratories are now available
which permit long-term inhalation studies designed for about 4ooo
rats, hamsters and mice to be conducted under barrier conditions.

The only access route for the personnel to enter this area is
via special lock chambers with shower units. Supplies and all other
materials needed can be brought into the restricted area through
large-capacity autoclaves and peracetic acid or formaldehyde lock
chambers, which are integrated into the wall shown on the slide.
The SPF animals intended to be used in this experiment are brought
into the barrier area by a special animal entry unit which can be
hooked to the lock chamber wall.

The stainless steel inhalation chambers have a volume of about
12 m^3. They are integrated into the inhalation laboratory design
and are horizontally ventilated by the dilute emissions.
Depending on the animal species, the chambers will house 3oo to
6oo animals.
Hamsters and rats are kept individually, and mice are held in pairs
in stainless steel wire cages. A uniform distribution of the
horizontal flow of the exposure aerosol in the inhalation chambers
is established by the use of special baffles and perforated plates
covering the walls of the inlet and outlet ducts.
The bottom panel of the door frames of the two-wing chamberdoors
swing up. This allows a smooth insertion and withdrawal of the

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cage racks into and out of the chambers.

The inhalation laboratory is hermetically sealed off from the area for the measuring equipment behind the chambers. This technical section is not incorporated into the barrier area.
The ceiling and the rear wall of the chambers are part of the hermetic seal separating the animal area from the measuring equipment. This division of space permits us to take measurements in the inhalation chambers without the inconvenience that the technical personnel and their instruments have to enter the barrier area housing the animals. The air pressure outside the exposure chambers is adjusted so that the inhalation chambers have a slightly lower pressure than the room housing the animals and a slightly higher pressure than the measuring station.

Above the space for animal handling there is a working stage accessible from outside where additional supply and exhaust lines for emissions and clean air are installed. Each of the i inhalation chambers used in this experiment may be connected to its own mixing box located directly above the inhalation chamber. It facilitates the adjustment to any desired dilution of the exposure aerosol.

Engine bench

The exhaust emissions are produced by a VW Diesel engine connected by an automatic transmission to a fly wheel with an eddy-current brake. The engine is continuously computer controlled to simulate the US-72 Federal Test Procedure Cycle.

EXPERIMENTAL PROGRAMME

As in our pilot study[1], the total exhaust emissions and the emissions after removal of the particles are investigated in a long-term study. The experimental animals are exposed for about 18 hours/day and 5 days/week.

A preliminary experiment is scheduled to begin this month. It is designed to reveal the subchronic effects of two or three exhaust dilutions containing about 4, 8 or 16 mg of particles/m^3 for the three species of animals. After an exposure period of 2 - 3 months, a series of tests on clinical chemistry and hematology will be conducted as well as an investigation of some lung lavage liquids. Furthermore, tests on pulmonary function and histopathology will be made at that time.

The subsequent long-term study is to be conducted with an appropriate dilution of the exhaust emissions so that, on one hand, it has as high a particle content as possible, but on the other hand, it does not substantially reduce the natural life span of the experimental animals. This particular dilution of exhaust emissions will be determined when the results of the preliminary experiment are available.

The long-term study will be conducted with a total of 39 test groups consisting of 96 animals each (Fig.1)

TESTGROUP	TOTAL EXHAUST	EXH. WITHOUT PARTICLES	CLEAN AIR
HAMSTER	96	96	96
HAMSTER + DEN, S.C.	96	96	96
HAMSTER + B(A)P, I.TR.	96	96	96
HAMSTER *	96	96	96
RAT	96	96	96
RAT + DPN, S.C.	96	96	9C
RAT *	96	96	96
MOUSE	96	96	96
MOUSE + B(A)P, S.C. 1. DOSE	96	96	96
MOUSE + B(A)P, S.C. 2. DOSE	96	96	96
MOUSE + B(A)P, I.TR. 1. DOSE	96	96	96
MOUSE + B(A)P, I.TR. 2. DOSE	96	96	96
MOUSE *	96	96	96

DEN : DIETHYLNITROSAMINE
B(A)P: BENZO(A)PYRENE
DPN : DIPENTYLNITROSAMINE
 * : FOR BIOCHEMICAL AND PHYSIOLOGICAL MEASUREMENTS
S.C. : SUBCUTANEOUS
I.TR.: INTRATRACHEAL

Fig. 1. Number of animals in the different exposure groups with and without additional treatment.

Of all three species of animals, 192 of each will be exposed to the three types of atmosphere: that is total exhaust emissions, exhaust emissions without particles, and clean air. One half of each group will be used to obtain a series of measurements on clinical chemistry, hematology and physiology while the other half will remain exclusively for the carcinogenicity investigation following exposure to diluted exhaust emissions alone.

According to the design of our new animal test model, a number
of the animal groups will be treated with a known carcinogen in
addition to the exhaust exposure. This will induce a specific basic
tumor incidence rate in the respiratory tract. In case of the
hamsters, the tumors are induced by subcutaneous injection of
diethylnitrosamine (DEN) or intratracheal instillation of benzo(a)-
pyrene (B(a)P). In the rats, this is achieved by subcutaneous
injection of dipentylnitrosamine (DPN) and in the mice by sub-
cutaneous injection or intratracheal instillation of B(a)P. A
systemic carcinogenic effect after subcutaneous injection of B(a)P
in newly born mice with the lung as the primary target organ is
well documented. The latency period in this case is only about 6
months.[2]

The purpose of the increased basic tumor rates in the
respiratory tract of three animals species induced by various
methods is to reveal as to whether a syncarcinogenic or co-
carcinogenic effect of Diesel exhaust, as it was observed in our
pilot study, can be reproduced on a larger scale. Furthermore, the
study should show whether the same effect can also be observed in
the other animals species and under the influence of other
carcinogens. Finally, the question is to be answered whether the
additional effect is related to carcinogenic properties of the ex-
posure aerosol or can be brought about also by non-carcinogenic
inhalation burdens. This problem will be investigated in connection
with concurrent studies on the inhalation of gasoline engine exhaust
and the effluents of domestic coal-burning stoves.

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SECTION 7

EXPOSURE AND RISK ASSESSMENT

POTENTIAL HEALTH RISKS FROM INCREASED USE OF DIESEL LIGHT DUTY VEHICLES

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INTRODUCTION

The potential health risks for people who may be exposed to increased levels of diesel light duty vehicle emissions in the future are generally expected to be similar to the risks from exposures to other combustion products in the atmosphere. They include risks of developing respiratory functional diseases, cancers of the respiratory tract and cancers of other organs. Extensive toxicology research programs are currently attempting to determine if light duty diesel vehicle emissions have physical or chemical properties that would make them significantly more toxic than other combustion products in the environment. To date, however, no unique compounds have been identified in diesel emissions that add substantial new concerns to those already raised by existing levels of these pollutants in urban air. Therefore, the goal of this report is to identify the potential contribution of future light duty diesel vehicle emissions to existing atmospheric pollutant levels and to estimate an upper limit for their potential health risks.

DIESEL VEHICLE MARKET FORECAST

The projected increased use of diesel light duty vehicles was stimulated by the Federal Corporate Average Fuel Economy requirement for 1985.[1] By 1985 manufacturers of light duty vehicles must attain an overall fleet average of 27.5 miles per gallon of fuel. Vehicles equipped with diesel engines currently achieve more miles per gallon of fuel than those with gasoline engines because of their higher efficiencies and because diesel fuel contains about 15% more energy than an equal volume of gasoline. Diesel vehicles have also been popular with consumers because diesel fuel costs have traditionally been less than those for gasoline.

In the future, however, increased demand for diesel fuel or other middle distillate products including heating oil could raise its cost relative to gasoline. Current refinery processes produce about twice as much gasoline per barrel of crude petroleum than diesel fuel. Significant changes in refinery

processes aimed at producing more diesel fuel relative to gasoline are likely to result in cost penalties.[2] The efficiency of diesel engines is also likely to be affected by the application of emission control technology. Between 1965 and 1975 emission control technologies reduced the efficiency of gasoline engine vehicles by about 25%.[3] Similar losses of efficiency may occur with diesel vehicles as emission control devices are added. However, new engineering advances and reductions in vehicle sizes are currently improving fuel efficiency.

In addition to fuel economy, fuel supply and vehicle costs, other factors will influence future use of diesel light duty vehicles. These include consumer experiences with routine maintenance, frequency of repairs and convenience of operation. All of these factors will influence the market forecast, but because adequate information is not available, we have assumed that diesel fuel supply limitations will restrict diesel light duty vehicle use to 20% of the total light duty vehicle fleet.

After 1995, the total annual distance traveled by light duty vehicles in the United States has been projected to be about 3×10^{12} km.[4] Therefore, diesel vehicles are expected to travel about 6×10^{11} km per year.

EMISSIONS FROM DIESEL VEHICLES AND OTHER SOURCES

Diesel vehicle exhaust contains five catagories of pollutants for which national air quality standards have been promulgated. These include total suspended particles, sulfur oxides, nitrogen oxides, hydrocarbons and carbon monoxide. A summary of these emissions as measured in the exhaust of current light duty diesel vehicles is given in Table 1. The ranges of diesel vehicle emission rates include both small and large automobiles that were driven on a variety of test cycles. The total projected emissions for the entire diesel light duty vehicle fleet after 1995 are also given. Values for the projected fleet emissions given in parentheses result from using the current proposed federal emissions standards applicable to light duty vehicles instead of the mid-ranges of the measured values. These values are given whenever adherence to the emissions standards would result in lower estimates than those projected from the measured values. Also listed in Table 1 are the current EPA estimates of emissions into the environment during 1977 from all diesel engines and from all area sources and point sources.[11] The projected diesel light duty vehicle emissions for 1995 are 20% to 60% of the current diesel engine emissions and they are less than 3% of the current emissions of these pollutants from all sources.

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TABLE 1

CURRENT AND PROJECTED EMISSIONS OF REGULATED POLLUTANTS FROM DIESEL VEHICLES
AND FROM ALL AREA AND POINT SOURCES IN THE UNITED STATES.[5-11]

	Diesel Light Duty Vehicles		EPA Estimated 1977 Emissions	
	Current Vehicle Emission Rates (g/km)	Projected Fleet Emissions: 1995 (thousand tons)	All Diesel Vehicles[a] (thousand tons)	All Sources (thousand tons)
Particles	0.1 -0.6 (0.12)[b]	200 (90)[b]	350	15000
Sulfur Oxides	0.01-0.5	100	430	30000
Nitrogen Oxides	0.5 -2.0 (0.62)	700 (350)	3500	25000
Hydrocarbons	0.1 -0.6 (0.25)	200 (150)	530	30000
Carbon Monoxide	0.3 -1.5 (2.10)	500	1900	110000

[a]Includes heavy duty vehicles, off-highway vehicles and railroad engines.
[b]Projected values assuming emission control advances are made to achieve
 conformance with proposed future federal emission standards.

Because the emissions of particles, vapors and gases from diesel light duty
vehicles are expected to be only a few percent of current emissions, they are
not likely to produce measurable changes in the levels of these pollutants in
the environment. Mathematical modeling studies of their dispersion in the
atmosphere also support this conclusion.[12] The modeling studies were done
with two computer simulation models; one was used to project typical urban
concentrations of photochemical reactant gases and the second model was used
to project diesel particle concentrations in cities of different sizes and
population densities.

The modeling studies of photochemical reactant gases were done by Joyce
Penner, Michael MacCracken and John Walton at the Lawrence-Livermore National
Laboratory using the LIRAQ computer model.[13] Pollution sources, topography
and weather patterns typical of the San Francisco Bay Area were used. When
20% of the light duty vehicle emission source term was changed to represent
diesel emissions, no significant changes were projected in the air
concentrations of nitrogen oxides, carbon monoxide, sulfur oxides, ozone or
hydrocarbons. These results even applied for simulated weather conditions
that favored production of high atmospheric oxidant levels.

Modeling studies of the atmospheric dispersion of diesel exhaust particles in urban environments were done with a computer model based upon Gaussian plume atmospheric dispersion characteristics that was extended to represent area sources.[12] Results of these studies, projected for 20% diesel light duty vehicles after 1995, indicated that the average concentration of diesel particles in U.S. cities would be about 0.2 $\mu g/m^3$. A histogram of the projected air concentrations of diesel particles for cities with more than 25,000 people is shown in Figure 1. These studies also projected that the largest cities would average about 2 $\mu g/m^3$. At the present time, air concentrations of particles in large cities average about 100 $\mu g/m^3$ so that the small projected increase of 2 $\mu g/m^3$ due to diesel particles is consistent with their small projected contribution to the total particle emissions in the United States as shown in Table 1.
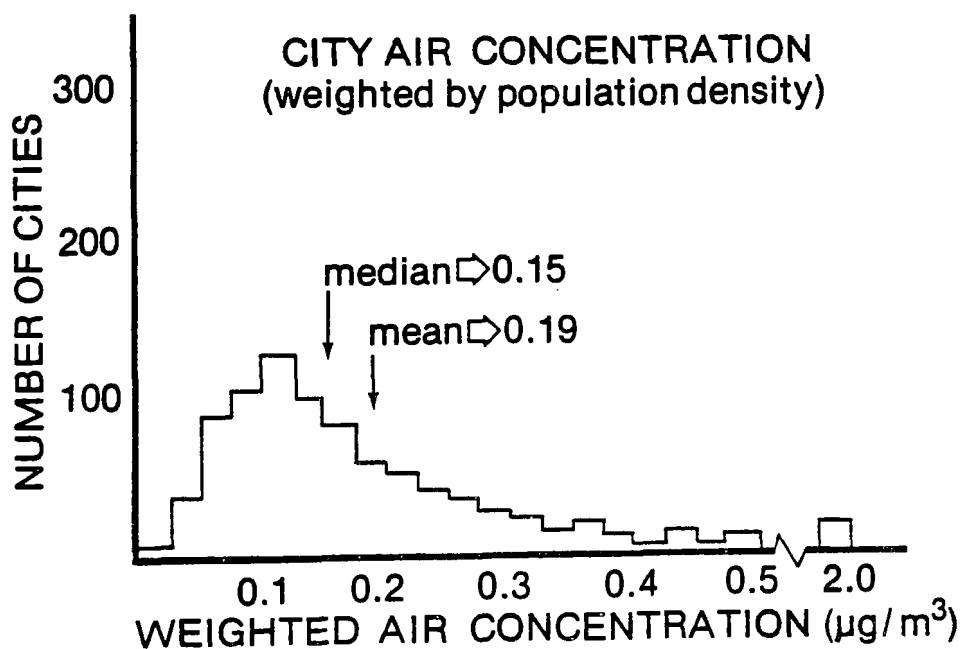
Fig. 1. Projected distribution of average air concentrations of particles from diesel light duty vehicles in cities having populations of more than 25,000.

The average concentrations of diesel particle emissions in urban environments projected from the computer modeling studies do not reflect the potential high concentrations that could occur in congested urban areas where there is restricted air circulation. Urban street canyons are known to have higher concentrations of automobile emissions than less congested areas, although few quantitative studies have been done to determine human exposure levels near central metropolitan streets. Some studies that have been done above streets in downtown Manhattan, Nashville, San Jose and St. Louis were summarized in a report of the U.S. Department of Transportation.[2] A mathematical relationship was also developed for projecting air concentrations of non-interacting pollutants based upon the measured relationships between levels of carbon monoxide in automobile exhaust and street canyon air concentrations. The overall model equation for projecting these air concentrations is the following:

$$\text{Pollutant Concentration } (\mu g/m^3) = \psi \times EF \times TC$$

where; ψ = pollutant concentration index calculated as the concentration in $\mu g/m^3$ divided by the product of vehicle emission rates (g/mi) and the traffic count (veh/hr),

EF = pollutant emission factor (g/mi),

TC = street canyon traffic count (veh/hr).

Empirical values for ψ at street level, in the units defined above, were between 0.04 and 0.06 for typical meteorological conditions and between 0.08 and 0.18 for unfavorable conditions. Assuming that diesel light duty vehicles will meet future emissions standards, the projected air concentrations of particles are those given in Table 2 for a vehicle traffic count of 2000 vehicles per hr.

Gasoline vehicles burning unleaded fuel have only about 1% of the particle emission rates of diesel vehicles, although, both types of vehicles emit similar amounts of nitrogen oxides and other gases. Having 20% light duty diesel vehicles is likely to raise current street canyon atmospheric particle concentrations from 100 $\mu g/m^3$ to 120 $\mu g/m^3$. If more than 20% diesel vehicles are used in urban transportation or if their particle emission rates are greater than 0.12 g/km, then they could add up to 100 $\mu g/m^3$ to the air in street canyons. Because diesel vehicles and gasoline engine vehicles emit similar amounts of nitrogen oxides and other gases, little effect of increased diesel vehicle utilization is likely to be observed in the levels of these pollutants.

TABLE 2

PROJECTED AIR CONCENTRATIONS OF PARTICLES IN URBAN STREET CANYONS AND PARKING
GARAGES OF MAJOR METROPOLITAN AREAS (ASSUMING 20% DIESEL LIGHT DUTY VEHICLES)

	Total Particles $\mu g/m^3$	Diesel Light Duty Vehicle Particles $\mu g/m^3$
Ambient Urban Background	100	2
Urban Street Canyon[a]		
Typical Meteorology		5
Unfavorable Meteorology		20
Urban Parking Garage[a]		30

[a]Urban background concentrations must be added to the projected street
canyon concentrations to project total concentrations at street level. These
totals may also provide the background air concentrations to be added to the
projected urban parking garage emissions.

Particle concentrations in urban garages were also modeled in the U.S.
Department of Transportation report.[2] Their model consisted of a simple box
configuration with vehicles as pollutant sources inside of the box. The
garage volume, ventilation rate and vehicle activity were the important model
parameters. Results of their studies indicated that for 20% diesel vehicles,
particle concentrations inside of garages could average 30 $\mu g/m^3$ above
that of outside air and during peak traffic, particle concentrations could
increase to 500 $\mu g/m^3$, Table 2.

ESTIMATION OF LUNG CANCER RISKS
Concern for the potential health risks to people exposed to diesel vehicle
emissions was stimulated by observations that diesel particle extracts were
mutagenic to bacteria cells grown in culture. Chemical analyses of diesel
particle samples revealed the presence of known carcinogenic compounds
including polycyclic aromatic hydrocarbons. However, previous studies of
London transit workers who were exposed to high levels of diesel bus emissions
between 1950 and 1974 failed to show any increased risk of developing lung
cancers or other health effects.[14] Unfortunately, the results of these
studies were confounded by the absence of smoking information in these

populations, the mobility of workers, their changing ethnic background and the lack of follow-up after retirement. To date, no direct observations of increased cancer risks in people exposed to diesel engine emissions are available. However, several very diverse attempts have been made to infer upper limits for these risks. These attempts have made use of bacteria and mammalian cell mutagenesis assays, skin painting studies and studies of inhaled or instilled diesel particles in laboratory animals. Many studies have attempted to determine the relative potency of diesel particle extracts as compared to other surrogate combustion products for which human health effects have been documented. These include cigarette smoke, coke oven emissions and atmospheric particulate pollution.

The Diesel Impacts Study Committee of the National Research Council recently completed a review of many of these studies and their review is an important source of biological effects information used in this cancer risk evaluation.[15] Much of the data summarized in the report describes studies of the mutagenic potential of diesel particle extracts as compared to benzo(a)pyrene, cigarette smoke condensate, coke oven emissions, roofing tar and gasoline engine particle extracts. Although the different in vitro test systems all provided a measure of the relative ability of these agents to transform cells genetically, no quantitative relationships can be developed from these studies alone that would predict their carcinogenic potential in human exposures. Quantitative relationships can not be developed because of the difficulties in extrapolating between effects on cells in vitro and human carcinogenesis. These include the following. Extracts of the particle samples were usually obtained with organic solvents for these tests. When biological fluids or surrogates were used for the extraction, the mutagenic activity of the test substance was markedly decreased. Therefore, mutagens associated with the particles may not be readily available for contact with lung cells after inhalation and deposition. Also, different test systems showed different measures of cell transforming ability for the extracts. Some of the mutagenesis tests with different substances depended upon activation by added biological enzymes, but others did not. The relative potency of some of the samples depended upon the types of engines and fuels that were used and upon the mechanism required for cell transformations.

The National Research Council report reviewed studies that used skin painting, inhalation or intratracheal instillation; many are still in progress. Results of the skin painting studies reported by Slaga et al.,[16] showed that chemical compounds, known to be in diesel particle extracts, are

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complete carcinogens. However, none of the inhalation studies reported to date have demonstrated that diesel emissions are carcinogenic to laboratory animals.

One conclusion that can be drawn from the National Research Council report is that diesel emissions have not been shown to be more mutagenic or carcinogenic than cigarette smoke or coke oven emissions on a unit particle mass basis. Diesel particle emissions were also shown to be less than 1% as mutagenic as benzo(a)pyrene. Although not discussed in the National Research Council report, another study has shown that soot collected from urban air was only about 2% as mutagenic as benzo(a)pyrene.[17] The urban soot, like diesel particles, contained both direct acting and indirect acting mutagenic compounds. Therefore, urban soot appears to be similar to diesel particle emissions in its mutagenic potency.

In the following evaluation of lung cancer risks in people exposed to diesel vehicle particle emissions, it is assumed that the concentrations of particles in rural and urban air, in air near coke ovens and in cigarette smoke can be used as an index of lung cancer risks in the exposed populations.[12] The information displayed in Figure 2 shows the reported annual lung cancer risks for groups of smokers and non-smokers living in rural and urban areas and for coke oven workers. It also indicates their exposure air concentrations of particles averaged over all of the air breathed by an individual. These were obtained by calculating the total amount of particles inhaled from cigarette smoke, urban air, rural air or air near coke ovens during one year and dividing by the total amount of air breathed in that year. Also shown in Figure 2 is the projected range of exposures to future light duty diesel vehicle emissions for people living in the United States.

The annual lung cancer risk for each population was divided by the average exposure air concentration to obtain the cancer risk factors shown in Table 3. Assuming that the exposures of people to airborne particles are reasonable indices of their lung cancer risks, the risk factors show that smokers have lower cancer incidences per unit mass inhaled than nonsmokers and coke oven workers. Although large quantities of particles are inhaled by cigarette smokers, they are more likely to deposit in the upper airways and to be cleared more quickly than the particles inhaled by nonsmokers and coke oven workers. For the purpose of estimating lung cancer risks for people exposed to diesel particles, an annual lung cancer risk factor of 150 cancers per 100,000 persons per mg/m^3 of air over the lifetime of individuals was taken as an upper estimate of the risk. Using this risk factor along with the air

concentration information shown in Figure 1 and urban population statistics
for the United States, we estimated that diesel light duty vehicle particle
exhaust is not likely to result in more than 30 lung cancers per year after
1995. For reference purposes, about 100,000 lung cancer deaths currently
occur in the United States each year. Thus, on the average, the lung cancer
risk is increased by 0.5% per $\mu g/m^3$ of diesel particles.
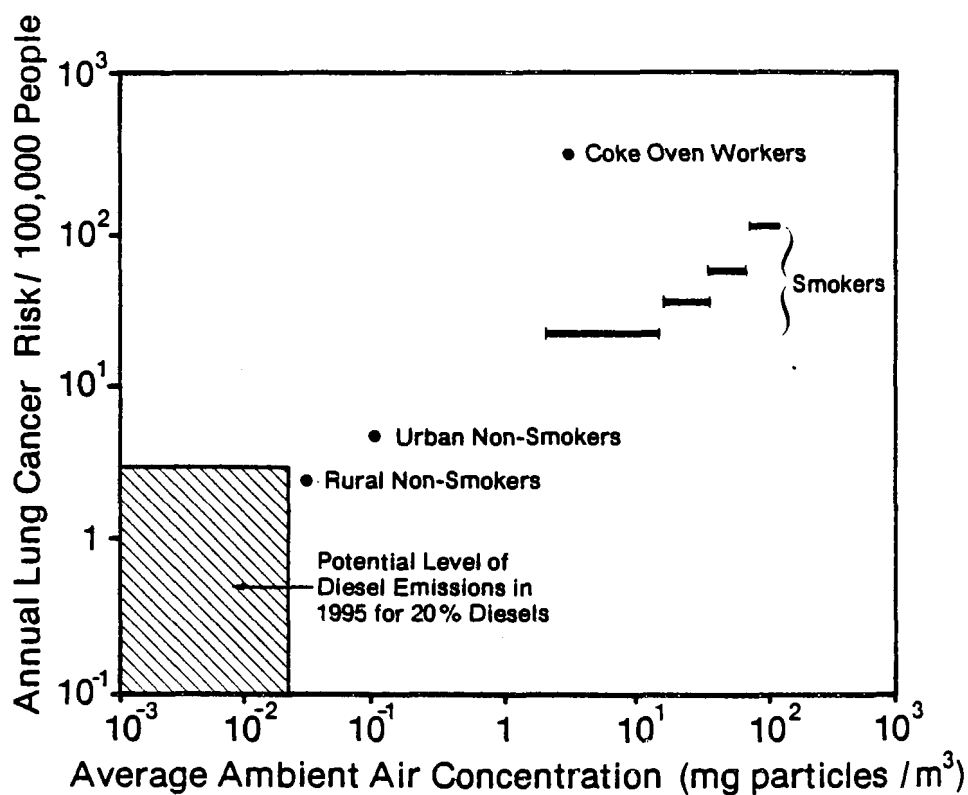
Fig. 2. Measured annual lung cancer risks for various populations compared to
average air concentrations of particles.

TABLE 3

SUMMARY OF POPULATION EXPOSURES TO AIRBORNE PARTICLES AND ANNUAL CANCER RISKS

Study Population	Average Air Concentration mg Particles/m^3	Annual Lung Cancer Risk Per 100,000 People	Annual Cancer Risk Per 100,000 People Per mg/m^3
Rural Nonsmokers	0.03[a]	3[b]	100
Urban Nonsmokers	0.1	7	70
Smokers (cigarettes/day)[c]			
1- 9	2-16	26	3
10-19	18-35	47	2
20-39	36-71	80	2
40 +			
Coke Oven Workers	3[d]	400[e]	130

[a]Bond[18]
[b]Haenszel et al.[19]
[c]Surgeon General[20], Hammond[21], Kahn[22]
[d]Smith et al.[23]
[e]Redmond et al.[24]

Two additional analyses of the carcinogenic risks from exposures to diesel emissions were reported during the past year. The first analysis was contained in a report by Harris to the Diesel Impacts Study Committee of the National Research Council.[25] This analysis was mainly based upon studies of London bus garage workers. Their lung cancer incidences were compared to other transit workers including engineers, bus drivers, conductors and subway motormen. Although the studies failed to show a definite increased lung cancer risk in the garage workers, Harris calculated an upper limit for their lung cancer risks based upon statistical considerations. The upper 95% confidence limit on the increased lung cancer risks was 1% per $\mu g/m^3$ particulate exposure.

A second study has been completed by DuMouchel and Harris[26] that estimates lung cancer risks from diesel emissions based upon the results of laboratory studies of mutagenesis and viral cell transformation produced by diesel particle extracts. The relative potency of the diesel particle extracts in these test cell systems was estimated as compared to roofing tar vapors and coke oven emissions. The results of epidemiology studies were used

to estimate the absolute lung cancer risks per unit of exposures to roofing tars and coke oven emissions. By this technique DuMouchel and Harris estimated the upper 95% confidence limit for exposures of people to diesel particles to be 1.8% increase in lung cancer risks per µg of particles/m^3 of air over the entire lifetime. As shown in Table 4, all of the estimates of lung cancer risk to people exposed to diesel particle emissions are reasonably similar.

The above calculations of lung cancer risks apply to the entire population of the United States. It is also important to consider the individual risks for people who might have unusually high exposures in or near city street canyons, such as police officers, utility workers and some office workers. Garage workers may also be exposed to high levels of diesel emissions in enclosed work areas. Using the models for street canyon pollutants, it appears that some of these people could be exposed to diesel exhaust particle concentrations on the order of 20 µg/m^3 of particles. The individual risk of lung cancer for various projected exposure levels is shown in Table 5. Although the added risks from dieseT emissions appear to be small for most of these groups, it should be noted that nonsmokers working or living near heavily polluted street canyons could double their risks of developing lung cancer.

TABLE 4

SUMMARY OF THE PROPORTIONAL INCREASED RISK OF LUNG CANCER

Data Sets Used	Proportional Increased Risk of Lung Cancer Per µg/m^3 Particulate	Reference
Cigarette Smokers, Urban Residents and Coke Oven Workers	0.5%[a]	Cuddihy, et al. [12]
London Garage Workers	1.0%[b]	Harris[25]
London Garage Workers, Roofers and Coke Oven Workers	1.8%[b]	DuMouchel and Harris[26]

[a]Largest estimate
[b]Upper 95% confidence limit

TABLE 5

COMPARISON OF INDIVIDUAL ANNUAL LUNG CANCER RISKS AND ADDITIONAL RISKS FROM DIESEL EXPOSURES FOR VARIOUS POPULATION GROUPS.

Population	Measured Annual Lung Cancer Risk (cancers/year)		Diesel Particle Concentration ($\mu g/m^3$)	Estimated Added Annual Risk from Diesel (cancers/year)
	Nonsmoker	Smoker[a]		
Street Canyon residents			20	$1\text{-}3 \times 10^{-5}$
Highest city average exposure	7×10^{-5}	8×10^{-4}	2	$1\text{-}3 \times 10^{-6}$
Average city exposure			0.2	$1\text{-}3 \times 10^{-7}$
Rural	3×10^{-5}		0.02	$1\text{-}3 \times 10^{-8}$

[a]Average U.S. smoker of 1 1/2 packs per day.

SCOPE OF RISK ANALYSIS

This analysis considered the potential lung cancer risk from increased exposure to diesel particles and projected a small increase in the risk for U.S. residents. It should be kept in mind that this risk estimate was based upon the assumption that future diesel vehicles will meet the proposed emission standard for particles of 0.12 g/km and that consumers will continue to maintain their vehicles to meet this standard. The current measured diesel vehicle particle emissions have often been several times this level. Should this proposed standard be exceeded in the future, the calculated risk would be proportionately higher.

The analysis did not directly consider the possible interactive effects between increased exposures to diesel exhaust particles and other occupational or environmental pollutants that could influence the risk of lung cancer. People who were included in the epidemiology studies that formed the basis of this analysis also had exposures to a multitude of pollutants in the environment, in homes and in workplaces. Therefore, this analysis assumes that their exposures are typical of future population exposures. Gaseous emissions of diesel vehicles are similar to those of gasoline engine vehicles, however, these could change with use of certain emissions control options that might increase emissions of oxides of nitrogen while decreasing particle emissions.

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It should also be kept in mind that the potential health risks associated with gaseous emissions alone may exceed the risk related to particulate emissions. This is especially true for respiratory function impairment. Most assessments of the health risks from gaseous emissions have assumed threshold values for the health-effect relationships with the threshold levels being of the same order of magnitude or higher than current ambient levels of these pollutants. Thus, the models do not provide a basis for predicting possible health risks from small incremental increases in emissions of exhaust gases.

SUMMARY

Diesel light duty vehicles are expected to comprise 20% of the total light duty vehicle fleet in the United States within 15 years. Their use should grow because of a perceived advantage over gasoline engine vehicles in fuel costs, but it will be limited by the capacity to produce sufficient diesel fuel economically. Chemical analyses of diesel emissions have identified potentially toxic gases including nitrogen oxides and carbon monoxide along with particles that contain known carcinogenic compounds including poly-aromatic hydrocarbons. Diesel particle extracts have been shown to be mutagenic to cells in culture, to cause cell transformations and to induce tumors in the skin of mice. Further studies have also shown that diesel particle extracts are not markedly different from extracts or condensates of cigarette smoke, coke oven emissions, or urban soot in their ability to cause these biological effects per unit mass.

Atmospheric concentrations of particles emitted by diesel vehicles were projected for major cities in the United States using a computerized atmospheric dispersion model. This model predicted that with 20% diesel light duty vehicles the average city particulate pollution levels would increase by about 0.2 $\mu g/m^3$ over existing levels. The largest cities would be expected to have an overall increase of 1 to 2 $\mu g/m^3$ but in urban street canyons and parking garages diesel particles may add 20 to 30 $\mu g/m^3$ to the existing particulate concentrations.

By using the results of previous epidemiological studies of smokers, coke oven workers and urban residents, we obtained an upper estimate of lung cancer risk that would be expected in people exposed to diesel exhaust particles. The risk estimator was taken to be 0.0015 cancers per year per mg/m^3 lifetime exposure to diesel particles. Combining this risk factor with projected future air concentrations of diesel particles in urban environments, we

estimated that less than 30 lung cancers per year could be related to the projected increased use of diesel light duty vehicles in the United States.

ACKNOWLEDGMENTS

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HEALTH EFFECTS OF EXPOSURE TO DIESEL FUMES AND DUST IN TWO TRONA MINES

by

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The industrial environment is often a useful situation in which to study the effect of health hazards, as workers usually receive higher exposures than does the general population. This is particularly so for miners exposed to diesel fumes underground, since the restriction on ventilation acts to concentrate the fumes. This study involves 700 workers engaged in Trona ($Na_2CO_3 \cdot NaH CO_3 \cdot 2H_2O$) mining. These miners were given chest radiographs, asked questions on chest symptoms, smoking and work history, and given spirometric tests. In addition, comprehensive industrial hygiene surveys were undertaken at the two mines which were studied. The data availabe from these surveys is being explored for dose-response relationships between health indices and measures of diesel engine-related pollutants. This paper reports on some preliminary results.

The two mines were quite similar in character. Although one had opened in 1949 and the other in 1967, they were similar in size and employed just over 100 diesel units underground each. Total dust levels were high in 1976 (13 mg/m^3), but NO_2 levels were low (0.1 ppm), probably because of the high ventilation velocities and generally low horsepower of the units. Diesels had been used underground for up to 10 years at the two mines.

About 680 white males were studied overall. Table 1 shows statistics of age and exposure. Although some workers had remained at work in the mines for many years, the predominant duration of exposure was low. This indicates a rapid turnover of workers; one cause of this may have been ill health arising from exposure to dust or diesel fumes. Mechanisms such as this can bias or obscure dose/response relationships in epidemiological studies.

In order to explore the possible effect of NO_2 on lung function, the data were analyzed separately by age group, first overall, and again with the omission of those with other dust exposure (359 workers). To do this, linear

least squares models were fitted to forced vital capacity (FVC), forced expiratory volume in one second (FEV_1) and flow at 50% of VC (FEF_{50}). In the older group, both with and without those with other exposure, lung function decline with age was unusually and significantly great in all smoking groups (-0.50 or worse, liters/yr for smokers). Despite this, no clear deleterious relationship between lung function and either dust of NO_2 exposure could be detected. In the young group, about the only variable to be significantly related to lung function was height.

Despite the superficially negative nature of these findings, it is believed that caution is necessary in the interpretation of these results. This is advised, not only because of the high rates of decline in lung function, but also because the short duration of tenure indicates the possibility of a powerful 'healthy worker' effect. Further analysis needs and will be undertaken on these data; this may show whether there is a problem in these two mines, and whether that problem is dust or diesel exhaust.

Table 1. Age and Exposure Statistics of Mine Workers

	Age < 25	(S.D.)	Age > 25	(S.D.)
Number	481			
Age (years)	38	(12)	22	(2)
Dust exposure (years)	5	(16)	2	(1)
Dust exposure (mg years/m^3)	74	(104)	24	(23)
Diesel exposure (years)	3	(2)	2	(1)
NO_2 exposure (ppm years)	0.4	(0.4)	0.2	(0.1)
Other dust exposure[a] (years)	6	(8)	1	(3)

[a]Principally in coal mining.

MUTAGENICITY AND CHEMICAL CHARACTERISTICS OF CARBONACEOUS PARTICULATE MATTER FROM VEHICLES ON THE ROAD

by

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Two experiments were conducted in the eastbound tunnel of the Allegheny Mountain Tunnel of the Pennsylvania Turnpike in 1979 to evaluate the bacterial mutagenicity of the organic solvent extracts of particulate emissions from heavy-duty Diesels and from (predominantly light-duty) gasoline-powered vehicles in highway operation. Filter samples (PTFE-Teflon-impregnated glass fiber and PTFE membrane) collected during periods dominated by Diesel traffic as well as periods dominated by gasoline-powered vehicles were Soxhlet-extracted with dichloromethane (CH_2Cl_2) followed by acetonitrile (CH_3CN). Concurrently collected ambient-air samples (in the ventilation intake fan rooms and at a tower on the mountaintop) were treated the same way in order to distinguish between vehicle and ambient contributions to the mutagenic activity of the tunnel samples, and also to compare mutagenic activity of vehicle and ambient particulate-matter extracts. Total tunnel air flow, traffic volume, and traffic composition were monitored to permit calculation of emission rates per unit distance driven, for Diesels and for gasoline-powered vehicles (e.g., Fig. 1).

Mutagenicity was determined by the *Salmonella typhimurium* plate incorporation assay [Ames test (1)] using several tester strains, with and without microsomal activation by S9 rat-liver homogenate. The number of revertant colonies per km travelled was calculated for each sampling run and plotted against traffic composition (e.g., Fig. 2) to obtain revertants/km averages for gasoline- and Diesel-powered vehicles separately. High performance liquid chromatography (HPLC) profiles were obtained on the CH_2Cl_2 and CH_3CN

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extracts. Gas-chromatographic (GC) molecular-weight distributions (retention-time distributions) were obtained on the CH_2Cl_2 extracts and resolved as above according to vehicle type.

The main findings are as follows:

(1) The Diesel-produced aerosol in the Allegheny Tunnel is similar to that encountered in dilution tubes, with respect to all criteria, viz., percentage extractable into CH_2Cl_2 (24 $\pm$ 3%), Ames mutagenicity in revertants per km travelled or revertants per μg of CH_2Cl_2-extracted material, HPLC fluorescence profile, and molecular-weight distribution.

(2) Expressed as revertants per μg of CH_2Cl_2-extracted material, the mutagenic activities of the Diesel-produced aerosol in the Allegheny Tunnel are of the same order of magnitude as the mutagenic activities of the ambient aerosol in the vicinity at Allegheny.

(3) Expressed as revertants per km travelled, the mutagenicity of the CH_2Cl_2 extract of the particulate emissions from Diesels is several times that from gasoline-powered vehicles.

Some of the mutagenicity results are summarized in Tables 1 and 2.

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Table 1. Mutagenicities, Thousands of TA98 Revertants per Kilometer Travelled; CH_2Cl_2 Extracts, Allegheny Mountain Tunnel 1979.

		Gasoline-powered Vehicles	Diesel Trucks (a)
Without S9	May/June	39 ± 24[b]	211 ± 113
	Aug/Sept	19 ± 10	80 ± 20
With S9	May/June	26 ± 14	181 ± 40
	Aug/Sept	12 ± 4	51 ± 7

[a]Average gross weight approximately 35 tons.

[b]Error quoted is the standard deviation.

Table 2. Mutagenicities, TA98 Revertants per Microgram of CH_2Cl_2-extracted Material, Allegheny Mountain Tunnel 1979.

		Vehicles			Ambient Air	
		Gasoline-powered	Diesel Trucks	Over-all	Fan Rooms	Tower
Without S9	May/June	3 ± 2	1.1 ± 0.6	1.3	0.9	-
	Aug/Sept	4 ± 3	0.4 ± 0.1	0.6	0.6	0.2
With S9	May/June	2 ± 1	0.9 ± 0.2	1.0	0.4	-
	Aug/Sept	2.4 ± 1.6	0.27 ± 0.04	0.4	0.4	0.08
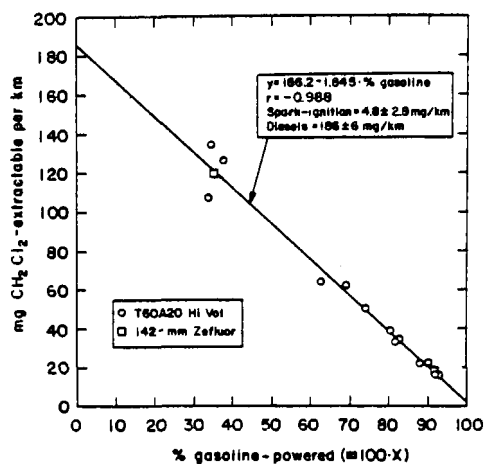
Figure 1. Plot of mg/km emission rate of CH_2Cl_2-extractable particulate matter vs. traffic composition, Allegheny Mountain Tunnel August/September 1979. Intercept at 0% gasoline-powered vehicles is the emission rate (186$\pm$ mg/km) from Diesels.
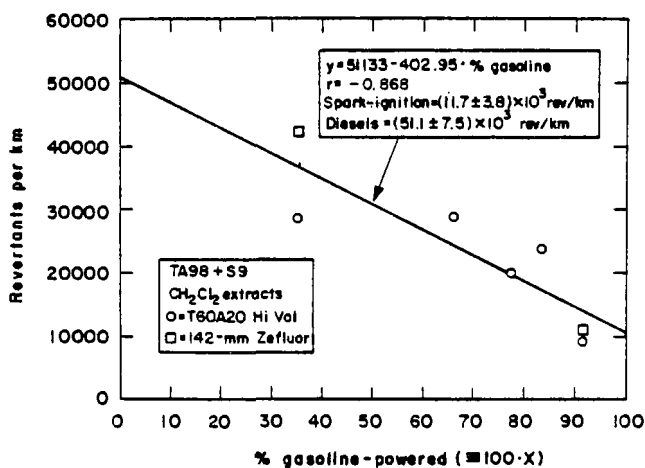
Figure 2. Plot of revertants/km vs. traffic composition, CH_2Cl_2 extracts, tester strain TA98 with microsomal activation (+S9), Allegheny Mountain Tunnel August/September 1979.

EMISSIONS OF GASES AND PARTICULATES FROM DIESEL TRUCKS ON THE ROAD (2)

by

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(A) Experiments have been conducted to estimate the diesel trucks emission rates at the Nihonzaka Tunnel. The Tunnel is a 4-lane dual tunnel (2 eastbound lanes through one tube, 2 westbound lanes through the other), 2 km long, on a slight grade upward (2.5%, 1.2 km), then downward (-1.84%, 0.8 km toward the east), located 170 km west of Tokyo. The vehicle traffic in the tunnel is very high with an average 1,107 - 1,148 cars/h; the percent of diesel trucks is 30 - 85%. Magnetic counts of eastbound traffic are by car length of 6 ± 0.5 m. (Over 6 m is almost diesel-truck.) The 90% of the diesel trucks is 6.5 - 22 t car weight. The 53% is 10 - 22 t. Intake fans above each of the tunnels force ventilation air into the tunnel through overhead louvers at 303 m^3/s. Air is drawn in also through the vehicle entrance portal by the ramming action of the traffic. All of the air leaves via the vehicle exit portal, at volumes averaging 380 m^3/s. Truck speed was 80 km/h. Sulphur content of fuel oil was 0.4%. Measurement procedures are almost similar to the Allegheny tunnel study by William R. Pierson and Wanda W. Brachaczek (1). The preliminary study was done in 1972; the present study was started in 1979, and will continue to 1981.

(B) Average emissions rates of diesel truck were found as in Table 1 and Table 2. Particulates are ~0.03 μm by electronmicroscope.

(C) The overall sulphur dioxide conversion to sulphate in the Tunnel was 2% (1980), 3% (1979). The measurements of the ambient are on the way. Sulfuric acid particulates of 2 - 10 micron spheres were observed on the thymol blue dye coated films exposed in the ambient 40 meters distant from the Tunnel portal, when relative humidity was +90%.

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Table 1. Diesel Gaseous Emission Rates on the Road (g/km)[a]

Year	1980-Oct.	1979-Oct.
NO	6.42 ± 9.7%	5.02 ± 7.4%
NO_2	0.62 ± 15 %	0.77 ± 6.5%
NO_x	7.03 ± 8.8%	5.79 ± 7.3%
CO	5.04 ± 17 %	--
SO_2	1.27 ± 20 %	1.14 + 27 %
T-HC	1.73 ± 9.7%	--
CH_4	1.08 ± 9.2%	--
NM-HC	0.63 ± 17 %	--

[a]In 1980, numbers of measurements: n=36.
In case of SO_2 measurement, n=12. Pearson's correlation coefficient, $p < 0.05$.
In 1979, n=24. In case of SO_2 measurement, n=8, $p < 0.05$.

Table 2. Diesel Particulates Emission Rates on the Road (g/km)

Year	1980-Oct.	1979-Oct.
Total particulates[a]	1.03 ± 4.9%	0.92 ± 5.4%
Sulphate particulates	0.041 ± 25 %	0.051 ± 19 %
Nitrate particulates	0.003 ± 37 %	0.003 ± 15 %
Ammoniate particulates	0.005 ± 44 %	0.004 ± 33 %

[a]Particulates of under -10 μm were measured by high-volume air sampler.
In 1980, numbers of measurement: n=36. Pearson's correlation coefficient,
$p < 0.05$.
In 1979, numbers of measurement: n=24, $p < 0.05$.

DIESEL BUS TERMINAL STUDY
EFFECTS OF DIESEL EMISSIONS ON AIR POLLUTANT LEVELS

BY

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INTRODUCTION

The New York City Bus Terminal Diesel Study was designed to measure, collect, chemically characterize, and bioassay diesel exhaust as it exists after becoming resident in the ambient atmosphere. Concentration levels of size fractionated particle mass, organic vapors and inorganic vapors were determined. Gram amounts of size fractionated particle samples were collected for detailed chemical analysis and bioassay (1) screening.

STUDY DESIGN

A semi-enclosed area of the NYC bus terminal in which approximately 1400 buses operate daily was used for the diesel exhaust collection site (indoor, Site #1); a second site (outdoor, Site #2) on 9th Avenue upwind of the terminal, was used as a particle and organic vapor background sampling and collection site. The background site was used for ambient control and for comparative chemical and physical characterization of the terminal diesel enriched pollutants and the unaffected outdoor pollutants. Background inorganic gaseous data were obtained from nearby ambient monitoring stations which were also unaffected by the terminal pollutants.

The terminal site was selected so that the majority of the ambient pollutant loading would be specifically concentrated diesel exhaust originating from diesel vehicles operating in a specified area. Street canyons have the disadvantage of being traffic pattern and meteorology dependent and are confounded by stationary sources and gasoline powered vehicles.

The traffic count of buses passing by the indoor site remained consistent throughout the study with the weekday daily count averaging 1,415 (1,336 minimum; 1,440 maximum) and the weekend daily count averaging 615 (579 minimum; 643 maximum). Monitoring and sample collection began July 16, 1979 and ran daily on a 24-hour schedule through July 30, 1979.

TSP hi vol (2), Dichotomous (2), and massive air volume (3) particle samples were collected; volatile organic compounds were collected on Tenax cartridges; and primary pollutant inorganic vapors were measured by continuous sensors.

The Tradescantia plant system (4) developed by Brookhaven National Laboratories for on-site detection of toxic air pollutants, was also operated at the indoor site. The Tradescantia system allows ambient air to be screened for the presence of mutagenic chemical vapors.

The majority of buses using the terminal were equipped with Detroit two-stroke heavy diesel engines.

RESULTS AND DISCUSSION

Results of aerometric measurements reveal that some pollutants were elevated considerably at the indoor site, while others there remained at background level. Particle measurements revealed very high levels of fine particulate matter being generated in the terminal building.

The average Total Suspended Particulate (TSP) 24-hour average level in the terminal was 325 $\mu g/m^3$ while the same TSP outdoors was 120 $\mu g/m^3$. Dichotomous measurements gave a 240 $\mu g/m3$ 24-hour average for indoor particles less than 2.5 microns in diameter compared to 60 $\mu g/m^3$ at outdoor Site #2. The 24-hour average coarse fraction of the dichtomous sample (2.5-15 microns) averaged 46 $\mu g/m^3$ indoors and 20 $\mu g/m^3$ outdoors. Weekend particle mass levels dropped considerably more indoors than outdoors. Results of 24-hour TSP sulfate, nitrate, and lead levels indoors were at the same approximate concentration as at background Site #2. The outdoor levels were influencing the indoor air for the three pollutants. Ratios of sulfate, nitrate, and lead to TSP were all lower indoors than at the outdoor background site.

The massive air volume size fractionating particle collectors were operated at both sites throughout the study. The amount of material collected is shown in Table 1.

Like the size distribution of the particle mass collected by the dichotomous samplers, the massive volume samplers show the majority of the elevated particulate mass at the indoor site to be aerosol in the fine size range.

Comparison of Daily Maximum Hourly Averages for the Gaseous Primary Pollutants

Real time continuous measurements of the gaseous primary pollutants revealed peaks to occur at 8.a.m. and 5 p.m. at the indoor site each day when bus traffic was at a maximum. More variation in maximum hourly averages occured at the indoor site than outdoors for all gases measured. A summary of gaseous pollutant measurements follows:

Sulfur dioxide (SO$_2$). Based on 15-day averages, there was no difference (statistically) between indoor and outdoor maximum hourly averages. Both the Mable Dean Bacon School (outdoor) site and the Central Park (outdoor) site SO$_2$ levels are equivalent to those found inside the terminal, thus indicating very low SO$_2$ emissions from the buses inside the terminal. There is no significant difference between weekday and weekend maximum hourly values inside the terminal.

Nitrogen dioxide (NO$_2$). For NO$_2$ levels, the data indicate more variation in maximum hourly averages for the indoor site than outdoors. The maximum hourly average indoor values (mean of 1.36 ppm) are on the average significantly higher than those for outdoors (mean of .09 ppm) based on 15-day average of maximum hourly values. Weekend maximum hourly values indoors are significantly lower compared to weekdays. The NO$_2$ mean of 1.36 ppm is 10 times higher than the maximum 24-hour level of the National Ambient Air Quality Standard.

Nitric oxide (NO). NO maximum hourly values indoors throughout the study were significantly much higher than those outdoors. This is consistent with other diesel exhaust products measurements where NO has been shown to be emitted at high concentration levels.

Ozone (O$_3$). There were no detectable O$_3$ levels indoors at the bus terminal during the study. Outdoors, the maximum hourly O$_3$ values ranged from 0.0 to .12 ppm with an average of .04 ppm over the sample period. With the NO levels exceeding 7 ppm and NO$_2$ levels exceeding 1.0 ppm, it is safe to assume all of the O$_3$ at indoor Site #1 was reacting with NO to form NO$_2$.

Carbon monoxide (CO). There was significantly more day-to-day variation in the maximum hourly CO values for the indoor site as compared to the outdoor site. This was apparently due to lower weekend values for indoor Site #1 maximum hourly averages. Averaged over the sampling period, the maximum hourly average for indoors (17.79 ppm) was significantly higher than the outdoor 10-day average of 2.6 ppm.

Total hydrocarbons (THC). No ambient THC data were available for comparison with indoor Site #1 levels. Peak hourly maximum averages for indoor Site #1 was at the 10 ppm level.

Comparison of Indoor Diurnal Patterns for the Gaseous Primary Pollutants

The indoor generated gaseous pollutants were also analyzed for diurnal variation of concentration. Weekday (Monday-Friday) hourly averages and standard deviations for the study period were computed; weekend (Saturday-Sunday) hourly averages with standard deviations were also computed. As described below for each gaseous pollutant, the diurnal concentration levels for weekdays (1400 buses) were always considerably higher than for weekends (600 buses) for all gaseous pollutants except SO$_2$.

Sulfur dioxide (SO$_2$). Indoor SO$_2$ diurnal patterns are similar for both weekends and weekdays. Both were influenced by peak hour traffic

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activity occurring at 8 a.m. and 5 p.m. each day. The indoor average 5 p.m. value was significantly higher during the weekday as compared to weekend levels. The ambient background SO_2 levels did not experience 8 a.m. and 5 p.m. peaks. As mentioned earlier, there were no significant differences between indoor and outdoor peak daily hourly averages for the duration of the study.

Nitrogen dioxide (NO_2). Diurnal patterns indicate significantly higher average levels for weekdays compared to weekends for the 8 a.m. and 5 p.m. indoor hourly averages. For weekdays the hourly averages range from .06 ppm during early morning hours (3-4 a.m.) to 1.55 ppm during 5 p.m. rush hour. For weekends the hourly averages range from .08 ppm during morning hours (3-5 a.m.) to 0.45 ppm during 5 p.m. averaging time. Had more ozone been present in the terminal, much higher NO_2 levels may have been expected.

Nitric oxide (NO). Trends indicate a higher indoor NO average level during peak hours 8 a.m. and 5 p.m. for weekdays compared to weekends. The values for weekday peaks were beyond the range of the instrument but are estimated to be approximately 10 ppm.

Carbon monoxide (CO). Indoor weekday 8 a.m. and 5 p.m. hourly averages were significantly higher than weekend averages during the same averaging times for weekends. During weekdays, hourly averages were slightly elevated compared to weekend averages for hours before 8 a.m. to after 5 p.m. This cannot be concluded about other gaseous pollutants examined in the study. The differences for CO were not significant on an hour-by-hour basis during this time period, even though the hourly averages at 8 a.m. and 6 p.m. were elevated above the rest of the hours in the day.

Total hydrocarbons (THC). Diurnal patterns indicate elevations in indoor hourly averages during rush hour activity (8 a.m. to 5 p.m.) for weekday measurements. The 5 p.m. measurement averaged 10.3 ppm during weekdays and is significantly higher than the weekend average of 3.6 ppm.

Effect of Buses on Particle Levels

A statistical analysis for describing the relationship between the bus activity and particulate levels both indoors and outdoors was performed. A paired t-test was used to statistically examine the difference between the indoor and outdoor sites for TSP, dichotomous (fine, coarse, and total), sulfate, nitrate, and lead. The inside measurements were on the average significantly higher (α = .05) than outside for TSP, (0-2.5u) and (0-15u) particles. These were the only significant differences. There was no significant difference between indoor and outdoor (2.5-15u) coarse fraction particles.

Correlation coefficients between daily bus activity and pollutant levels were calculated. Several correlations were determined to be significantly different from zero. These were specifically TSP indoors .84; (0-

2.5) indoors, -.74; (0-15μ) indoors, .87; sulfate indoors, -.65; sulfate outdoors, -.65.

An important observation is that the bus activity does not correlate very highly with indoor sulfate, nitrate, and lead. Indoor levels of these pollutants are essentially the same as outdoors. Obviously the bus emissions contribution to sulfate, nitrate, and lead are lower than levels of these pollutants already resident in the atmosphere.

CONCLUSIONS

Sulfate, nitrate, and lead emissions from the buses were at a low level. Sulfur dioxide from the bus emissions were also at a low level, since no significant difference between the indoor and outdoor SO_2 levels was found. Small particles below 2.5μ aerodynamic diameter, and the gaseous pollutants of NO, NO_2, THC, and CO were all emitted at high levels from the buses. The indoor site was somewhat shielded from ultraviolet radiation, and its absence could have an effect on the organic exhaust products found in the atmosphere (5). Ozone was below detectable limits due to its use in the production of NO_2.

TABLE 1. Amount of Size-Separated Ambient Particles Collected
by the Massive Air Volume Sampler

STAGE I (20-3.5μ)	Stage II (3.5-1.7μ)	Stage III (1.7-0μ)
Site #1 7.67 gm Indoors	1.72 gm	61.89 gm
Site #2 6.06 gm Outdoors	1.18 gm	14.68 gm

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DIESEL BUS TERMINAL STUDY:
CHARACTERIZATION OF VOLATILE AND PARTICLE BOUND ORGANICS

by

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The New York City Bus Terminal was selected as a unique source of heavy duty diesel bus engines. To evaluate emissions from these bus engines in comparison to ambient air a semi-enclosed site was selected inside the terminal where approximately 1400 diesel buses operate daily and a second site was selected outside and upwind of the terminal. Volatile organic compounds were collected on Tenax cartridges, recovered by thermal desorption and introduced into a high resolution gas chromatographic column for separation. Characterization and quantification of these compounds were accomplished by mass spectrometry measuring total ion current and mass fragmentography. Volatile chemicals selected for quantitation were benzene; toluene; xylenes; ethylbenzene; 1,1,1-trichloroethane; trichloroethylene; tetrachloroethylene; benzaldehyde; n-octane; and n-hepane. These were selected to represent chemicals associated with diesel engines, i.e., octane, heptane, benzaldehyde, chemicals not associated with diesels, i.e., chlorinated hydrocarbons and general aromatic chemicals which could be found in the atmosphere.

The quantitation of the volatile organic compounds collected over a two-week period showed that the chemicals which had consistently higher concentration inside were n-octane, n-heptane, 1,1,1-trichloroethane, xylenes, and toluene. The chemical which was higher outside was tetrachloroethylene while benzaldehyde, trichloroethylene, benzene, and ethylbenzene were about the same concentration. Week day (1400 buses/day) concentrations were higher for all ten chemicals than on weekends (600 buses/day).

Air particles were collected inside and outside (at the same site as the volatile samplers). Total suspended particulate (TSP) measurement was done by the standard Hi-Volume sampler method (1) and size fractionated particles were collected using a massive air volume sampler (MAVS) which separates the particles into three size ranges. Table 1 presents data on samplers, mass, organic extractable and benzo-α-pyrene analysis.

The smallest size range (1.7 μm and below) of all samples collected inside the terminal were combined into a single sample. This procedure was also followed for the outside samples. This was done to insure sufficient quantity of sample for chemical and biological analysis.

The air particle concentration, organic concentration and the percent organic extractables were, generally, considerably higher inside than outside the terminal while the benzo-α-pyrene concentration was higher outside than inside the terminal.

The air particle samples were subjected to a fractionation procedure to yield six fractions of various chemical properties and polarities. The acid fraction contains both weak (e.g., phenols) and strong (e.g., carboxylic acids) acids. The base fraction contains organic, Bronsted bases (e.g., amines). The neutral fraction is subdivided into three main fractions based on compound polarity. The non-polar neutral (NPN) fraction is comprised of compounds less polar than ∿ naphthalene. Paraffinic materials are characteristic of this fraction. The PNA fraction contains compounds of intermediate polarity, and is selective for condensed ring aromatics. All neutral materials with polarities greater than PNA hydrocarbons are found in the polar neutral (PN) fraction. Prior to the subfractionation of the neutral fraction, the latter must be dissolved in cyclohexane. All components are not soluble in this solvent. The insoluble material is collected as a separate fraction (CI), and is comprised of intermediate and highly polar compounds.

Spillover of various compounds into all fractions is a natural feature of solvent partitioning processes. Polar neutral material was removed from the PNA fraction by silica gel chromatography. The PNAs were chromatographed utilizing HPLC such that a fraction containing only PNA hydrocarbons was obtained (PNA-1). Other fractions (PNA 2-4) were collected that contained compounds of intermediate to high polarity.

Most fractions were directly analyzed by capillary GC/MS. The fractions enriched in polynuclear aromatic hydrocarbons (PNAs) were further purified by column chromatography, and the collected subfractions were analyzed by GC/MS. A portion of each sample, after fractionation, was prepared for bioassay by removal of the fractionating solvent and addition of dimethylsulfoxide (DMSO).

Comparison of the mass distribution of each chemical fraction inside and outside the bus terminal showed several significant differences. The organics from the outside air contained a higher percent mass of one of the PNA subfractions (PNA-3), the polar neutral fraction as well as the acids and bases. The non polar neutral fraction was present at a higher percent mass inside the bus terminal. This appears to be due to higher concentrations of alkanes from unburned fuel. Bioassay analysis of the non polar neutral fraction (2) suggests that this fraction may contain substantial amounts of polynuclear aromatic hydrocarbons.

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Table 1

SAMPLER particle size range	Particle Concentration µg Particle/M³		Organic Concentration µg Organics/M³		% Organic Extractable µg Organics/ 100 µg Particle		BaP µg BaP/g Particle	
	I	O	I	O	I	O	I	O
Hi-Vol (0-50 µm)	325.0	120.0	14.95	4.08	4.6	3.4	15.4	32.0
MAVS I (3.5-20 µm)	58.5	24.0	7.37	0.98	12.6	4.1	5.9	25.2
MAVS II (1.7-3.5 µm)	6.5	6.0	0.84	0.31	13.0	5.2	11.9	34.3
MAVS III (0-1.7 µm)	214.5	66.0	60.49	9.57	28.2	14.5	0.8	5.9

I = Inside
0 = Outside

DIESEL BUS TERMINAL STUDY: MUTAGENICITY OF THE PARTICLE-BOUND ORGANICS AND ORGANIC FRACTIONS

by

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Emission testing of both heavy-duty diesel engines and light-duty diesel cars using tunnel dilution and filtration to collect diesel particles has shown the organics associated with these collected particles to be mutagenic in the Ames Salmonella typhimurium assay (1). The mutagenicity of these organics has been shown to be dependent on fuel quality (2) as well as engine type (3). The organics associated with particle emissions from heavy-duty diesel engines have generally been less mutagenic than the organics from light-duty cars. Fractionation and bioassay studies suggest this is due to a greater concentration of nonmutagenic aliphatic compounds emitted from unburned fuel. Over 90% of the mutagenic activity has been observed in the polar neutral fractions not requiring metabolic activation (1). Mutagenicity studies of the organics associated with urban ambient air particles have also reported mutagenic activity in the organics extracted from particles (4,5).

In order to evaluate the impact of emissions from heavy-duty buses on the mutagenicity of ambient air, this study was designed to compare the mutagenic activity of the total extractable organics from size-fractionated air particles and chemical class fractions both inside and outside a diesel bus terminal.

The mutagenicity of the ambient air inside and outside the New York Port Authority Bus Terminal was compared using a microbial mutagenesis bioassay. Approximately 1400 diesel buses operate daily in the semi-enclosed bus terminal. Air particles were collected simultaneously using both the Massive Air Volume Sampler (MAVS) (6) and the standard Hi-Volume air sampler (Hi-Vol). The dichloromethane-extractable organics from these air particles were bioassayed in the Salmonella typhimurium plate incorporation assay (7) in TA98 with and without metabolic activation with minor modifications (3). The slope of the dose-response curve (rev/μg) was determined using a nonlinear model (8). The air particle concentration inside the bus terminal was nearly 3 times the outside concentration based on the Hi-Vol TSP. Comparison of the Hi-Vol and MAVS data showed the increased concentration of particles inside was due primarily to increased concentrations of particles less than 1.7 micron in size. These small (less than 1.7 micron) particles inside the terminal had a higher concentration of extractable organics than the small particles outside the terminal. Although both the small particle and organic concentrations

were lower outside the terminal, the mutagenicity of the organics from outside was significantly greater (nearly 10 times) than inside the terminal. Both direct-acting and indirect-acting mutagens were detected in these samples. The mutagenic activity of the air in revertants per cubic meter provides a direct comparison of the mutagenicity of the inside and outside air. Using data from either the Hi-Vol samples or the smallest particles from the MAVS, the outside air was approximately twice as mutagenic as the air inside the bus terminal.

Fractionation and mutagenesis bioassay of the organics from the less-than-1.7-micron particles were conducted to compare the chemical composition inside and outside. The mutagenicity (rev/μg) of each fraction and the mass percentage of each fraction were used to calculate weighted mutagenicities. The percent of the total mutagenicity attributable to each chemical fraction was determined and compared inside and outside the terminal. The diesel emissions inside the bus terminal increased the concentration of aliphatic hydrocarbons found in the non-polar neutral fraction. The higher mutagenicity in the outside ambient air appears to be due to higher concentrations of organic acids and direct-acting moderately polar neutral compounds. The highly polar neutral fraction showed more direct-acting mutagenic activity inside the terminal.

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NITRO DERIVATIVES OF POLYNUCLEAR AROMATIC HYDROCARBONS IN AIRBORNE AND SOURCE PARTICULATE

by

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INTRODUCTION

Direct mutagenic activity is observed in the Ames _Salmonella_ bioassay of organic extracts from ambient airborne particulate and source emissions. Nitro derivatives of polynuclear aromatic hydrocarbons (nitro-PNA), some of which are strong direct-acting mutagens, are considered to be probable contributors to this activity.

SAMPLING AND ANALYSIS

Particulate samples were collected on dexiglas filters and extracted with benzene-ethanol (80:20 v/v) with a Soxhlet apparatus. Automobile exhaust samples were obtained using a chassis dynamometer and dilution tube. An analytical method for nitro-PNA was recently developed which involves reduction of these compounds to the corresponding amino-PNA and their determination by HPLC with fluorescence detection.[1] With HPLC methods, the concentrations of 1-nitropyrene, 6-nitro-BaP, pyrene, and BaP were measured in samples of the various particulates. In all of these samples, part of the nitro-PNA may have resulted from the reaction of oxides of nitrogen found in emissions and in ambient air with PNA bound to the particles on the filter. For example, an increase in nitro-PNA was measured when diesel exhaust particulate was exposed to filtered diesel exhaust gases, suggesting a strong likelihood that nitro artifacts are formed during filter sampling.[1] The direct mutagenic activity was determined by the Ames Salmonella bioassay (Litton Bionetics, Kensington, MD) using tester strain TA-98 without metabolic activation. Each particulate extract was dissolved in DMSO and tested at five doses using triplicate plates with equal numbers of bacteria from the same starting culture. The slope of the initial linear part of the dose response curve was considered as the specific activity.

AMBIENT AIRBORNE PARTICULATE

Ambient particulate collected at an urban and a suburban site during Spring and Summer contained 0.2-0.6 ppm (i.e., ng/mg particulate) of 1-nitropyrene corresponding to airborne concentrations of 0.016 to 0.030 ng/m^3, and also contained 0.9-2.5 ppm 6-nitro-BaP corresponding to 0.04-0.28 ng/m^3. As shown in Figure 1, the fluorescence spectra of the nitro-PNA were scanned to determine their identity by comparison with authentic standards. The direct mutagenic activity of the suburban samples in the Ames bioassay was 0.15-0.56 revertants/microgram of particulate corresponding to 7-20 revertants/m^3 airborne mutagenicity. Based on its reported specific activity, the 1-nitropyrene in the particulate could account for less than 0.3% of the total activity.

AUTOMOBILE EXHAUST PARTICULATES

Samples were collected from a few of the numerous sources of particulate emissions including a 1981 2.5-L 4-cylinder catalyst car, a 1980 4.3-L 8-cylinder car with no catalyst using unleaded gasoline, a 1974 5.7-L 8-cylinder car with leaded gasoline, and 5.7-L 1980 8-cylinder diesel cars made by General Motors divisions. The catalyst car gave particulate with a lower concentration of 1-nitropyrene (0.63 ppm) than the noncatalyst cars (3.9-4.3 ppm) and production diesel car (8.0 ppm). The catalyst car particulate had 0.21 ppm 6-nitro-BaP, the noncatalyst cars 17-33 ppm, and the diesel car less than 0.4 ppm. On a per mile basis also, the catalyst car emitted less of the PNA, nitro-PNA, and direct mutagenic activity than the other vehicles. The level of direct acting mutagenicity seemed to be directly related to the total nitro-PNA concentration of the particulate and not to PNA concentrations.

An experimental (noncommercial) low emission diesel car was also tested. Compared to the production model of the 1980 diesel, the low emission diesel gave much lower particulate, PNA, nitro-PNA, and mutagenic emissions.

STATIONARY SOURCES

Particulate samples from a wood-burning fireplace did not contain levels of the nitro-PNA above the minimum detection limits (less than 0.1 ppm). The concentrations of pyrene and BaP found in these samples were low compared to automobile particulates and depended on how the samples were collected: averages of 3-4 ppm in particles collected from the raw flue gases with an EPA Method 5 sampling train (heated filter and impingers) compared to 30-60 ppm when collected from emissions diluted 25-fold with air. The increased levels of PNA in particulate from cool, diluted fireplace emissions suggests that much of the organics remain in the vapor phase in emissions sampled by the EPA method. Particulate emissions measured by this method should not be compared to vehicle emissions determined by the dilution tube method and may lead to

erroneous estimates of emissions from stationary sources (sampled from raw flue gases). Similarly, a particulate sample from a coal-fired boiler, collected from hot, undiluted flue gases, showed low or undetectable levels of PNA (less than 0.5 ppm) and nitro-PNA (less than 0.02 ppm).

CONCLUSIONS

Nitro-PNA are found in ambient airborne particles and various source emissions. Because of the very limited data from only a few of the possible sources and the complicating effects of differences in sampling methods, filter artifact formation, and atmospheric reactions, source allocation for PNA derivatives in ambient particulate is not feasible at the present time.

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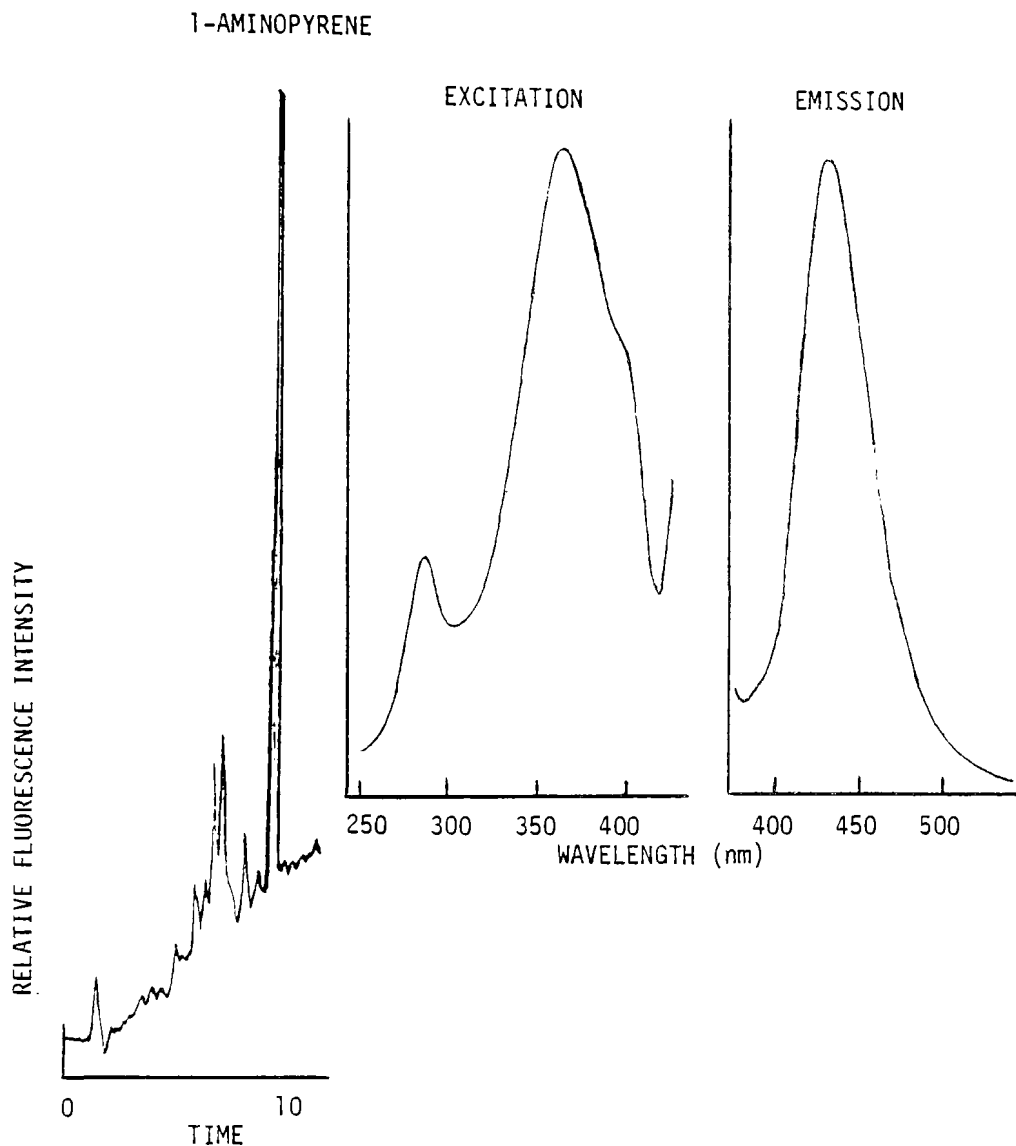
Figure 1. HPLC chromatogram of ambient particulate extract after treatment with a reducing agent (Conditions, see reference[1]) Fluorescence Detector -- excitation 365 nm, emission 430 nm. Stop-flow scanning gave the emission and excitation spectra shown, which match those of authentic 1-aminopyrene.

RISK ASSESSMENT OF DIESEL EMISSIONS

by

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The observations made a number of years ago, which have since been verified, to the effect that diesel exhaust particulates are mutagenic and contain agents that are recognized carcinogens, established the position that diesel particulates are likely to be carcinogenic in humans. However, the unanswered question is how potent are these particulates and what is the magnitude of the cancer hazard to the general population. In view of the absence of any direct animal experiments or epidemiologic data, an approach to risk assessment several years ago which seemed reasonable was to use the available epidemiologic data that involved exposure to combustion products having similarities to diesel particulates and to compare the relative potency of these materials with diesel exhaust particulates. The epidemiologic studies that were chosen involved cigarette smoking, coke oven emissions, and roofing tar. An extensive series of studies including mutagenesis, cell transformation, skin painting, inhalation, and intratracheal intubation have been undertaken to compare these materials with diesel particulates. The present status of the carcinogen risk assessment in terms of the epidemiologic and laboratory studies will be presented.

SECTION 8

POSTER PRESENTATIONS

MUTAGENICITY OF PARTICLE-BOUND ORGANIC CHEMICAL FRACTIONS FROM DIESEL AND COMPARATIVE EMISSIONS

by

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A variety of mobile and stationary sources emit particle-bound organics that have demonstrated mutagenicity. The objective of this study was to measure the mutagenicity of the chemical class fractions derived from the total extracted organics for diesel and several comparative emission sources.

The four sources of combustion organics were diesel engine exhaust particles, a cigarette smoke condensate, a coke oven main sample, and roofing tar emissions. The diesel exhaust particles were collected from a 1978 Oldsmobile 350 diesel vehicle operated on the highway fuel economy test cycle (HWFET) with No. 2 diesel (Union 76) fuel. The particles were collected on Pallflex T60A20 filters and the organics were removed by Soxhlet extraction with methylene chloride as previously described (1). The 2R1 Kentucky reference cigarette smoke condensate was generated according to the method of Patel (1977) at Oak Ridge National Laboratory (1). The coke oven main sample was collected from a separator located between the gas collector main and the primary coolers within a coke oven battery at Republic Steel in Gadsden, AL, about 60 miles northeast of Birmingham. The roofing tar sample was generated and collected using a conventional tar pot containing pitch-based tar, enclosed within a chamber and heated to 360° to 380°F, a normal temperature for commercial use. The evaporative emissions were collected using a small bag house fitted with Teflon filter bags (1). The solvents used to extract or condense the organics from each of these samples were removed by evaporation under nitrogen. The total extracted organics were class-fractionated into organic acids, organic bases, cyclohexane insolubles, polar neutrals, non-polar neutrals, and polynuclear aromatics (PNA). The PNA fraction was further fractionated chromatographically using gradient elution on high pressure liquid chromatography (HPLC) such that a purified fraction containing PNA hydrocarbons was obtained (PNA1) by elution with 2% dichloromethane (DCM) in hexane. Elution with more polar solvents resulted in three additional fractions (PNA2-4) that contained compounds of intermediate to higher polarity. Each class of organics was chemically characterized using GC/MS (2).

The chemical fractions for each emission source were bioassayed using the *Salmonella typhimurium*/microsomal plate incorporation test as developed by Ames et al. (3). Due to the large number of samples to be bioassayed at one time in this study and the limited amounts of some of the samples, only the mutant strain (TA98) of *Salmonella typhimurium* was used. The protocol described by Ames et al. (3) was followed with minor modifications (4). The data was analyzed using a non-linear model (5) to determine the slope of the dose-response curve. Weighted mutagenicities were determined for each fraction based on the mutagenicity model slope (rev/µg) and the percent of the total mass recovered from each fraction represented. The weighted mutagenicities were then used to determine the percent of mutagenicity attributed to each chemical fraction. Based on this data as summarized in Table 1 the following summary can be made:

1. Olds Diesel. Although the non-polar neutral fraction (NPN) represented the greatest percent of the total mass recovered upon fractionation, it accounted for very little (< 2%) of the mutagenic activity. From 45 to 50% of the mutagenic activity was found in the polar neutral fraction (PN). Polar neutral compounds having limited solubility in cyclohexane would appear in the cyclohexane insoluble fraction (CI), which contained from 15 to 31% of the mutagenic activity. Both of these fractions (PN and CI) contained direct-acting mutagens.

2. Cigarette Smoke. The cyclohexane insoluble fraction (CI) represented the greatest percent of the total mass recovered upon fractionation. The purified polynuclear aromatic fraction (PNA1) was the most active fraction; however, when the model slopes were weighted according to the percentage each fraction represented of the total organic sample, the basic fraction (BASE) accounted for the majority of the mutagenic activity in the presence of metabolic activation (57%), and a polar neutral PNA contaminant (PNA4) accounted for the majority of the mutagenic activity in the absence of metabolic activation (87%).

3. Coke Oven Mains. The greatest percent of the total mass recovered after fractionation was represented by the cyclohexane insoluble fraction (CI). The basic fraction (BASE) and the cyclohexane insoluble fraction (CI) contained the largest percentage of the mutagenic activity in the presence of metabolic activation (41% and 34%, respectively). A polar neutral PNA contaminant (PNA4) accounted for the majority of the mutagenic activity in the absence of metabolic activation (76%).

4. Roofing Tar. The chemical fractions representing the greatest percent mass were the non-polar neutrals (NPN) and a purified PNA fraction (PNA1). Although mutagenic activity was associated with several of the fractions, the cyclohexane insoluble fraction (CI) accounted for the majority (> 50%) of the mutagenic activity both in the presence and absence of metabolic activation.

This study demonstrated significant biological differences among the four emission sources. Within each source, the relative mutagenicity of each fraction was significantly different in the presence and absence of metabolic activation. The two sources which showed some similarities were the cigarette smoke and the coke oven mains. These sources had similar profiles in the percent of mutagenic activity attributed to each fraction both with and without metabolic activation; however, chemical characterization showed significant differences in the compounds identified in these two sources (2). Further chemical characterization of the constituents of each fraction is required to determine which specific chemicals are biologically active within a single source.

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Table 1. Percent of Mutagenic Activity Attributed to Each Chemical Fraction
from Comparative Sources (Reported as Percent of Weighted Slope)[a]

Fractions	TA98 Without Activation				TA98 With Activation			
	Olds Diesel	Cigarette Smoke	Coke Oven	Roofing Tar	Olds Diesel	Cigarette Smoke	Coke Oven	Roofing Tar
Acid	9.56	2.22	0.00	0.00	3.88	0.46	0.15	1.09
Base	4.63	0.00	0.00	0.00	3.66	57.39	40.89	5.91
PN	44.90	0.00	0.00	4.67	49.94	0.00	8.99	17.22
NPN	0.00	0.00	0.00	0.00	1.26	1.99	7.04	4.21
PNA1	0.00	0.00	0.00	0.00	0.19	0.20	4.31	4.79
PNA2	9.44	0.00	0.00	6.85	15.87	0.00	0.00	7.84
PNA3	0.74	10.84	24.14	0.03	9.67	0.18	2.90	0.14
PNA4	0.21	86.94	75.86	0.07	0.30	8.37	1.84	0.96
CI	30.53	0.00	0.00	88.38	15.23	31.41	33.87	57.83

[a]Percent of mutagenic activity (% M) determined by: weighted mutagenicity of
each fraction (model slope [rev/μg] x % mass of fraction) x 100 ÷ total
weighted mutagenicities of all the fractions.

SCANNING ELECTRON MICROSCOPY OF TERMINAL AIRWAYS OF GUINEA PIGS CHRONICALLY INHALING DIESEL EXHAUST (DE)

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The structural physiology of airways near gas exchanging alveoli was documented to establish any changes induced by DE exposure. Preliminary findings are published on effects of DEP inhalation on alveolar macrophages (1,2). Here scanning electron microscopy was used to reveal cell interrelations and to resolve distribution of DE particulates (DEP) along the terminal airway. Thirty guinea pigs inhaled either 0, 250, 750 or 1500 µg DE/m^3 for 110 hr/week for 2 weeks, 3 and 12 mon while fifteen rats were exposed for 10 weeks 6000 µg DE, 6 mon 750 µg DE and 12 mon 1500 µg DE. Peripheral airways were selected for study and photography when they were of sufficient length to provide structural information from terminal bronchiolus to alveolar out-pockets. Airways were evaluated without knowledge of the animal's history. The relative amount and distribution of deposited particulate, was graded on a scale of 1 to 5+. Decoding was done later followed by final interpretations. At least 10 terminal airways/animal were extensively photographed. DEP was identified as free individual particles, 0.1 + 0.03 µm, (Fig. 1). DEP was adherent to epithelium and irregular patches of particles were prominent at airway bifurcations (Fig. 1A). Proximal airway, characterized by an epithelium of secretory and ciliated cells, had even larger agglomerates of particulates especially in 12 mon 1500 and 10 week 6000 µg DE sets. These agglomerates consisted of various sized particles, only some of which had sizes appropriate for DEP. Quite likely some of the admixture was secretory granules and congealed proteins; which tend to be larger and more irregular than particles suspected of being DEP (Fig. 1B). Terminal bronchioles often were crowded with macrophages and granulocytes, exiting the lung. Surface domes were prominent on Clara cells which may be increased in number in DE sets. More pneumocyte II cell clusters occurred at bronchiolar-alveolar junctions in heavily exposed than in age-matched control animals. Broad expanses of terminal airway in DE exposed animals appeared relatively clean, but still contained more particulate than companion controls. Alveoli opening off terminal bronchioles had more particulates than other alveoli. The morphology and 0.1 µm size of the spherical particles and relative sparcity of such in controls suggests that this is a visualization of the DEP burden but is insufficient for absolute identification. However the highest DE exposure conditions were associated with the dustier terminal airways. (This study was aided in part by General Motors Research Laboratory, Warren, Michigan).

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Fig. 1A. Terminal airway of guinea pig exposed to 1500 µg DE for 12 mon. Note patches of particles whose individual size is 0.1 µm and could be DE deposits.
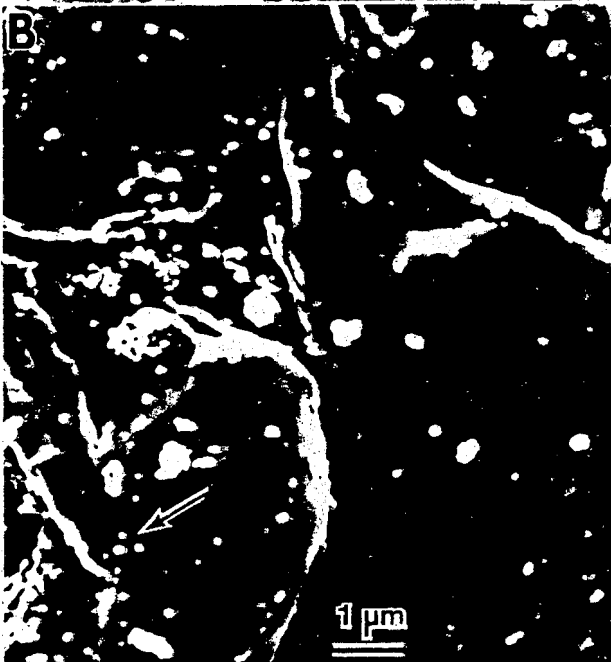
Fig. 1B. Terminal bronchiole adjacent to alveolus in rat exposed to 6000 µg DE for 2 mon. Small dust particles are 0.1 µm in diameter and probably DEP.

EMISSION OF DIESEL PARTICLES AND PARTICULATE MUTAGENS AT LOW AMBIENT TEMPERATURE

by

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Gaseous and particulate exhaust emissions from two 1980 model diesel-powered passenger cars, an Oldsmobile 98 and a Volkswagen Rabbit, were measured over the urban dynamometer driving schedule of the Federal Test Procedure (FTP) as a function of ambient temperature (23°F-84°F). Gaseous emissions analysis included total hydrocarbons (HC), carbon monoxide (CO), nitrogen oxides (NO_x), and fuel economy (MPG). All were measured as a function of the 3 individual test phases (i.e., cold transient-phase 1, stabilized-phase 2, and hot transient-phase 3) of the FTP. Particulate emissions analysis included total particulate matter, particulate organic emissions, and molecular weight distribution measurements (also measured as a function of FTP individual test phase), polynuclear aromatic hydrocarbon measurements including benzo-a-pyrene (BaP), nitropyrene, and pyrene, and Ames <u>Salmonella</u> bioassay of the particulate organics in strain TA-98. See figure detailing diesel emissions analysis scheme below:

DIESEL EMISSIONS ANALYSIS SCHEME

DIESEL EXHAUST ⟶ GASEOUS EMISSIONS
*HC (g/mi)
*CO (g/mi)
*NO_x (g/mi)
*MPG

PARTICULATE EMISSIONS

*TOTAL PARTICULATE MATTER (mg/mi)

EXTRACT WITH CH_2Cl_2
(DISCARD INERT CARBONACEOUS MATERIAL)

*PARTICULATE ORGANIC EMISSIONS (mg/mi)

*MOLECULAR WEIGHT DISTRIBUTION
(C_{12} - C_{38}, mg/mi)

AMES SALMONELLA BIOASSAY
(rev/μg, rev/mi)

POLYNUCLEAR AROMATIC HYDROCARBON ANALYSIS

BaP
(ng/mg, μg/mi)

NITROPYRENE
(ng/mg, μg/mi)

PYRENE
(ng/mg, μg/mi)

*MEASURED AS A FUNCTION OF FTP PHASE 1, 2, 3 AND COMPOSITE FTP.

The data trends may be conveniently divided into two general categories, gaseous emissions and particulate emissions. Gaseous emission trends: HC, CO, and NO_x emissions increased slightly ($0.10 < r^2 < 0.75$) with decreasing test temperature. Oldsmobile HC emissions ranged from 0.20 to 0.29 g/mi, CO emissions from 1.1 to 1.4 g/mi, and NO_x emissions from 1.2 to 1.4 g/mi; Volkswagen HC emissions ranged from 0.26 to 0.32 g/mi, CO emissions from 1.0 to 1.2 g/mi, and NO_x emissions from 1.0 to 1.2 g/mi. Fuel economy was more temperature dependent ($r^2 > 0.80$) with the Oldsmobile's decreasing from 21.6 to 18.0 mpg and the Volkswagen's decreasing from 45.7 to 41.0 mpg.

Particulate emission trends: total particulate matter and particulate organic emissions (i.e., the CH_2Cl_2 extractable particulate matter) increased with decreasing test temperature. Comparison of overall FTP data at 82°F (median high temperature) versus 45°F (median low temperature) indicates that Oldsmobile total particulate emissions increased from ~567 to 739 mg/mi (+30%) and particulate organics increased from ~94 to ~153 mg/mi (+63%). For the Volkswagen, total particulate emissions increased from ~361 to ~423 mg/mi (+17%) and particulate organics increased from ~72 to ~101 mg/mi (+40%). Molecular weight distributions of the particulate organics in the C_{12}-C_{38} carbon number range, determined by gas chromatography, indicated that much of the organic matter associated with the particulate appears to be uncombusted diesel fuel. This is evident when comparing a lower temperature FTP to a higher temperature FTP. Using the Oldsmobile for example, at 32°F, 61% of the overall FTP particulate organic emission rate of 127 mg/mi is attributable to C_{13}-C_{22} while at 82°F, only 40% of the particulate emission rate of 83 mg/mi is attributable to C_{13}-C_{22}. This uncombusted diesel fuel effect is even more pronounced in the FTP test phase 1 molecular weight distributions: at 32°F, 68% of the Oldsmobile organic emission rate of 137 mg/mi is attributable to C_{13}-C_{22} while at 82°F, only 35% of the particulate emission rate of 105 mg/mi is attributable to C_{13}-C_{22}.

Polynuclear aromatic hydrocarbon (PAH) analysis of selected FTPs indicated decreasing (r^2 ~0.60) BaP, nitropyrene, and pyrene emissions (in ng/mg extract) with decreasing FTP test temperature. Overall Oldsmobile BaP emissions ranged from 0.5 to 1.1 µg/mi (average 0.7 ±0.2); nitropyrene emissions ranged from 6.6 to 13.6 µg/mi (average 9.8± 2.2); pyrene emissions ranged from 82.1 to 133.7 µg/mi (average 106.6± 7.4). Overall Volkswagen BaP emissions ranged from 15.5 to 20.2 µg/mi (average 18.2± 1.5); nitropyrene emissions ranged from 4.2 to 12.6 µg/mi (average 8.3± 2.8); pyrene emissions ranged from 260.4 to 354.5 µg/mi (average 293.9± 33.7).

Ames activity levels, in terms of revertants per microgram of particulate organic emissions, correlated moderately ($r^2 = 0.73$) with FTP test temperature indicating decreasing mutagenic activity with decreasing test temperature. Activity levels also correlated moderately (r^2 ~ 0.73) with PAH emissions indicating decreasing mutagenicity (rev/µg) with decreasing PAH emissions (ng/mg extract). Mutagenic activity was greater without metabolic activation (-S9). For the Oldsmobile, activity with metabolic activation ranged from 1.1 to 1.6 rev/µg (average of 1.3 ± 0.1) and without metabolic activation from 2.4 to 3.8 rev/µg (average of 3.4 ± 0.9). Corresponding

Oldsmobile rev/mi x 10^3 ranges and rates were 105 to 185 (average of 152 ± 29) with S9 and 297 to 475 (average of 383 ± 66) without S9. The Volkswagen displayed slightly greater mutagenic activity than the Oldsmobile. Activity with metabolic activation ranged from 1.8 to 2.9 rev/µg (average of 2.2 ± 0.3)and without metabolic activation from 3.2 to 5.7 rev/µg (average of 4.5 ± 0.80. Corresponding Volkswagen rev/mi x 10^3 ranges and rates were 163 to 226 (average of 192 ± 23) with S9 and 341 to 426 (average of 385 ± 28) without S9.

CONCLUSIONS

1. The regulated gaseous emissions (HC, CO, and NO$_x$) of diesel-powered passenger cars were slightly temperature dependent with decreasing FTP test temperature slightly increasing HC, CO, and NO$_x$ emissions. Fuel economy was moderately temperature dependent with decreasing FTP test temperature decreasing fuel economy.

2. Total particulate matter and particulate organic emissions were moderately temperature dependent. Decreasing FTP test temperature increased total particulate and particulate organic emissions.

3. Increases in particulate organic emission rates at low FTP test temperatures may be primarily attributed to uncombusted diesel fuel.

4. There appeared to be moderate correlation between polynuclear aromatic hydrocarbon emissions and FTP test temperature with PAH emissions decreasing with decreasing test temperature.

5. There appeared to be moderate correlation between Ames test mutagenic activity (without metabolic activation) and FTP test temperature with mutagenic activity decreasing with decreasing test temperature. There also appeared to be moderate correlation between mutagenic activity and PAH emissions with mutagenicity decreasing with decreasing PAH emissions.

THE DESIGN OF THE CCMC'S LONG-TERM INHALATION PROGRAM TO INVESTIGATE THE POSSIBLE TOXICOLOGICAL EFFECTS OF DIESEL AND GASOLINE ENGINE EXHAUST EMISSIONS

by

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The health effects program of the Committee of Common Market Automobile Constructors (CCMC) on diesel and gasoline engine emissions is presented in another poster. Part of this program, the long-term inhalation study, is being carried out by Battelle-Geneva, and in this poster we present the design of the equipment and the experimental protocol.

Four different types of emissions—diesel (D), filtered diesel (DF), gasoline (G), and gasoline with convertor (GC)—are generated by three engines (VW Rabbit 1.5 litre diesel and two Renault R18 1.6 litre gasoline) running on the FTP (US-72 hot start cycle). Two species of animals (Syrian hamsters and Fischer-344 rats) will be exposed for up to 24 months, 16 hours per day, 5 days per week.

These emissions can be diluted to three dose levels: high (H), medium (M), and low (L). From this 4 x 3 matrix of exhaust types and dose levels, three have been omitted as being of little potential interest. An indicative matrix of dose levels selected for exposure are shown in Table 1.

Table 1. Matrix of Dose Levels

Dose Levels	D	DF	G	GC
H	8.3	8.3	3.6	3.6
M	2.8	2.8	1.2	1.2
L	0.92	-	-	-

The figures given in the above table are the calculated mean exhaust gas concentrations (% volume) when the exhaust gases from one cycle are diluted by the same volume of air for all engines. In this example the dilution air volume per cycle for the high dose levels is 300 Nm^3 (equivalent to 40 Nm^3/mile). The M and L dose levels have been set at 1/3 and 1/9 of the H levels.

Such dose levels are considered to be directly comparable across the table since they are based on equal mileage. This comparison takes into account the different internal dilution taking place in the engines and also the different fuel efficiencies of the automobiles being compared.

In Table 2, the high dose levels, HD and HG, are expected to correspond to the following concentrations of the regulated components (running the gasoline engine at lambda = 1).

Table 2. High Dose Level and Concentration Correspondence

Components	Units	Diesel (HD)	Gasoline (HG)
Exhaust gas in air	%	8.3	3.5
Particulate matter	mg/m^3	5.5	-
CO	ppm	20	203
No$_x$ (NO$_2$ equivalent)	ppm	15	49
THC	mg/m^3	9.2	37

The figures given in these tables are all calculated from the data supplied by the automobile manufacturers. They are currently being evaluated in our system and, if necessary, the flow rate of air to the dilution tunnels will be modified with a view to keeping concentrations of the biologically critical components in HD and HG at as high a level as is considered compatible with a chronic study.

Although slight differences exist in the distribution systems for each engine, the basic principle remains the same and is described below for one engine.

The exhaust gases are injected from the tail pipe directly into a dilution tunnel where they are mixed with a constant flow of conditioned air (filtered and dried to a water content of 7 g/kg air). The air is dried to compensate, at least partially, for the high water vapour content of the gasoline engine emissions so that condensation does not take place in the dilution tunnel, and to ensure that the relative humidity in the inhalation chambers is not too high.

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A fraction of the diluted exhaust gas is drawn off from the mixing tunnel into a buffer tank where it is held for about one minute. The need for this tank is being investigated, and it will be removed from the system if not required. Its role is to attenuate the high peak values of NO_2 and CO that are produced by the engines over short periods during the US-72 cycle to a level at which they do not appreciably affect the breathing pattern of the animals.

The dilution tunnels are run at the high dose level. The medium and low dose levels are achieved by further diluting the high dose level with air. The dose levels are computed directly from flow rate measurements using rotameters.

The high dose level streams will be continuously monitored for CO and NO_x for safety purposes. The concentrations of the regulated components and certain non-regulated components will be checked in the inhalation chambers at regular intervals.

Battelle-Northwest designed Hazleton-1000 inhalation exposure chambers will be used, each chamber housing one treatment group of 72 male and 72 female rats or 156 male and 156 female hamsters. The control groups (fresh air) will contain 288 rats and 624 hamsters.

Initial and interim sacrifices will be made on 8 animals of each sex from each group after 0, 6, 12, and 18 months of exposure. These animals will be used for respiratory physiology. haematology, urinalysis, and blood chemistry investigations.

Complete autopsies will be carried out on all animals in the study and the animals in the highest dose levels and the control groups will be subjected to a histopathological examination of the respiratory tract. Any anomalies detected during autopsies will also be subjected to a histopathological diagnosis. Other organs will be stored in formalin and be available for further examination if required.

System testing of the experimental facility and some short-term exposures at the high dose levels were carried out up to August 1981. The plan to start up the long-term exposure using staggered intakes of animals during the months of September to December 1981 has unfortunately been delayed by several months due to an accident. Rebuilding and testing of part of the facility is now necessary before the long-term exposure can be started.

CHRONIC INHALATION ONCOGENICITY STUDY OF
DIESEL EXHAUST IN SENCAR MICE

by

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A large number of Sencar mice were used in an investigation to assess the long term potential inhalation oncogenicity of automotive diesel emissions. After exposure of parental mice from before mating on through gestation, the offspring continued in exposure 8 hours daily for 15 months to an atmosphere of diluted automotive diesel engine exhaust. Exhaust dilution was controlled so as to provide a particulate level of about 6 mg/m^3 in exposure chamber atmosphere through the parental phase and until the young were mature (10 wks. of age), at which time this concentration was adjusted to about 12 mg/m^3. Subgroups for testing initiation (A), promotion (B), and whole carcinogen (C) potentials of diesel exposure were administered, respectively, weekly i.p. injections of promoter (butylated hydroxytoluene) for about 1 year, an initial single i.p. injection of initiator (urethan), and neither promoter or initiator. Matching controls were exposed to purified air. Each subgroup initially numbered 260, equally divided by sex. Additional negative, positive, and vehicle control groups were used.

Over all groups, survival was 13 percent less in diesel-exposed than in control mice (75 vs 65 percent), the initiation test group males being the most affected. Survival was least in initiation-test mice, due apparently in large part to consequences of frequent i.p. BHT-in-oil injections. In all subgroups except A females survival was greater in control than in diesel-exposed mice. In general, males were more susceptible than females to reduced-survival effects of diesel exposure, and survival effects were least severe in mice receiving diesel exhaust only (i.e., not receiving promoter or initiator).

In periodically weighed sample groups, body weight gain was depressed in diesel-exposed mice of both sexes in all subgroups compared to corresponding clean air controls, the effect ranging from 11 to 24 percent of control mean weight gain. Similarly, mean terminal body weights taken for all survivors showed lower values (ranging 7 to 17%) for both sexes in all subgroups of diesel-exposed compared to control mice.

Histological results showed a small but not statistically significant overall increase in lung (alveolar bronchiolar) tumor rate (primarily adenoma) in surviving diesel-exposed compared to control mice. However, for several types of respiratory lesions there were consistenly and greatly increased incidences in diesel-exposed compared to control mice. These lesions included: alveolar macrophages, black alveolar pigment material, perivascular and peribronchial mononuclear cells, focal fibrosis, alveolar interstitial thickening, rhinitis (females), and black pigment in mediastinal lymph nodes. Predictably, serositis was a common lesion in mice of the BHT-in-oil injected groups of both control and diesel atmospheres.

SPECIES DIFFERENCES IN DEPOSITION AND CLEARANCE
OF INHALED DIESEL EXHAUST PARTICLES

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Deposition of inhaled diesel particles and their subsequent clearance from the gas exchange regions of the respiratory tract may play important roles in the question of potential health impact of diesel emissions. The initial particulate deposition in the lungs depends on the physical characteristics of the particles and the airways morphometry may also affect the regional deposition within the lungs. For example, the narrow nasal passages can shift the particle deposition in the nasal region proximally for particles larger than a micron in small experimental animals, and the final deposition patterns in the lungs will be significantly different from those of larger species or man. Although diesel particles are not large enough to deposit by impaction in the upper respiratory tract, species differences can still exist in alveolar clearance mechanisms, clearance pathways and kinetics. Table 1 compares the estimated initial particulate dose to the lungs in different species exposed to 0.1 μm particles at 250 μg/m^3 for an hour. Although the absolute particulate burden by weight is highest in man, the immediate local dose to the lung tissues is expected to be five times higher in the dog and guinea pig. The relative dose is even higher in rats and hamsters by at least a factor of ten.

Male Hartley guinea pigs and Fischer 344 rats were exposed in a nose–only inhalation chamber to radioactive diesel exhaust particles. The particles, tagged in the insoluble carbonaceous core with ^{14}C, were generated by combustion of (1-^{14}C)-n-hexadecane in a single cylinder diesel engine operated at full load [1]. The ^{14}C activity in the lungs and lymph nodes were determined for groups of exposed animals sacrificed immediately after the 45-minute exposure and others at scheduled intervals for an extended period of time. Although the initial lung deposition efficiencies and mucociliary clearance half-times were comparable in both species, the amount of inhaled diesel particles cleared from the upper respiratory airways in the guinea pig accounted for only 17% of the initial lung burden, compared to 34% in the rat. Furthermore, the alveolar clearance of diesel particles in the guinea pig was extremely slow, with more than 80% of the initial dose retained after 105 days (Figure 1). The pulmonary clearance half-time for inhaled diesel particles in the guinea pig is estimated to exceed 300 days which strongly contrasts with 60-80 days in rats (determined by fitting experimental data collected so far to two- or three-phase clearance models.) The differences observed in this study demonstrate a greater long-term retention of inhaled diesel particles in the guinea pig possibly caused by slower clearance processes in the deep lung of this species. The actual biological dose to the respiratory epithelium would also be

different in both species. This clearly indicates the difficulty in comparing studies on potential health effects of inhaled diesel particles among different species and in extrapolating experimental animal data to man.

TABLE 1. ESTIMATED INITIAL LUNG DEPOSITION DOSE IN
DIFFERENT SPECIES AFTER INHALATION OF 0.1 µm
PARTICLES AT 250 µg/m^3 FOR 1 HOUR

Species	Body Weight (g)	Minute Volume (mL)	Deposition Efficiency (%)	Lung Wt (g)	Particulate Burden (µg)	Particles/g Lung Tissue (µg/g)
Man	70K	7000	25	1000	26	0.025
Dog	12K	3100	27	80	12	0.15
G. Pig	400	125	20	3.0	0.4	0.15
Rat	250	150	17	1.5	0.4	0.25
Hamster	92	61	20	0.4	0.2	0.50

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Lung Retention of Inhaled Diesel Particles
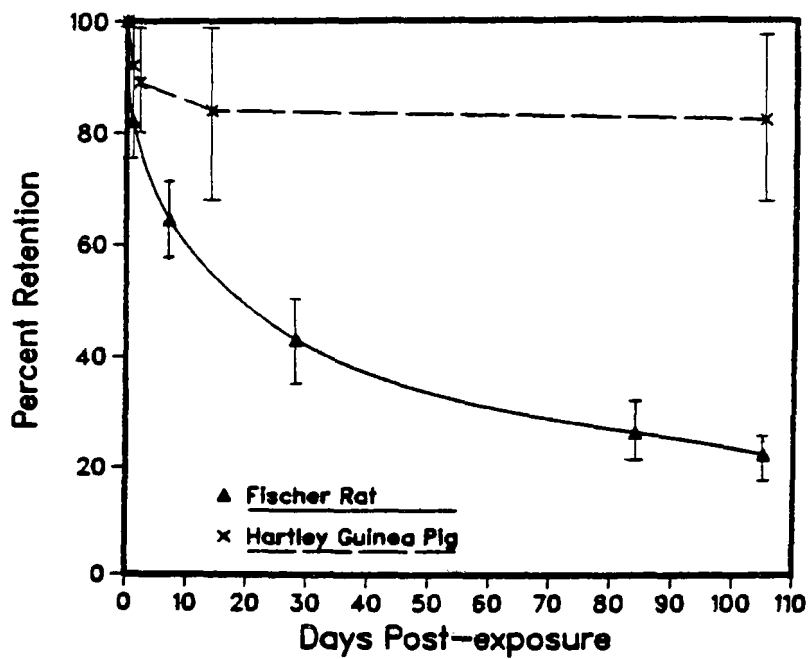
SPECIES COMPARISONS OF BRONCHOALVEOLAR
LAVAGES FROM GUINEA PIGS AND RATS EXPOSED IN VIVO
TO DIESEL EXHAUST (DE)

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This ultrastructural, biochemical and functional study involved 93 Hartley guinea pigs and 63 Fischer rats divided into control and DE exposed sets of 3 animals. Preliminary reports on certain aspects of this study have been published (1-3). Clean air or DE exposures of 250, 750, 1500 and 6000 µg DE particulates/m^3 were inhaled for a few days or chronically up to 18 mon. Broncholavage with Dulbecco's phosphate buffered saline provided free cells, which in controls, were 89-100% macrophages. Viability according to dye exclusion was 90-99% in all specimens. The absolute number of alveolar macrophages increased 1.4 to 2 times control values (7.63 ± 1.11 million cells) by 6 and 12 mon exposure to 750 and 1500 µg DE in guinea pigs. In a short term study in rats after 2 mon 6000 µg DE macrophage number was 5 times controls. Macrophage size also increased, excepting 250 µg DE sets. Maximum diameters and surface areas were measured on scanning electron micrographs of cytocentrifuged lavage specimens. Calculated macrophage volume increased up to 4 times the controls. After the in vivo DE exposure there were ultrastructural signs of macrophage activation. DE particulates were phagocytized without cytotoxic effects since phagosomal membranes remained intact and macrophage lactic dehydrogenase activity (a signal of cell lysis) was not in lavage fluids. Cytofluorometry revealed partial blockade of macrophage phagocytosis of ex vivo fed latex. Macrophages from 12 mon 750 and 1500 µg DE/m^3 exposures had reduced staining for acid phosphatase while the cell free lavage fluids showed 2-5 fold increases in that enzyme. Comparison of equivalently dosed animals revealed similar magnitudes of change in macrophage number and granulocyte recruitment. Upon DE exposure granulocytes became a significant percent of the free cell population by 1 mon at 6000 µg DE, 2 mon at 750 and 1500 µg DE and 12 mon at 250 µg DE. In rat lavages neutrophilic granulocytes appeared contrasting with eosinophilic granulocyte mobilization in guinea pigs. Lymphocytes appeared in lavages of both species after 2 mon. While rats appear to be less responsive to the DE challenge, both species show elevations of albumin, IgG and total protein in the longer duration and higher exposure sets (Table 1). Both granulocyte emigration and elevations in proteins at the high DE doses are features of a classic inflammatory response, but the 250 µg DE exposures even to 18 mon elicited few and generally insignificant changes over controls. Defense capabilities of these healthy rodent species appear adequate to cope with chronic DE challenges at the tested concentrations.

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Table 1. Comparison of Dose-Duration Effects of Diesel Exhaust Exposure on Protein & Enzyme Content of Acellular Broncholavage Fluids

ANIMALS (#) & CONDITIONS	TOTAL PROTEIN mg/ml	ALBUMIN mg/ml	IgG mg/ml	ACID PHOSPHATASE n M/hr/mg prot
GUINEA PIGS (58)				
CONTROLS (16)				
2 WK-18 MON	4.78 ± 1.35	1.55 ± 0.66	0.14 ± 0.10	7.97 ± 4.58
250 µg DE (15)				
2 WK & 2,4,6 MON	4.82 ± 0.93	1.27 ± 0.65	0.12 ± 0.10	10.32 ± 5.07
12 & 14.5 MON	4.74 ± 1.01	1.41 ± 0.66	0.26 ± 0.17	12.60 ± 6.74
18 MON	10.57 ± 4.19	3.95 ± 2.59	0.72 ± 0.19	11.54 ± 5.85
750 µg DE (14)				
2,6 & 8 MON	7.89 ± 2.64	2.19 ± 0.94	0.39 ± 0.27	16.41 ± 3.25
12 MON	11.50 ± 3.77	5.00 ± 2.61	0.54 ± 0.21	29.18 ± 7.94
1500 µg DE (13)				
2 WK, 2 & 6 MON	8.55 ± 0.80	2.17 ± 0.17	0.25 ± 0.07	13.72 ± 3.07
12 MON	17.88 ± 5.64	4.75 ± 0.90	0.92 ± 0.43	61.76 ±23.48
RATS (38)				
CONTROLS (12)				
2-18 MON	1.94 ± 0.21	0.34 ± 0.19	0.05 ± 0.01	19.10 ±12.34
250 µg DE (11)				
2 MON	1.99 ± 0.45	0.28 ± 0.15	0.04 ± 0.02	29.24 ±15.58
12 & 14.5 MON	1.84 ± 0.18	0.28 ± 0.16	0.06 ± 0.01	28.30 ±10.88
18 MON	2.75 ± 0.08	0.33 ± 0.08	0.11 ± 0.03	23.40 ± 3.56
750 µg DE (9)				
2, 5 & 8 MON	2.38 ± 0.15	0.41 ± 0.18	0.07 ± 0.01	27.26 ± 6.95
12 MON	3.55 ± 0.41	0.89 ± 0.26	0.08 ± 0.0	42.14 ± 6.7
1500 µg DE (6)				
2 MON	2.06 ± 0.23	0.59 ± 0.41	0.04 ± 0.03	43.07 ± 2.98
12 MON	7.26 ± 1.09	4.18 ± 2.67	0.27 ± 0.02	70.93 ±10.82

CHEMICAL CHARACTERIZATION OF MUTAGENIC FRACTIONS OF DIESEL PARTICULATE EXTRACTS

by

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Projected increased use of diesel-powered automobiles has stimulated considerable interest in research on health effects of the particulates and identification of deleterious compounds adsorbed to the particulates. Diesel particulates are highly respirable and may present significant inhalation health hazard. It is now well recognized that organic extracts of diesel emission particulates exhibit significant mutagenicity as detected by Ames _Salmonella_ bioassay and several other short-term mutagenicity assays. A great deal of effort has been directed to identification of known as well as hitherto unrecognized mutagens in the particulate extracts.

We have applied Ames _Salmonella_ reversion assay to determine mutagenic potencies of diesel particulate extracts and to aid in isolation of mutagenic fractions for in-depth chemical characterization. In this presentation I will discuss the chemical characterization of mutagenic fractions employing a combination of complementary analytical techniques. Extracts of particulates collected from three vehicles run on a chassis dynamometer-dilution tube have been examined. On-line high performance liquid chromatography-mass spectrometry (HPLC-MS), HPLC-ultraviolet spectroscopy, and gas chromatography-MS provided definitive characterization of a number of compounds in mutagenic fractions. Several polar derivatives of polycyclic aromatic hydrocarbons (PAHs) including some nitrated PAHs have been identified. Some NO_2-PAHs are presently known to be bacterial mutagens. However, it is likely that several polycyclic carbonyl compounds detected in the extracts may also be mutagenic.

PRELIMINARY REPORT OF SYSTEMIC CARCINOGENIC STUDIES ON DIESEL AND GASOLINE PARTICULATE EMISSION EXTRACTS APPLIED TO MOUSE SKIN

by

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Emission particulates may constitute a potential health hazard to persons constantly exposed. We are determining if emission components given by skin application might cause carcinogenesis in other organs. To the skin of SENCAR mice, we applied dichloromethane extracts from particulates collected by filtration of cooled diluted emissions from Oldsmobile (OLDS) (1 mg/mouse), Nissan (10 mg/mouse), and Volkswagen (VW) (10 mg/mouse) diesel, and Mustang V-8 (3 mg/mouse) gasoline fueled engines. Appropriate controls including benzo(a)pyrene (BP) (0.054 mg/mouse), 12-O-tetradecanoylphorbol-13-acetate (TPA), and aging untreated mice (CONTROLS) were maintained. In each treatment group, 40 male and 40 female 6-week-old mice were treated for 52 weeks following one of two protocols: 1) A single initiation dose of the compound was followed twice weekly by applications of TPA (2 µg); or 2) the test compound was applied twice weekly (OLDS only, 4 mg/mouse/week). Surviving mice were killed 52 weeks after initiation and examined grossly; tissues from 20 different organs were routinely taken for histologic examination. The doses chosen were those that gave maximal tumor-initiating activity (1,2).

Survival and tumorigenesis for the experimental groups are shown in Table 1. The numbers of surviving mice were significantly reduced by TPA alone and all experimental groups given TPA reflected this treatment with decreased survival as compared with controls. The only group that was further reduced in survival by the test compound was Nissan + TPA, which had the lowest survival. Lung tumor incidences varied with treatment groups but were not different from controls and TPA lone. Tumors of other organs were observed randomly in the treated groups but showed no consistent increased incidences associated with treatments; tumors were found in the mammary gland, uterus, pituitary gland, cervix, and liver. No evidence of leukemogenesis was seen in killed animals, although 35% (6/17) of dead males had leukemias in BP + TPA group. In mice

that died prior to the kill date significant numbers exhibited squamous cell carcinomas of the skin with 10 to 25% metastases to regional lymph nodes and lungs in Nissan mice. We have also observed high incidences (40 to 60%) of amyloidosis, primarily in the spleen and liver, and pyelonephritis and papillary necrosis in the kidneys of mice given TPA with or without test compounds as initiators. The relationships and pathogenesis of these diseases are now under investigation. The sex effect, which shows a difference in survival as well as tumorigenesis, is not consistent between treatment groups, and its relationship to the tumor process is unclear at this time. When OLDS was given repeatedly over the 52-week treatment period (protocol 2), tumor incidences were not different from untreated controls; it was not effective as a complete carcinogen with this dose and protocol.

Further analysis of remaining treatment groups and complete of observations on mice through 24 months of age will provide information about temporal advancement and tumor incidence modifications by various treatments.

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Table 1. Survival and Tumor Induction in SENCAR Mice Surviving 1 Year Given Diesel and Gasoline Particulate Emission Extracts to the Skin

Treatment	Sex[a]	No. surviving mice (%)	Tumors					
			Skin					
			T-positive (paps) (%)	No. paps/ No. surviving	T-positive carcinomas	Lung (%)	Misc. (%)	Leukemias (%)
Untreated controls	F	100	0	--	0	3	0	3
	M	97	0	--	0	0	0	0
TPA	F	67	19	0.23	0	4	0	0
	M	71	4	0.04	4	4	4	0
BP + TPA	F	81	74	3.10	0	6	13	0
	M	56	100	4.70	14	4	0	0
OLDS + TPA	F	74	27	0.38	3	14	3	0
	M	95	42	0.67	3	19	0	6
Mustang + TPA	F	80	31	0.46	9	3	0	0
	M	71	12	0.12	0	8	0	0
Nissan + TPA	F	47	94	4.50	11	0	5	0
	M	46	89	3.40	5	5	0	0
VW + TPA	F	68	54	1.00	8	0	4	0
	M	75	50	1.00	3	3	0	0
OLDS (no TPA)	F	97	0	--	0	0	3	0
	M	97	0	--	0	3	3	0

F = female; M = male.

INFLUENCE OF DRIVING CYCLE AND CAR TYPE ON THE MUTAGENICITY OF DIESEL EXHAUST PARTICLE EXTRACTS

by

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Solvent extracts of particles collected from the exhaust of diesel trucks and automobiles are known to be mutagenic in bacterial test systems. To reasonably predict the potential health hazard of diesel exhaust emissions, differences in toxicity likely to be produced by different cars, or under various driving conditions, were studied. Extracts of exhaust particles collected from Oldsmobile, Peugeot, Fiat, Mercedes, Audi and Volkswagen diesel automobiles were evaluated for mutagenicity in standard and dinitropyrene-resistant <u>Salmonella</u> tester strains.

EXPERIMENTAL

Diesel exhaust particles were collected from the exhaust of cars operated on a climate-controlled chassis dynomometer at the Bartlesville (Oklahoma) Energy Technology Center. Cars were acquired new from the dealer or on loan from the manufacturer and allowed a 4000 mile break-in period before testing. All test vehicles were operated according to the EPA Federal Test Procedure (FTP). To study the influence of driving cycle, exhaust samples were also collected while driving the Oldsmobile on the Highway Fuel Economy Test (HFET) and the New York City Cycle (NYCC). The exhaust was diluted in a tunnel sized to cool the air/exhaust mixture to below 125°F, and the particulate portion collected on 40 x 40 inch Pallflex T60A20 filters. All tests were conducted with the same standard #2 diesel fuel.

The organic material associated with the exhaust particles was extracted by ultrasonication in dichloromethane and the extract evaporated to dryness under nitrogen. A portion of the extract was fractionated by high pressure liquid chromatography (HPLC) on a Biosil A column and eluted in a gradient from 94% isooctane to 100% dichloromethane. Only the gamma-1 fraction (1)

was evaluated in these studies. The extracts were evaluated for mutagenicity in Salmonella strains TA 100, TA 98, or TA 98-1,8-DNPR, without the addition of a liver enzyme homogenate. Five concentrations of each sample were tested in triplicate, and the results reported as the slope of the dose-response curve (revertants/µg) calculated by linear regression analysis. Since results of mutagenicity testing reflect only the genetic toxicity of the material extended from the filters, the results were normalized for differences in the amount of extractable material associated with the particles (extractable fraction) and particle emission rates. This provided an estimate of the amount of mutagenic activity emitted from the exhaust per mile of vehicle operation (revertants per mile).

RESULTS

Influence of Car Type - Extracts of exhaust particles collected from all six cars demonstrated direct, dose-related increases in mutagenicity in TA 100 (Table 1). The amount of dichloromethane extractable material associated with the exhaust particles (extractable fraction) produced by the cars varied markedly, and was inversely related to mutagenic potency of the extracts. The particle emission rates varied by about 3-fold in the six cars. Normalizing the mutagenic potency for extractable fraction and particle emission rates yielded "revertants per mile" values of a different ranking than that shown by the revertants per µg value.

Influence of Driving Cycle - Operating the Oldsmobile on highway, urban and congested urban driving cycles did not markedly influence the mutagenicity of exhaust particle extracts but dramatically changed the particle emission rate and extractable fraction (Table 2). Increasing the extent of stop and start driving increased the particle emission rate but decreased the amount of extractable material associated with the particles. Thus estimates of mutagenicity emitted from the exhaust (revertants per mile) were similar for the three driving cycles.

Mutagenicity in a Nitroreductase-Deficient Strain of Salmonella - Because of the occurrence of nitro-substituted polycyclic aromatic hydrocarbons (PAH) in diesel exhaust extracts (1) and the extreme potency of nitro-PAH in Salmonella, presumably due to their high nitroreductase activity (2), the extracts were evaluated in a strain shown to be resistant to the mutagenicity of some nitro-PAH. Mutagenicity of the gamma-1 HPLC fractions of the extracts, previously shown to contain nitro-PAH (1), were markedly lower (20-60%) in TA 98-1,8 DNPR than the standard TA 98 tester strain.

CONCLUSIONS

The similar mutagenic potencies of extracts of particles collected from six different diesel cars operated on the same fuel suggest that engine design has very little influence on mutagenicity of the particle associated organic materials. Driving cycle also did not significantly alter mutagenicity of the particle extracts. The large variability in extractable fraction and particle emission rates between different cars, and in the same car operated on different driving cycles, emphasizes the need to include these variables

when estimating the quantities of potentially hazardous materials emitted from the exhaust. The lower response of nitro-PAH containing fractions of the extracts in a nitroreductase deficient <u>Salmonella</u> strain is difficult to interpret since it is not known if the enzymes which activate nitro-PAH to mutagenic metabolites in <u>Salmonella</u> are unique to bacteria. (Research performed in part under DOE Contract Number DE-AC04-76EV01013.)

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Table 1. Influence of Car Type on Mutagenicity of Diesel Exhaust
          Particle Extracts

Test Vehicle	Rev/μg Extract (TA-100)	Extractable Fraction (%)	Particle Emissions (g/mi)	Revertants per mile (x 10^3)
Fiat 131	6	71	.34	1500
Peugeot 504	13	29	.21	800
Audi 5000	13	43	.51	2900
Oldsmobile D-88	17	20	.38	1300
VW Rabbit	16	26	.17	700
Mercedes 300	15	13	.26	500

Federal Test Procedure, hot start used for all tests

Table 2. Influence of Driving Cycle on Mutagenicity of Diesel
          Exhaust Particle Extracts

Driving Cycle[a]	Average Speed (mph)	Rev/μg Extract (TA-100)	Extractable Fraction (%)	Particle Emissions (g/mi)	Revertants per mile (x 10^3)
HFET	50	15	35	.22	1100
FTP	20	16	21	.33	1100
NYCC	7	13	12	1.23	1900

[a]Oldsmobile Detla-88 used in all tests

CCMC'S HEALTH EFFECTS RESEARCH PROGRAM

by

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1. GENERAL

In the past decade much progress has been achieved in cleaning
the air by a concerted effort of the governments and the automotive
industry. With more health effect data becoming available, further
legislative measures involving more stringent car emission standards
become more and more questionable. Before stricter standards for
emissions of motor vehicles are legislated, resources for research
programs on health effects must be deployed on an increased scale.

Already in 1974 in the U.S. the National Academy of Sciences (NAS)
established that there were virtually no results available relating to
the effects of automobile emissions. Since then the situation has not
altered greatly. It is only with the concern about cancer with regard
to diesel-powered cars that larger research programs have now been
started in the U.S.

Although a limited number of research investigations have shown
no carcinogenic effects of human exposure to diesel-engined vehicles'
exhaust gases up to now, the European car manufacturers have also
initiated a large scale research project on "an investigation into
possible toxicological and carcinogenic effects of diesel and gaso-
line engine exhaust emissions". The project is sponsored by the
Committee of Common Market Automobile Constructors (CCMC).

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     S. Wallman (Volvo), J.H. Weaving (BL)

2. OBJECTIVES

This program is considered to be a first step in what will
probably be a continuing investigation by the European industry on
the potential hazards associated with the emissions from automobiles.

The objectives of this first CCMC program are

- to compare potential toxic and carcinogenic effects
  of diesel and gasoline engine emissions,
- to check out the presumed beneficial effect of the
  catalytic converter of gasoline engines,
- to check out the often assumed relative harmlessness
  of the gaseous fraction of the diesel emissions,
- to investigate the mutagenic properties of diesel
  and gasoline engine particulates and condensates.

3. PROGRAMS

The project is divided into two parts:

(1) a "long term" inhalation exposure of rats and hamsters
    to gasoline and diesel engine exhausts;

(2) a "short term" program of in vivo and in vitro tests on
    the effects of particulate extracts and condensates from
    gasoline and diesel engine exhausts.

3.1 Long Term Program

The "long term" inhalation program has been contracted
out by the CCMC to the Geneva Division of the Battelle
Memorial Institute.

With about 6000 hamsters and rats the combined effects of
different concentrations of total exhaust from gasoline engines
with and without catalyst, and a diesel engine with and without
particulate matter, removed by filtration, operating according
to the U.S. FTP cycle, are being investigated to determine the
dose/response relationship, and to make a carefully controlled
comparison of diesel and gasoline engines, with respect to
toxicity and carcinogenicity. The running time of the project
is 3 years and costs will amount to 4 million U.S. dollars.

The program is described in detail in another paper in this Symposium.

3.2 Short Term Program

The "short term" program, including three main approaches, is being conducted in separate laboratories in England, France and Italy.

In different short term in vivo and in vitro tests with bacteria, mammalian cells, rats and mice, the effects of particulate extracts and condensates from gasoline and diesel engine exhaust, collected at Fiat, Italy, are being investigated in an endeavour to identify the mutagenic components.

The running time of this project is three years and the costs will amount to 0.73 million U.S. dollars.

Three main approaches were taken:

3.2.1 "in vitro" assays - detection and identification of mutagens (Microtest, University of York, United Kingdom)

The four basic preparations (particulate and condensate, both diesel and gasoline) in total and additionally fractionated in accordance with the EPA procedure, are subjected to a modified AMES procedure (bacterial mutation assay) using five strains of salmonella typhimurium (TA 1535, TA 1537, TA 1538, TA 98 and TA 100). The preparations are also tested for the ability to elicit unscheduled DNA synthesis (UDS) in cultured human fibro-blast, (DNA repair assay using Hela cells in culture) and in vitro transformation in rodent cells (mammalian cell mutation assay using mouse lymphoma L 5178 Y cells). All these assays have been combined with a liver monoxygenase enzyme fraction.

The tests are also carried out with extracts of the particulate fractions.

3.2.2 skin painting tests - detection and identification of promoters (Institute of Scientific Research on Cancer (CNRS), Paris, France)

In the short term work sebaceous gland and hyperplasia tests are performed with the different exhaust preparations. Investigation is being undertaken to separate potential cancer initiators and cancer promoters.

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Female 45-day old Swiss mice housed individually and randomised between the different groups receive, on well delineated skin areas previously clipped (3 days before), 0.05 ml of acetone solution of the studied substance. The treatment is repeated on alternate days up to a total of 3 skin applications (more applications may be necessary). Eight days after the first treatment, the mice are killed and the areas of treated skin fixed, sectioned and stained for histological examination. The thickness of the epidermis and the number of sebaceous glands present are determined in 2 to 4 microscopic fields of each of 6 sections that are cut from each skin specimen. The microscopic examination is carried out on code-numbered slides which carry no details of treatment given.

For each term or treatment, 25 to 30 mice are being used. To control the model and to evaluate the relative activities of the test compounds, adequate positive (benzo-a-pyrene and forbol ester-tpa) and negative (solvent) control groups will be provided for each assay.

3.2.3 in vivo tests - possible extraction of mutagens from particulate matter in the whole animal (bioavailability) (University of Naples, Department of Biochemistry, Italy)

An important aspect of the CCMC short term project is the bioavailability of any mutagen or possible carcinogen and although inhalation is the preferred test method, due to the long term nature of this process, additional short term tests are being conducted with animals.

These tests consist of assimilating the sample (diesel particulates) into an appropriate medium, such as corn oil, bovine serum albumen and human blood serum and injecting this into the peritoneum and subsequently subjecting the urine to appropriate AMES assays to test for mutagenicity. These tests are being carried out using male and female Sprague-Dawley rats. Three animals for each dose are being used.

FRACTIONATION AND IDENTIFICATION OF ORGANIC COMPONENTS IN DIESEL EXHAUST PARTICULATE

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Diesel exhaust particulate, generated using production model passenger car engines on a chassis dynamometer, was extracted from Teflon-coated glass fiber filters with methylene chloride and fractionated using either a solvent partition scheme partition scheme or low pressure liquid chromatography (LPLC). The solvent partition scheme(1) generated two acid, two base, a cyclohexane insoluble, polar neutral, non-polar neutral, and PNA fractions. The LPLC scheme generated fractions which elute from a silica gel (Lobar) column with 10% CH_2Cl_2/90% hexane (Fraction F1 and part of F2), 50% CH_2Cl_2/50% hexane (part of F2 and F2A), 100% CH_2Cl_2 (F3 and F4), 10% CH_3OH/90% CH_2Cl_2 (F5 and F6), 50% CH_3OH/50% CH_2Cl_2 (F7 and F8) and 100% CH_3OH (F9 and F10). A hexane-insoluble (HI) fraction was analyzed using the same preparative LC technique. The fractions generated by both schemes were analyzed by normal phase HPLC, glass capillary GC/MS/DS (electron impact [EI], chemical ionization, and negative ion chemical ionization [NICI]), direct probe NICIMS, direct probe EI high resolution MS, FTIR and $(GC)^2$/FTIR.

Including isomers, 52 polycylic aromatic hydrocarbons and alkyl derivatives, 35 PNA-ketone and di-ketone derivatives, 20 aromatic aldehydes and cyclic anhydrides, 26 nitrogen-containing PNAs (including nitro PNAs) and 30 other compounds (including alkanes and some possible background contaminants) were identified.

The compounds of particular interest are the various PNA ketones (e.g., fluorenone) and the nitro PNAs(e.g., nitropyrene) and their alkyl-substituted homologs. These compounds are listed in Tables 1 and 2. The nitro PNAs are of interest in light of recent findings that some of them are highly mutagenic.(2-4) The nitro PNAs were found in the more mutagenic fractions. Thus, it may be speculated that these compounds are contributing much or possibly most of the mutagenic activity to these fractions and therefore to the diesel exhaust particulate itself.

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Table 1. POLYCYCLIC KETONES AND DIONES IDENTIFIED IN NISSAN DIESEL EXHAUST PARTICULATE

Identified Compound	Number of Isomers Identified	Analysis					Fraction(s) Containing Compound Identified
		GC/EIMS	GC/NICIMS	HRMS	GC/FTIR	Other	
naphthoquinone	1	x					F4
9-fluorenone or $C_{13}H_8O$ [2] isomer	1	x	x				F4;F3;F2
methylfluorenone isomer or $C_{14}H_{10}O$ isomer	3	x	x				F4;F3;F2
anthrone or phenanthrone isomer	1	x					F2
C_2-alkyl fluorenone isomers or $C_{15}H_{12}O$ isomers	4	x					F3;F2
C_3-alkyl-fluorenone isomers or $C_{16}H_{14}O$ isomers (tent)	4	x	x				F3;F2
C_4-alkyl fluorenone isomers or $C_{17}H_{16}O$ isomers (tent)	2	x	x				H1;F2
xanthone (tent)	1	x					F3
anthraquinone	1	x			?[3]		F3;F2
4H-cyclopenta(def)phen-anthrene-4-one (tent)	1	x					F3;F2
benzanthrone isomers	3	x					F3;F2
methyl-4H-cyclopenta(def)-phenanthrene-4-one isomer (tent)	2	x					F2
benzofluorenone isomers (tent)	2	x					F2
$C_{18}H_{12}O$ ketone isomers [3] (tent)	2	x	x				F2
$C_{18}H_{10}O_2$ dione isomer [4] (tent)	1	x	x				F2
6H-benzo(cd)pyrenone isomers or $C_{19}H_{10}O$ isomer (tent)	3	x	x				F2
C_2-alkyl-4H-cyclopenta(def)phenanthren-4-one isomer (tent)	2		x				F2
C_5-alkyl fluorenone isomer or $C_{18}H_{18}O$ isomer (tent)	1		x				F2

[1] See text for fraction identifications.
[2] Other possible isomers include perinaphthenone and benzoindenone isomers.
[3] Tentative identification.
[4] Possible isomers include naphthacenone, triphenylenone, chrysenone, and methylbenzanthrone isomers.
[5] Possible isomers include di-ketones of naphthacene, chrysene, and triphenylene.

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Table 2. NITROGEN CONTAINING AROMATICS IDENTIFIED IN NISSAN DIESEL EXHAUST PARTICULATE

Identified Compound	Number of Isomers Identified	Analysis					Fraction(s) Containing[5] Compound Identified
		GC/EIMS	GC/NICIMS	HRMS	GC/FTIR	Other	
N-phenylnaphthylamine isomer	1	x			x		F4;F2;F5;F6;F3;F8;F1G
C_2-alkyl-N-phenylnaphthyl-amine isomer (tent)	1	x					F5
benzo(c)cinnoline[2]	1	x					F5;HI
methylbenzo(c)cinnoline[2] isomers	3	x					F5
$C_{13}H_9O$ isomer[3] (tent)	1	x					F6
nitroanthracene isomer or nitrophenanthrene isomer	1	x	x		x		F1G;F2
methylnitroanthracene or methylnitrophenanthrene isomers (tent)	4	x	x				F1G;F2
C_2-alkyl nitroanthracene or C_2-alkyl nitrophenanthrene isomers (tent)	7	x	x				F1G
C_3-alkyl nitroanthracene or C_3-alkyl nitrophenanthrene isomers (tent)	4	x					F1G
nitropyrene isomer or $C_{16}H_9NO_2$ isomer	1	x	x		x		F2
methylnitropyrene isomer or nitrobenzofluorene isomer (tent)	1	x	x				F1G;F2
$C_{18}H_{11}NO_2$ isomer[4]	1	x	x				F2

[1] See text for fraction identifications.
[2] It is possible that these are polycyclic ketones of the formulas $C_{13}H_8O$ and $C_{14}H_{10}O$. However, their mass spectra more closely resembled those for benzo(c)cinnoline in standard spectra. These compounds were also later eluting than 9-fluorenone and its alkyl homologs. Further elucidation of these compounds is currently underway for fraction F5 of the refractionated HI sample by means of GC/FTIR and HRMS to determine whether these are indeed benzo(c)cinnolines.
[3] This eluant gave a mass spectrum similar to that of acridine or benzoquinoline, but only a trace quantity of the compound was present.
[4] Possible isomers include nitrochrysene, nitronaphthacene, and nitrotriphenylene isomers.
[5] Fraction F1 was further fractionated to yield subfractions F1A through F1G.

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EFFECT OF CHRONIC DIESEL EXPOSURE OF PULMONARY PROTEIN SYNTHESIS IN RATS

by

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There is evidence that when rats are subjected to acute exposure to cigarette smoke, hepatic protein synthesis is inhibited and the extent of inhibition is positively correlated with the dosage of smoke. The present study has been undertaken to determine the effect of diesel smoke on pulmonary protein synthesis. For this study, male Fischer rats have been exposed to diesel exhaust (6 mg/m^3) for 2, 4 and 8 weeks. At the end of the desired exposure periods, the lungs have been removed respectively from the exposed and time-matched control rats and placed in an isolated lung apparatus.

The apparatus was devised by modifying the design of Fisher et al., (2) for the perfusion of lung excised from rats. Briefly, for perfusion of each lung, the animal was anesthetized with pentobarbital intraperitoneally and trachea was cannulated and connected to a Harvard respirator. At this point, by means of a pressure transducer placed in the in-flow route of the respirator, we recorded in situ ventilation pressure for 3-4 minutes in an eight channel recorder. This allowed us to compare in vivo tracheo-bronchiolar air resistance under inspiratory pressure of 10 cm H_2O with that of the resistance when the lungs would be under ex vivo condition. Following this procedure, thoracotomy was conducted and the lungs were excised by carefully separating pulmonary artery from the aorta. Once the pulmonary artery was clearly dissected out from the other mediastinal structures, heparin (0.5 units/g) was injected through this. After a few minutes of circulation of heparin, the pulmonary artery was separated from the right ventricle and the open end was cannulated for delivery of perfusate at 15 ml/min by means of a Harvard peristaltic pump from the reservior. While perfusate was going through the lungs, a small incision was made in the left artrium so that the perfusate might flow freely and wash blood out of the pulmonary vascular bed. When the lungs were cleared of blood, they were transferred to a water-jacketed perfusion chamber maintained at 35°C. During the transfer, we kept respiring the isolated lung while interrupting the perfusate only for a few seconds. The peristaltic pump was then switched on and upon perfusion of the isolated lungs the perfusate freely drained into the perfusion chamber and from there by means of Tygon tubing back to the reservoir. Thus, a fixed volume of

perfusate continuously recirculated through the lungs for the entire duration of the experiment. Moment to moment performance of the preparation was monitored by recording continuously: pH and pO_2 of the perfusate before it entered the lungs and the pressures required for tidal ventilation and for delivery of perfusate at 15 ml/min, through the pulmonary artery.

One exposed and one control Fischer rat was tested in one day, and two such runs were conducted each week. It required approximately two hours to complete one perfusion experiment which consisted of 30 minutes of equilibration followed by one hour of ^3H leucine incorporation. During the equilibration period, the lungs were perfused by recirculation with 50 ml Leibovitz solution and watched for any change in pH, $pO2$, ventilation and perfusion pressures. If all these parameters looked normal, which was the case for all the 24 experiments needed to complete the series, the perfusion medium was changed to fresh 50 ml Liebovitz solution containing approximately 1 µCi/µ mol/ml of ^3H-leucine. Thus, the perfusion technique made it possible to provide a constant supply of substrate, ^3H leucine, with a desired specific activity all throughout the incorporation period.

At the end of ^3H leucine incorporation period, samples were taken for autoradiography and, then, the rest of the lungs were subjected to homogenization in 0.02 KH PO_4 and TCA precipitation of protein which was washed with polar and non-polar solvents. The washed protein residue was dissolved in 4.0 ml of 2.0 N NaOH at 50°C. The protein was determined by the method of Sedmak and Grossberg (3). Total pulmonary DNA was determined by using the modified diphenylamine technique of Burton (4) following precipitation of DNA. Radioactivity obtained from incorporated ^3H-leucine was measured in 1.0 ml samples of the dissolved protein using a Beckman LS-100 counter. Because there were differences in quenching between the diesel exposed (very dark due to presence of diesel particles) and control samples, internal standard (^3H tolune) was used to correct the observed CPM before expressing the results in DPM which was converted to nmole ^3H-leucine by using the known specific activity. Results were normalized on the bases of mg protein as well as mg DNA as obtained per gram of lung tissue. The results of ^3H leucine incorporation as shown in table 1 revealed that 8 weeks of exposure of male Fisher rats to 6.0 mg particulates/m^3 of diesel engine exhaust had no significant effect on the lungs to incorporate ^3H-leucine into the TCA insoluble protein. Similar results were obtained after 2 and 4 weeks of exposure to diesel exhaust in comparison to respective time matched controls. Also, electron microscopic autoradiographic grain counts as obtained from the diesel exposed vs. control rats showed no particular difference among various groups.

Table 1. Incorporation of ^3H-leucine by the perfused lungs as obtained from rats after 8 weeks of exposure and their time matched controls.

Experiment	nmoles leucine per mg protein	nmoles leucine per mg DNA	Protein/DNA
8 weeks of air exposure	3.53 + 0.23*	41.3 + 3*	11.8 + 0.4*
8 weeks of diesel	3.30 + 0.48	46.3 + 6	13.9 + 1.1

* Means + S.E.

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THE EFFECT OF EXPOSURE TO DIESEL EXHAUST ON PULMONARY PROTEIN SYNTHESIS

by

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Previous work performed in collaboration with the Biomedical Research Division of General Motors Corporation had indicated that exposure of rats to diluted diesel exhaust for periods of up to one year did not induce the activity of microsomal benzo[a]pyrene-oxidizing enzymes in lung tissue. Several explanations for this observation, including the possibility that exposed animals are unable to respond to inducing agents, have been suggested. In this report, we will discuss the ability of animals which have been exposed to exhaust in the concentration of 6 mg/m^3 of diesel particles to synthesize pulmonary proteins as judged by in vivo ^{3}H-leucine incorporation and to respond to 3-methylcholanthrene by the induction of pulmonary oxidative metabolism of benzo[a]pyrene.

THE RAPID ANALYSIS OF DIESEL EMISSIONS USING
THE TAGA 6000 TRIPLE QUADRUPOLE MASS SPECTROMETER

by

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Because of the increasing consumption of diesel fuels by cars, trucks, heavy equipment, and industry, the environmental scientist is concerned with the atmospheric loading of toxic combustion products and their detrimental biological effects.

The conventional analysis of diesel fuel combustion products is very time-consuming and difficult, since the quantity of toxic pollutants such as polycyclic aromatic hydrocarbons (PAH) and their nitro derivatives is low, and since they are often associated with other contaminants (for example, unburned diesel fuel). The analysis entails sample trapping, extraction, substantial clean-up, and determination by capillary column gas chromatography combined with mass spectrometry.

Using MS/MS, the particulate extract can be directly analyzed for nitropolycylic aromatic hydrocarbons without sample clean-up. The sample is deposited unto a temperature program direct insertion probe operated at atmospheric pressure, and is desorbed over a period of twenty minutes. In the negative mode, parent ions which yield a daughter ion of m/z 46 [NO_2^-] can be scanned, or target compounds can be quantitated by integrating the desorption curve or the m/z 46 daughter ion in the multiple ion monitoring mode. Calibration plots for nitropyrene in spiked samples of diesel particulate extract are linear (r = 0.99), and the extrapolated detection limit for nitropyrene in the diesel extract is in the ppb range.

Since the vapor phase emission of diesel engines are also of interest, the exhaust has been sampled directly by the TAGA 6000 MS/MS. In this study, emissions of a diesel-powered vehicle were transported through a heated (150°C) Teflon pipe at 2 L/sec, and a small portion of this flow was admitted directly into the atmospheric pressure ion source. The mass spectrometric analysis was based on:

    (i)   the identification of particular compounds in the exhaust gas;
   (ii)   the rapid screening of the exhaust gas for nitro compounds;
  (iii)   the analysis of the exhaust gas for specific nitro-PAH.

PREPARATION OF DIESEL EXHAUST PARTICLES AND EXTRACTS
AS SUSPENSIONS FOR BIOASSAY

by

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A bioassay program is being conducted at IITRI to evaluate the acute toxicities and carcinogenic potentials of diesel engine exhaust components, cigarette smoke condensates and organic solvent extracts of roofing tar volatiles and coke oven emissions. The test materials were administered to hamsters by the intratracheal route. Administrations in both the acute toxicity and carcinogenic potential bioassay experiments have been completed.

The test materials were supplied through the U.S. EPA Biomedical Research Branch. The diesel engine exhaust components supplied were a whole particle exhaust consisting of carbonaceous soot with adsorbed liquid and gaseous species, and a dichloromethane extract of the whole particle exhaust. The cigarette smoke condensate was supplied as a concentrated solution in acetone. Both the roofing tar and coke oven emission extracts were supplied as dichloromethane solutions.

The intratracheal administration route required preparation of stable suspensions and emulsions of the test materials in fluids compatible with the hamster respiratory tract fluids. Saline was the obvious suspending fluid to be used but additional ingredients were required to enable suspension of the particles and the solvent-free extracts. Examination of the as-received whole particle exhaust revealed that very large (up to 150 μm) diesel particle aggregates were present. These large particles hindered suspension preparation and were not suitable for intratracheal suspension. Therefore, a research program was conducted to develop methods for preparing saline suspensions of the whole particle exhaust in particle size ranges amenable to intratracheal instillation and saline emulsions (liquid-liquid suspensions) of the various extracts.

For the short-term acute toxicity studies, a simple wet ball milling

method was developed which both reduced the size of the diesel engine exhaust particles and suspended them in saline. The hydrophilic nature of the diesel particles required that they first be wetted with propylene glycol in the glass milling jar before the saline suspending fluid and the glass milling beads were added. Gelatin was also added to the saline to serve as a protective colloid.

Simple hand emulsifying techniques were used to prepare the diesel engine exhaust extracts and the other extracts condensate as stable emulsions in the gelatin-saline fluid for the short-term acute toxicity studies. Standard glass tissue grinders proved to adequately emulsify the four types of organic extracts, once the solvent had been removed and they were wetted with propylene glycol and a surface active agent. To maintain the emulsion stabilities for more than 30 minutes, gum arabic was also added to the saline to provide a stronger protective colloid.

The greater materials requirements for the larger scale carcinogenic potential bioassay experiments required development of a semi-mechanized method to prepare the emulsions of the test materials. Trial emulsion preparations with a Polytron^R tissue homogenizer proved successful and a unit was purchased. The Polytron^R is a high speed mixing device which employs both mechanical shear action and ultrasonics to accomplish homogenization of liquid samples. Various types of homogenizing probes are available and provide a wide range of shear and ultrasonic energies. One probe design provides sufficient shear energy to reduce particle sizes of soft solid materials such as the diesel engine exhaust particles.

Thus, for the long-term carcinogenicity bioassay experiments, development of protocols to prepare the suspensions and emulsions with the Polytron^R were conducted. The whole particle diesel engine exhaust suspensions were easily prepared as stable suspensions in gelatin-saline, once the particles had been wetted with propylene glycol. Particle concentrations as high as 25 mg/ml were attainable. The primary advantages of the Polytron^R milling over ball milling to prepare the particle suspensions were shorter preparation times (one hour versus 10 days elapsed time), elimination of glass milling contaminants, and reduction of the reagglomeration tendency after milling was completed. The diesel engine exhaust extract was also easily prepared as an emulsion in gelatin-saline once the extract had been wetted with propylene glycol (after solvent removal). The use of the high energy Polytron^R to emulsify the diesel extract as well as the other organic extracts in saline eliminated the necessity of adding a surface active agent. However, gum arabic was still required as an additional protective colloid to maintain emulsion stability.

Assay and characterization methods for the prepared suspensions were also developed. While the emulsions could be characterized microscopically, no practical assay methods could be developed.

COMPOUNDS IN CITY AIR COMPETE WITH ^{3}H-2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN FOR BINDING TO THE RECEPTOR

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It is well known that filter collected urban particulate matter contain compounds which are mutagenic in the Ames' Salmonella assay in the absence of rat liver metabolic activation, showing that these compounds are different from conventional polycyclic aromatic hydrocarbons (PAH). In the present study, we have shown that such particulate matter also contains compounds with an affinity for the rat liver receptor protein which specifically binds 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The content of conventional PAH cannot account for the degree of binding.

Urban particulate matter used in the investigation was collected on glass fiber filters by high volume sampling at roof top levels in central Stockholm and a suburban site. Collection, Soxhlet extraction with acetone, extract preparation in dimethyl sulfoxide (DMSO) and mutagenicity testing of the extracts in the Salmonella assay have been described elsewhere. The affinity of the particulate fraction of the air samples to the rat liver receptor protein was measured by competition for the binding of ^{3}H-TCDD, as described below.

Liver cytosol was prepared and diluted to 3.5 $A_{280-310}$/ml 2-2.5 mg protein/ml). Each experiment consisted of a series of ten incubations of one ml of cytosol with 1.5 nM ^{3}H-TCDD. Two experiments were carried out in the presence of 20 μl of DMSO (total binding). Two incubations contained 150 nM radioinert 2,3,7,8-tetrachlorodibenzofuran (TCDBF) (non-specific binding). The remaining six incubations contained varying amounts of the extract of the particulate fraction of an air sample. 2.5-20 μl of the extracts, dissolved in DMSO, was added to the incubation mixture and the volume of DMSO was then adjusted to 20 μl/incubation. After incubation at 0°C for 60 min, the incubations were treated with dextran-coated charcoal and the amount of ^{3}H-TCDD bound to the receptor in each of the ten incubations was measured by isoelectric focusing carried out in polyacrylamide gel as described previously.

The non-specific binding of ^{3}H-TCDD was obtained from the two incubations in the presence of 150 nM radioinert TCDBF and was subtracted from the total binding in each of the other incubations (= specific binding). The specific binding of ^{3}H-TCDD in the presence of extracts of the air samples was expressed as a percentage of the specific binding in the control incubation (^{3}H-TCDD + DMSO). The relative binding affinity for the individual air samples was calculated from log-logit plots of the competition for TCDD-binding, where logit $b = \ln(b/1-b)$. The log-logit plots were calculated using linear regression analysis. Each extract was analysed 3-4 times at a suitable dilution.

An unused air filter (blank filter) was extracted in the same manner as

the other samples and tested for competition for receptor binding. The results were expressed as an equivalent volume of air. Benzo(a)pyrene and TCDBF were dissolved in dioxane and the competition for binding to the receptor measured.

The results are shown in Table 1, in which are given the mutagenic effects in Salmonella TA 98 and TA 100 in the absence of mammalian metabolic activation and the relative binding affinities expressed as the concentration of air sample extract that competes for 50% of the TCDD-binding to the receptor (ED_{50}; m^3 air/ml cytosol). Log-logit plots for the different air samples indicated that they competed for the same binding site. There seemed to be a gross correlation between the binding affinity and the pollution level as measured by mutagenic effects. Samples collected in the summer had higher ED_{50}'s than samples collected in the winter and spring.

Several compounds are known to bind to the receptor protein including many PAH present in urban particulate matter. Some of the particulate samples were analysed for PAH. Assuming that all PAH present at concentrations above 0.1 ng/m^3 have the same binding affinity for the receptor protein as benzo(a)pyrene $(B(a)P)$, it can be calculated that the ED_{50} value should be 1.7, 15.3 and 0.07 m^3 of air for sample 148, 173 and S-258, respectively. The observed values were 0.035, 0.14 and 0.015 m^3, i.e. known PAH may account for about 2.4, 0.8 and 22.4% of the binding, indicating that other types of compounds are of major importance.

TCDD and TCDBF have the highest binding affinities for the receptor among investigated compounds. Two samples have been analysed for TCDD and TCDBF, and the concentrations were below the detection levels of 2 pg/m^3. Consequently, these compounds cannot account for the binding.

The affinity of a compound for the TCDD receptor is well correlated to the magnitude of aryl hydrocarbon hydroxylase (AHH) induction caused by that compound. Strains of mice with high AHH inducibility are more susceptible to pulmonary cancers caused by 3-methylcholanthrene than strains with low inducibility, indicating a link between AHH activity and appearance of pulmonary tumors. The rat lung has a high content of receptor protein, and the capability of the human lung to metabolise B(a)P indicates the presence of the receptor protein also in this tissue. Although TCDD has been shown to be a potent carcinogen in chronic feeding studies, it is apparent that TCDD shows no or very little mutagenic activity in in vitro bacterial test systems such as the Ames test and a very low covalent binding to DNA in vivo. In a recent study, however, it was shown that TCDD is a potent promoting agent for hepatocarcinogenesis initiated by diethylnitrosamine. TCDD and possibly also other compounds with an affinity for the same receptor may thus better be described as cocarcinogens and tumor promoters rather than carcinogens. The presence of compounds with affinity for the receptor in urban particulate matter may be of importance with regard to the health implications of urban air pollution. This type of compounds may or may not be similar to the components that are mutagenic in the absence of mammalian metabolic activation in the Ames Salmonella assay.

Conclusions

Acetone extracts of filter-collected urban atmospheric particulate matter contain compounds which can displace ^{3}H-2,3,7,8-tetrachlorodibenzo-p-dioxin from the rat liver receptor protein. The concentration of conventional polycyclic aromatic hydrocarbons or chlorinated dioxins and dibenzo-furans cannot account for more than 0.8-22% of the displacement.

Table 1. Competition for TCDD receptor-binding and mutagenicity in Salmonella TA 98 and TA 100 in the absence of mammalian metabolic activation by extracts of filter-collected urban particulate matter. Samples were collected at roof top levels for 24 h starting about 6 a.m. Samples 148 and 174 were collected at a suburban site 22 km NNW Stockholm and the others in central Stockholm. The ED_{50} is the concentration of competitor that competes for 50% of the specific binding of ^3H-TCDD. The ED_{50}'s (in nM) for 2,3,7,8-tetrachlorodibenzofuran (TCDBF), benzo(a)pyrene (B(a)P), β-naphthoflavone (BNF), 3-methylcholanthrene (3-MC) and benz(a)anthracene (BA) are given for comparison. The mutagenic response is highest in the absence of mammalian metabolic activation and decreases by addition of S9 from rat liver; the decrease is dependent on the amount of S9 added. Samples 258, 264 and 268 were tested for mutagenicity both prior to (Feb. 1980) and after (Sept. 1980) the completion of the TCDD receptor analyses; there were no detectable changes in the mutagenic response.

Sample	Receptor affinity[a] ED_{50} (m^3 air/ml cytosol)	Mutagenic response (revertants/m^3) TA 98	TA 100	Sampling date and site
149	0.049 ± 0.014	19	11	79 04 10 inner city
148	0.035 ± 0.017	14	8	79 04 10 suburban
173	0.137 ± 0.073	3	n.d.	79 07 05 inner city
174	0.302 ± 0.147	1	n.d.	79 07 05 suburban
S-258	0.015 ± 0.007	59	79	80 02 04 inner city
T-262	n.d.	92	102	80 02 05 inner city
S-264	0.039 ± 0.010	30	20	80 02 06 inner city
T-268	0.049 ± 0.026	9	7	80 02 07 inner city
Blank filter[b]	2.18 ± 0.640	<0.2	<0.4	
TCDBF	2.69 ± 1.91 nM			
B(a)P	18.21 ± 9.88 nM			
BNF	7.0 nM			
3-MC	2.9 nM			
BA	3.8 nM			

[a] Values represent the means $\pm$ standard deviation (three to four determinations).

[b] Blank filter extracted with the same volume of acetone. The competition is expressed as an equivalent volume of air.

n.d. Not determined.

GC/MS AND MS/MS STUDIES OF DIRECT-ACTING MUTAGENS IN DIESEL EMISSIONS

by

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While the direct-acting mutagens in diesel emissions have not been un-equivocally identified, evidence has accumulated showing that nitro-PAHs (polycyclic aromatic hydrocarbons) may be a major source of mutagenic activity (1,2). We have found that diesel fuel-PAHs are devoid of mutagenic activity in <u>Salmonella</u> test strains. After reaction with NO_2, the nitro-fuel-PAH mixtures are direct-acting, frameshift mutagens (200-1000 revert-ants/µg) in TA98, but were not active in TA1535, a base-pair substitution indicator strain (2). The cytotoxicity to CHO cells also increases after reaction with NO_2(2). These biological responses are very similar to those observed with diesel soot extract fractions although the activity of diesel extracts is much lower.

The fuel-nitro-PAH mixtures have been found useful as a positive control and as a reference mixture for MS/MS (triple stage quadrapole mass spectrom-etry) analyses. Although the fuel-nitro-PAHs are not resolvable by capil-lary GC/MS, the fuel-PAHs before reaction with NO_2 are readily separated and identified by GC/MS. Since the fuel-PAHs extracted with DMSO (Me_2SO) are readily identified by GC/MS, correlations can be made with the nitro-PAH masses observed in MS/MS spectra of nitro-PAH mixtures. These are used for MS/MS interpretation since MS/MS does not differentiate between isomers of the same molecular weight in most cases. When possible, these interpreta-tions should be confirmed by independent methods (1).

MS/MS was carried out to compare the relative intensities of ions in samples of widely different mutagenic potencies. In this way, it may be possible to estimate which of the nitro-PAHs detected in diesel soot ex-tracts (1) may make major contributions to the total mutagenic activity.

The MS/MS analyses involved: R-NO$_2$$\rightarrow$ R-NO$_2$-H$^+$ (ionization under isobutane chemical ionization conditions) $\rightarrow$ R-N$^+$O (collisionally induced dissociation by collision with N$_2$ in quadrupole #2). The MS/MS instrument was a modified Finnigan 3200, which has been described previously(3). The first quadrupole was scanned from 80 to 350 amu with a scan time of 1.3 sec. The second quadrupole was operated (with RF voltage only) as a collision chamber. The third quadrupole was scanned the same as #1, but 17 amu behind it. In this way, only ions which lost 17 amu in passing through the collision chamber were detected. The instrument was tuned with 1-nitropyrene (parent-H$^+$ ion m/z 248) and the N$_2$ pressure adjusted for maximum m/z 231 daughter ion (m - 17). Extract samples (40 µg) were vola- tilized into the source using a thermal desorber (temperature programmed from 50 to 350° C in 10 minutes).

MS/MS analyses were done on selected samples with an APCI/MS/MS (triple quadrupole MS/MS with an atmospheric pressure chemical ionization source) at the laboratories of Sciex, Inc., Toronto, Canada. The reaction monitored was:

$$R-NO_2 + O_2^- \dashrightarrow R-NO_2^- \xrightarrow[Ar]{CID} R + NO_2^-$$
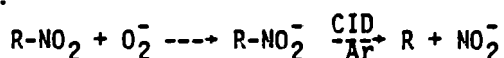
The first quadrupole was scanned over a 100 to 300 amu range or single ion monitoring was done at m/z 247$^-$. The third quadrupole was set for con- stant daughter scans at m/z 46$^-$. The corona discharge current was 6 µamp constant current at 4 to 6 kV and an ion energy of 65 eV. Thus in this reaction, only negative ions of 100 to 300 molecular weight were de- tected which yielded NO$_2$ on collision with argon gas.

Diesel soot extracts were fractionated with DMSO to yield aliphatic, aromatic and polar fractions for MS/MS analyses. This fractionation method has been described previously (4-6). The aromatic fraction from a single- cylinder Swan diesel engine was used for comparison with an Oldsmobile ex- haust aromatic fraction because of differences in mutagenic activity. The aromatic fraction recovered by DMSO fractionation of diesel soot extracts typically contains 50-80% of the direct mutagenic activity of different ex- tracts and is concentrated 5- to 10-fold in specific activity. The aromatic fraction of diesel exhaust is very similar to the γ fraction isolated by Schuetzle et al. (1) in containing mononitro-PAHs and dinitro PAHs of 2 ring PAHs.

The mutagenic activities of the extracts in TA98 (no S-9) under standard Ames bioassay conditions (7) were: nitro-fuel-PAHs--435 rev/µg; Swan diesel soot aromatic fraction--57 rev/µg; Olds diesel soot aromatic frac- tion--21 revertants/µg. These samples, differing by about 20-fold in mutagenic specific activity, were compared by MS/MS. The unfractionated extracts were low in the intensity of certain (M - 27) ions, the polar frac- tions contained only a minor part of the total mutagenicity (< 20%) and the aliphatic fractions contained very low intensities of nitro-PAH ions.

Figure 1 shows that the even-mass ions (dinitro-PAHs) appeared to cor- relate with increased mutagenic activity of the three extracts, while odd- mass ions (mononitro-PAHs) negatively correlated with mutagenic activity

with the exception of m/z 171. The even mass ions of particular signifi-
cance and their tentative identification were: m/z 252, dinitrophenan-
threnes; m/z 256, dinitromethylbiphenyls; m/z 230, 244 and 258, dinitro-
naphthalenes containing 2, 3 and 4-methyl groups. These compounds might be
particularly important in the total mutagenicity of diesel soot extracts.
Nitropyrenes (m/z 231) did not significantly correlate with differences in
the mutagenicity of these samples, although these compounds may account for
5-10% of the total mutagenicity(4).

Figure 1 also shows that the same nitro-PAH (M - 17) masses were present
in all three types of samples. The fuel-nitro-PAHs, having been treated
with excess NO_2, appeared to be lower in mono-nitro-PAH type masses and
higher in the even masses (dinitro-PAHs) discussed above. Since most of the
nitro-PAH ions present in diesel exhaust are also present in fuel aromatic
fractions treated with NO_2 and these masses are relatable to the PAHs
present in the fuel burned by the two engine types, this suggests that the
fuel PAHs contribute to the exhaust nitro-PAHS. One possible mechanism is
reaction of unburned fuel PAHs with NO_2.

Studies involving the addition of pyrene to the fuel for the single
cylinder diesel engine was performed to further test the hypothesis that
fuel PAHs may contribute to the formation of nitro-PAHs in exhaust soot ex-
tracts. It was found that addition of 0.01 to 1.0% w/v pyrene to diesel
fuel (less than 0.01% pyrene by GC/MS) resulted in increased pyrene/phenan-
threne ratios in soot extracts. With no addition of pyrene, this ratio was
less than 2, but increased to 12 with 1% pyrene addition to the fuel. The
mutagenicity in TA100 increased 2 to 3 fold by 0.01 to 0.1% pyrene addition,
but decreased somewhat with 1% pyrene addition. MS/MS analyses of these
extracts by APCI/MS/MS showed increased ion intensities of mono- and
dinitropyrenes in the soot extract for pyrene additions up to 0.1% in the
fuel, but the intensities of dinitropyrenes decreased at 1% pyrene addition
to the fuel. (Supported in part by U.S. Department of Energy under DOE
Contract No. DE-AC04-76EV01013.)

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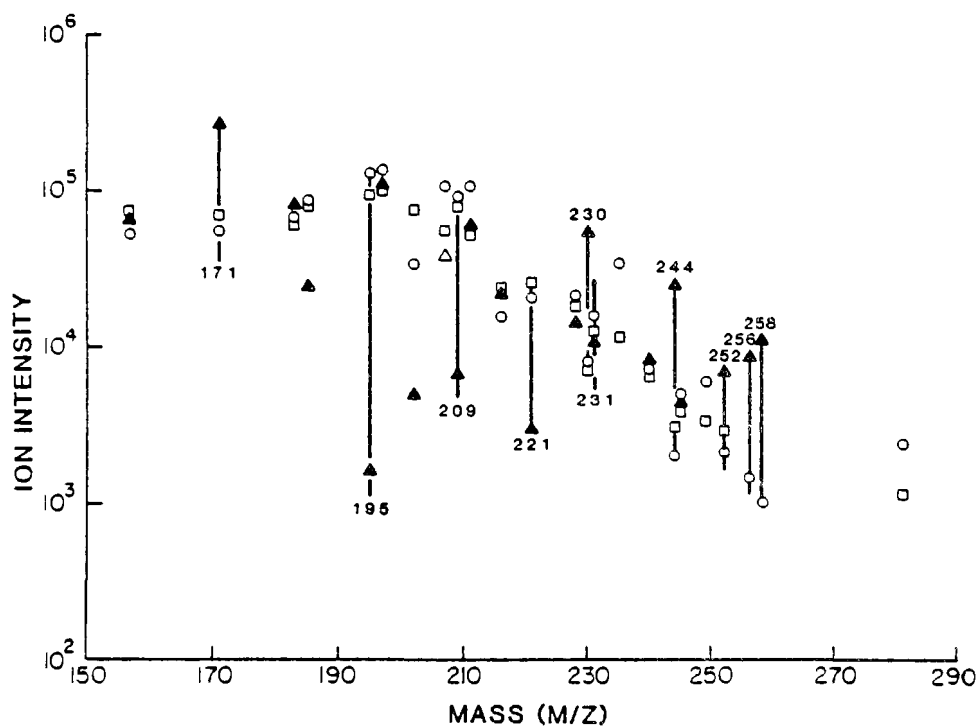
FIGURE 1

Figure 1. MS/MS Comparisons of Nitro-PAHs from Diesel Fuel and Exhaust
Filter Extracts. --△-- Nitro-fuel-PAHs; --□-- Aromatic
Fraction from Swan Engine Exhaust Soot Extracts; --O-- Aromatic
Fraction, Olds Exhaust Soot Extracts. The masses represent the
(M - 17) ions, the parent nitro-PAH being 17 amu more. The ion
intensity is the total ion counts summed over the entire run,
usually 300 scans.

RESEARCH PLANS FOR DIESEL HEALTH EFFECTS STUDY

by

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1) PAST RESEARCHES ON AUTOMOBILE EXHAUST IN JAPAN AUTOMOBILE RESEARCH INSTITUTE, INC. (JARI)

JARI was granted research contracts from the Japan Automobile Manufacturers Association, Inc. (JAMA) for studying the reduction of automobile exhaust and their health effects around 1970 when automobile exhaust were a wide public concern in Japan on the grounds that they may cause atmospheric pollutions typically exemplified by the phenomenon of photochemical smogs.

JARI started research on these contracts in 1971 including field surveys using a mobile smog chamber, tracing of photochemical reactions using a stationary smog chamber and chemical analysis of emission components. A behavioral assessments on animals were started in 1975, and basic researches on health effects of NO_x and O_3 were started in 1976. Preliminary researches on diesel emissions were started around this time. Small-scale animal inhalation system with modified Rochester type inhalation chambers were designed and tested. Studies on the health effects of diesel emissions to rats were done preliminary for one month and then three months. Investigations on respiratory system revealed that morphological changes in early stage of exposure are attributed mostly depend to the gaseous components, and it seems that particulate matters amplify the changes depending on the particulate concentrations.

The facility for Ames test was completed in 1980. A preliminary Ames test was conducted on extracts from diesel particulates using Salmonella Typhimurium TA100 and TA98. It was found that PAHs contained as neutral fraction components showed a relatively high mutagenicity. Examinations of sampling methods which would allow to obtain artifact-free diesel particulates are currently conducted comparing results of Ames tests and those of chemical analysis.

2) HEALTH EFFECTS RESEARCH PROGRAM (HERP)

In recent years, the health effects of diesel emissions has become an important subject in Japan. However, the research works in this field is still in an embryonic stage. Under these circumstance, JAMA referred this subject to some researchers of automobile engineering, medical and chemical fields. As a result, HERP has been drafted in 1981.

An outline of the HERP is presented below.

Period : From 1981 to 1985

Organization (Committee of HERP)

Chairman : Dr. Atsushi WATARI
President of the JARI
Prof. Emeritus of Univ. of Tokyo

Chairman of Steering Committee : Dr. Noburu ISHINISHI
Prof. of Hygiene & Public Health
Kyushu University

Subcommittees : 1. Diesel Exhaust Generation and Sampling
2. Analysis and Custoday
3. Inhalation Studies
4. Small Animal Experiment
5. Culture Cell Experiment (I)
6. Culture Cell Experiment (II)
7. Mutagenicity Test using Microorganisms
8. Miscellaneous including environmental assessment

Secretariat : JARI Officier

Research items :

Facilities for particulate generation, sampling and analysis and a full-scale facility for the inhalation experiment will be built at JARI. Preparations are underway to complete these facilities in 1982.

It is planned that JARI will participate principally sampling, analysis, storage and delivery of diesel tars and the inhalation experiment. The various in vitro and in vivo tests on diesel emission samples will be conducted in some research institutes and laboratories of the national and private universities.

Two types (large and small) of diesel engines will be used in the project considering that properties of emission materials might depend on diesel engines.

Chronic toxicity tests and carcinogenicity tests will be conducted by the inhalation for over two years. Intratracheal instillations, skin painting and other tests of the extracts from diesel particulates will be conducted

on small animals. Mutagenic test of the extracts from diesel particulates
will be conducted on culture cells and microorganisms (such as the <u>Salmonella</u>
<u>Typhimurium</u>).

NEURODEPRESSANT EFFECTS OF UNCOMBUSTED DIESEL FUEL

BY

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INTRODUCTION

Studies were conducted to characterize the short term neurotoxic effects of the inhalation of uncombusted diesel fuel vapors. Since diesel fuel contains indentified harmful hydrocarbon constituents which may exert neurological effects, the studies in this research effort were designed to screen for neurotoxic effects of the uncombusted diesel vapors. Mice were exposed at concentrations of 0.204 mg/1, 0.135 mg/1 and 0.065 mg/1 of uncombusted diesel vapor for 8 hours/day, 5 consecutive days.

METHODS

Three groups of mice were maintained throughout the experiment: an exposure chamber group, a control chamber group, and a vivarium control group. Ten mice each were in the exposure chamber and the control chamber groups; five mice were in the vivarium control group. Conditions of the exposure and control chamber groups were identical except for the presence of the uncombusted diesel vapor.

Five tests were selected to identify changes in performance which are related to interference of the nervous system. These tests were: the square box activity test, used to evaluate activity of the mice by suggesting either depression or stimulation of activity; the rota rod test which indicates alterations of the integrity of neuromuscular junctions and coordination; the inclined plane test, which serves to evaluate neuromuscular junction integrity of neuromuscular strength or paralysis; the corneal reflex test, used to screen for spinal cord depressant activity; and the hot plate test, a test of analgesic response. General observations were made during testing and for 30 minutes after mice were returned to their cages. The tests were administered to exposure and control chamber groups 24 hours prior to the first day of exposure, after completion of each day of exposure, and 24 hours after the last day of exposure. Results of the tests were compared between the exposure chamber and control chamber groups and expressed as percent of control within standard error. Exposure was conducted using an inhalation chamber which exposed mice primarily via the respiratory system with minimal ingestion and cutaneous exposures. Vapor generation was directly from complex liquid state to the vapor state and varied $\pm$ 10% for the duration of the study.

RESULTS

Square Box Activity Test: Mice exposed to 0.204 mg/l showed 50 to 90% less activity than the control chamber mice. Those mice exposed to 0.135 mg/l demonstrated activity comparable to that of the control chamber mice, while mice exposed to 0.065 mg/l had increased activity as high as 150 percent as that of the control chamber mice. Comparison 24 hours after removal from exposure to the diesel vapor resulted in similar values between exposure and control chamber groups.

Rota Rod Test: When compared to the control chamber group, mice exposed to 0.204 mg/l initially demonstrated a slight increase in performance which drastically deteriorated as exposure continued. Mice exposed to 0.135 mg/l had a slight increase in performance followed by a slight decrease in performance when compared to control chamber mice. Those mice exposed to 0.065 mg/l showed no relative difference in performance between exposure and control chamber mice. Comparison after 24 hours of recovery from last exposure indicated little change between exposure and control chamber mice.

Hot Plate Test: Results of the exposure to 0.204 mg/l indicated an initial increase in heat sensitivity followed by tolerance to heat. At 0.135 mg/l a slight increase in heat sensitivity was observed for the entire exposure. Exposure to 0.065 mg/l identified a substantial increase in heat sensitivity for the exposure mice as compared to the control mice in the chambers. No relative difference could be observed between exposure and control chamber groups 24 hours after termination of exposure.

Corneal Reflex Test: No difference in response was noted between groups for any concentrations.

Incline Plane Test: Both the exposure and control chamber groups had negative results at each concentration.

All test result variations are depicted in Figure 1.

General Observations: All mice exposed to 0.204 mg/l displayed severe discoloration of the tail indicating vasodilation after three days of exposure. Severe dehydration was observed in all mice. Grooming habits deteriorated after day two of the exposure. While walking, five (50%) of the mice displayed tremors through day three. Half of the mice with tremors died at day three or sooner; the rest continued to have tremors but recovered.. A weight loss of 30% was observed in the exposure group. The mice in the exposure chamber group were generally less active than the control chamber group when returned to their cages.

At an exposure of 0.135 mg/l, five (50%) of the mice displayed tail discoloration after 3-4 days of exposure; slight dehydration was also apparent. Tremors were evident in three (30%) of the mice while in motion; however, no deaths occurred. Grooming was poor but less so as compared to the 0.204 mg/l exposure group.

In mice exposed to the 0.065 mg/1 concentration no appreciable difference were displayed between the exposure and control chamber groups.

CONCLUSIONS

The exposure of the mice identified general trends in the effect of uncombusted diesel vapor on the nervous system. Comparison of exposure to control chamber groups suggests a positive central nervous system involvement. Exposure appears to be concentration and duration dependent. At the concentration 0.204 mg/1 depression of the inhibitory neuron occurs followed by extensive depression of the stimulatory neuron. Concentrations of 0.135 mg/1 appear to have little effect when compared to controls and suggest depression of inhibitory neuron and slight depression of the stimulatory neurons while the concentration of 0.065 mg/1 causes depression in the inhibitory neuron.
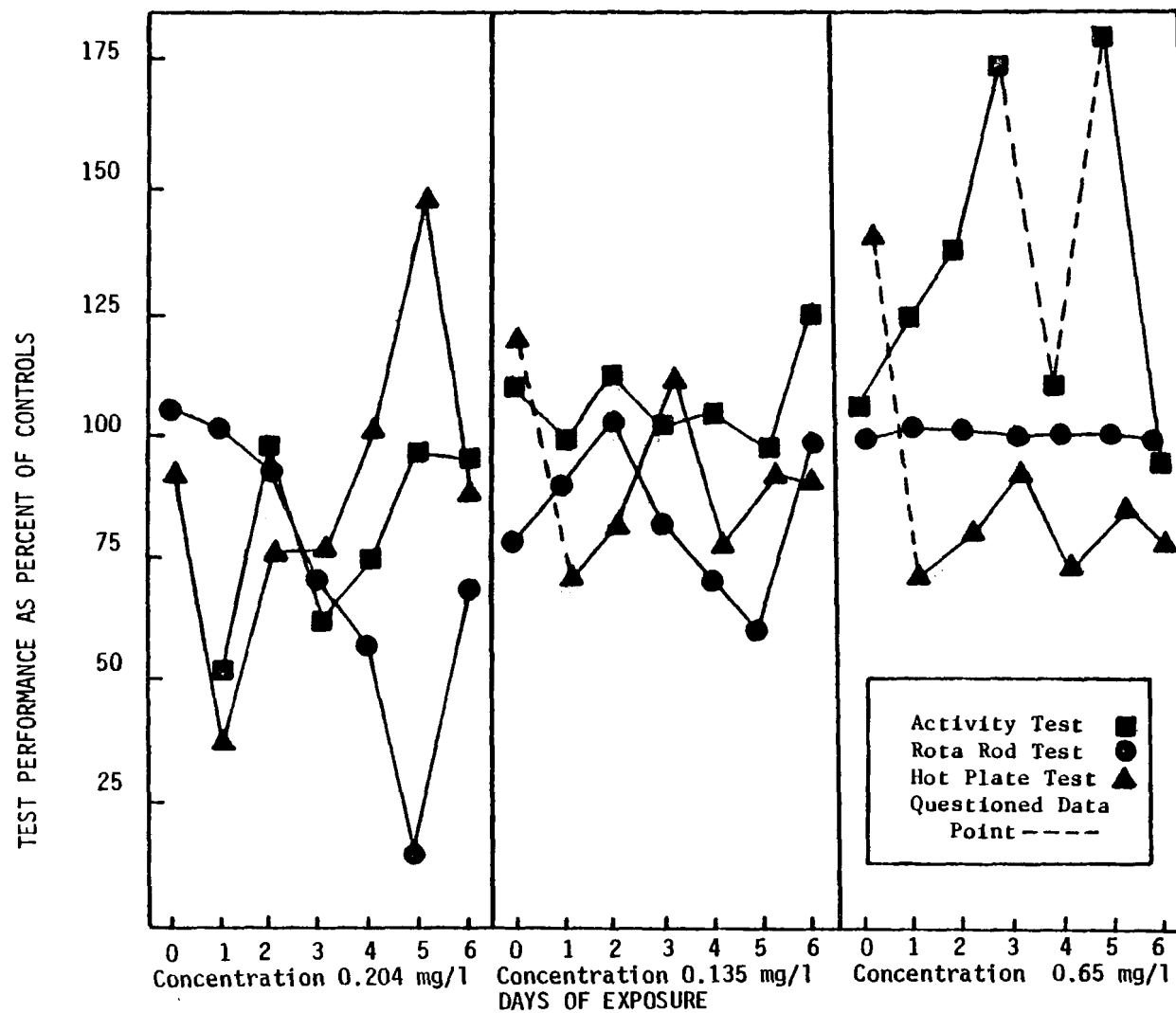
Figure 1. Results of Screening Tests as Percent Control for All Three Concentrations of Uncombusted Deisel Vapor

EVALUATION OF THE RELEASE OF MUTAGENS AND 1-NITROPYRENE FROM DIESEL PARTICLES IN THE PRESENCE OF LUNG MACROPHAGE CELLS IN CULTURE

by

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Diesel particles have been shown to contain organic components which are mutagenic in short-term bioassays (1). Nitroaromatics including 1-nitropyrene now appear to account for a portion of the mutagenic activity observed in these organics (2). It is important to determine whether these potentially carcinogenic components are released from particles under physiological conditions. We have shown in a previous study (3) using the Ames Salmonella typhimurium plate incorporation assay that serum and lung cytosol were very effective in removing mutagenic activity from diesel particles (3). This was determined by measuring the activity extractable from the particles before and after treatment of the particles with serum or cytosol. Direct addition of serum or lung cytosol to these organics reduced the mutagenic activity detectable by 80 to 90%. The objective of this study was to evaluate the removal and release of mutagenic activity and 1-nitropyrene from diesel particles in the presence of lung cells in culture. The lung cells used in these studies were alveolar macrophages obtained from rabbits by lung lavage. The diesel particles used in this study were obtained from a Datsun Nissan 220C, 4-cylinder passenger car previously described (3). The particles were sonicated in tissue culture medium (M199 with Hank's Salts) at 37°C for 30 min to deagglomerate and disperse the particles. Particle size analysis showed the majority of the particles to be 2.0 to 2.5 μm after this treatment.

Rabbit alveolar macrophage (RAM) cells were lavaged and cultured with the diesel particles according to previously published procedures (4) except that the final serum concentration was reduced to 10% and the culture time was increased to 40 h. In brief, RAM cells were added to individual wells of cluster dishes containing suspensions of the diesel particles from 15 to 1,500 μg/ml. Under these conditions, at concentrations of diesel particles above 75 μg/ml, over 95% of the particles were phagocytized after 20 h of culture. After 40 h of culture, cells were harvested by trypsinization. The final exposure conditions for the mutagenesis and 1-nitropyrene analyses were selected to maximize cellular exposure to the diesel particles while minimizing cellular toxicity. The final concentration of diesel particles selected was 375 μg of particles/ml. At this concentration, RAM cells engulf over 95% of the particles and after 40 h of exposure, less than 7% cell lysis was observed and cell viability was 63% of the control cultures.

In order to evaluate the effect of the lung macrophage cells on the removal of mutagens and 1-nitropyrene from diesel particles, particles were exposed to the culture medium at 375 μg/ml with and without lung macrophages and cultured for 40 h. After incubation the medium control treatment dishes were combined and the medium was separated by centrifugation from the particles which were washed once with water. The harvested macrophages were separated from the culture medium and sonicated to release engulfed particles. Dichloromethane and methanol (DCM:MeOH, 1:1) were used to extract the medium, free particles, engulfed particles, cell sonicate, and water washes. Each of these fractions was analyzed for 1-nitropyrene by LC/fluorescence (5) and compared to untreated particles extracted with DCM:MeOH.

Each of the fractions was also assayed for mutagenicity in the Ames Salmonella typhimurium plate incorporation assay in TA98 as previously described (3). The media, cells, and washes were assayed without extraction and due to a high background of activity in both the medium and cells, no detectable activity was observed as a function of the treatment group. Mutagenicity was detected in the DCM:MeOH extract of the macrophage-engulfed particles; however, it was only 2% of the mutagenicity originally present on the particles. Comparison of this activity with that of the media control particles, in which 6% of the mutagenicity was recovered, showed that the presence of macrophages decreased the mutagenicity 62%.

Nitropyrene analysis of the medium control group showed 96% recovery of the 1-nitropyrene with 31% of the nitropyrene found in the medium and washes and 66% remaining on the particles. Significantly less 1-nitropyrene was recovered in the macrophage treatment group (76%). Since over 95% of the particles were phagocytized and only 27% of the original 1-nitropyrene was recovered from the engulfed particles, it appears that the macrophages may have metabolized the 1-nitropyrene to a nondetectable form.

Comparison of the recoveries shows a greater loss of mutagenicity than 1-nitropyrene suggesting that other compounds detected in these particles may contribute more to the mutagenicity than the 1-nitropyrene alone.

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BACTERIAL MUTAGENICITY OF A DIESEL EXHAUST EXTRACT AND TWO ASSOCIATED NITROARENE COMPOUNDS AFTER METABOLISM AND PROTEIN BINDING

by

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Previous work has demonstrated that nitroarenes are associated with organic extracts from diesel exhaust (1,2,3). This study was designed to characterize two nitroarenes and to determine whether these compounds demonstrate the same type of mutagenic response as diesel exhaust organics. An extract of the particulate from exhaust of a VW diesel automobile, 2,7-dinitrofluorenone, and 1-nitropyrene were tested in the Salmonella mutagenicity assay using strain TA98 and two nitroreductase-deficient strains (TA98FR1 and TA98/1,8DNP$_6$) to establish a proper dose for a multivariant experiment. Each of the three samples was tested with and without a 9000g liver homogenate (S9) prepared from Aroclor 1254-induced rats. In the multivariant experiment, the following treatments (both with and without the NADPH-generating system) were used:

(a) no activating system (-S9),

(b) microsomes derived from the original S9 by centrifuging for 90 min at 100,000g,

(c) the cytosol fraction of the original S9, and

(d) boiled S9.

In addition, the samples were tested in the presence of both boiled and unboiled bovine serum albumin (BSA). Salmonella tester strains TA98 and TA98FR1 were used in the multivariant study. These treatments were used to determine the similarities of the three samples in the presence and absence of treatments with differing enzymatic and protein binding capabilities. This work is an extension of similar efforts by Wang et al. (4) and Pederson et al. (5).

The results using the diesel exhaust sample are seen in Table 1. When compared to the untreated (no activating system) situation, all of the treatments with one exception gave reduced mutagenic activity. Only the cytosol fraction gave an increase in activity. In comparison to the non-activated situation the cytosol activation gave a relative increase to 111%

for strain TA98 and to 164% for strain TA98FR1. In addition, it was noted that TA98 was the strain most responsive to the diesel exhaust extract, followed by TA98FR1, and then TA98/1,8DNP$_6$. 1-Nitropyrene activity was decreased by half in TA98/1,8DNP$_6$ but almost abolished in TA98FR1.

As in the diesel exhaust particulate sample, the mutagenic activity of 2,7-dinitrofluorenone was reduced in the majority of the different treatments. An increase to 170% of the untreated situation was seen when using the cytosol fraction with strain TA98FR1.

A reduction in the mutagenic activity of 1-nitropyrene was observed in all of the treatments without the generating system. However, in contrast to 2,7-dinitrofluorenone and the diesel exhaust particulate sample, a twenty-fold increase in the mutagenic activity of 1-nitropyrene was seen with the microsome treatment when using the generating system and strain TA98FR1. Smaller increases in activity were also observed with the S9 and cytosol fractions when using this same strain and generating system. The mutagenic activity of 1-nitropyrene also was increased by the microsome fraction with the generating system in strain TA98. This microsomal activation of 1-nitropyrene, in addition to the response pattern obtained with the nitroreductase-deficient tester strains, indicates that 1-nitropyrene may not be the major mutagenic component in this sample of extractable organics from VW Rabbit Diesel particle emissions.

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Table 1. Mutagenic Activity of Diesel Exhaust Particle Extracts Using Different Treatment Conditions

Treatment	Without Generating System		With Generating System[a]	
	TA98	TA98FR1	TA98	TA98FR1
Untreated	1039 (100)[b]	788 (100)[c]	1001 (100)	680 (100)
S9	467 (45)	242 (31)	696 (70)	649 (95)
Microsomes	781 (75)	488 (62)	826 (82)	557 (82)
Cytosol	687 (66)	332 (42)	1110 (111)	1118 (164)
Boiled S9	595 (57)	369 (47)	577 (58)	305 (45)
BSA	354 (33)	264 (34)		
Boiled BSA	343 (33)	232 (29)		

[a]NADPH-generating system: NADPH, G-6-P, $MgCL_2$, and KCL.
[b]Average net revertants per plate at 100 µg organic extract.
[c]Relative response to untreated sample expressed as percent.

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CHARACTERIZATION OF PARTICULATE EMISSIONS
FROM IN-USE GASOLINE FUELED MOTOR VEHICLES

by

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One of the primary tasks of those concerned with the study of environmental
quality is estimating population exposure to air pollutants and determining
the risk associated with exposure. Mobile sources generally contribute
significantly to the population's exposure to hydrocarbons (HC), carbon
monoxide (CO), oxides of nitrogen (NO_x), and fine particulate matter.
Much of the mobile source data available in the literature has been
obtained from well-maintained engineering test vehicles. However, emissions
from consumer-operated vehicles can vary considerably from well-maintained
vehicles.

Because of growing interest in diesel power for light-duty motor vehicles,
it has become necessary to study particulate emissions comprehensively
to determine the potential impact of dieselization on the public health.
Assessment of exposure and risk requires knowledge of the emission rates
and composition of diesel particulate matter and of the gasoline particu-
late matter being replaced by diesels.

Our study examined particulate emissions from twenty consumer-operated,
light-duty gasoline fueled cars and trucks. The emissions characteristics
observed were compared with those previously reported by Gibbs, et al.,
for nineteen consumer-operated, light-duty diesels (1).

A test fleet of twenty light-duty gasoline passenger cars and trucks was
obtained from local residents and rental agencies. Four of the vehicles
were fueled by leaded gasoline; the remaining sixteen fueled by unleaded

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gasoline. Vehicles from model years 1970 to 1981 were represented and ranged in mass from 907 kg (2,000 lbs) to 2,268 kg (5,000 lbs) and accumulated mileage from 565 km (353 mi.) to 130,490 km (81,559 mi.). A variety of emission control configurations were represented, including oxidation catalysts, oxidation catalysts with air pumps, and three-way catalysts. All vehicles were tested as received.

Three gasoline fuels were purchased locally for the program; two leaded and one unleaded. Road load simulation was achieved with a Clayton CTE-50-0 direct-drive, water brake dynamometer. The vehicles were tested with a daily routine involving a cold-start Federal Test Procedure (FTP) cycle followed by repetitive Highway Fuel Economy Test cycles (HWFET). The FTP simulates city driving after the car has not been started for at least twelve (12) hours. The HWFET simulates highway driving after the car has been warmed up.

A positive-displacement pump Constant Volume Sampling (CVS) system collected the exhaust gases and allowed dilution air to flow through the sample train in order to maintain the dilution exhaust temperature at or below 52° C (125° F). Before entering the CVS system, the dilution exhaust flow passed through three 20 x 20 inch filters for collection of particulate matter (2). Pallflex T60A20 PTFE glass fiber filters were used for collecting the particulate.

The 20 x 20 filters were soxhlet extracted for eight hours with dichloro-methane (DCM) to remove the soluble organic fraction (SOF). The extracted organics were examined for nitro-pyrene (NO_2-P), benzo(a)pyrene (BaP), and pyrene (Py) content by high pressure liquid chromotography (HPLC),and bioassayed with Ames Salmonella strain TA-98 for mutagenic potency. The activity (revertants/ microgram SOF) was determined as the slope in the linear portion of the dose-response curve. Five organic doses, 0 (solvent blank) to 100 ug, were used to define the dose-response curve.

A review of the regulated emission rates indicate that the test fleet included properly functioning vehicles and vehicles with a variety of emission control malfunction conditions. HC emission rates ranged from 0.07 to 24.5 g/mi., CO had a range of 0.08 to 60.95 g/mi., and NO_x was from 0.37 to 6.43 g/mi.. Comparison with the diesels indicate that the gasoline fleet average emission rates exceed the diesel rates and a broader range was seen in the gasoline data.

The total particulate emission rates from leaded gasoline vehicles were 2.7 times greater during the HWFET than the FTP. On the other hand, HWFET total particulate emission rates from unleaded gasoline vehicles were 81% of the FTP value. Overall, the leaded vehicles emitted more particulate than the unleaded vehicles during both cycles, 3.2 times more during the FTP and 10.8 times more during the HWFET.

The light-duty diesel total particulate emission rates reported by Gibbs, et al., compared to the gasoline vehicle rates as follows:
    (FTP) diesel ~5.9 x leaded rate; 19.1 x unleaded rate.
    (HWFET) diesel ~ 1.3 x leaded rate; 13.5 x unleaded rate.

Fleet average DCM soluble particulate organic emission rates during the FTP and HWFET compared with the light-duty diesels as follows:
(FTP) diesel ~ 5.9 x leaded rate; 8.6 x unleaded rate.
(HWFET) diesel ~ 3.4 x leaded rate; 6.9 x unleaded rate.

Nitro-pyrene emission rates were similar for leaded and unleaded gasoline vehicles. Diesels emit about 20 to 30 times as much NO_2-P as gasoline vehicles.

Gasoline vehicle BaP emissions were greater during cold-start FTP driving than during either hot-start FTP or HWFET driving. Functioning catalyst systems appeared to effectively reduce polynuclear aromatic hydrocarbon (PAH) emissions. During the cold-start FTP, BaP emission rates from leaded vehicles exceeded BaP rates from diesels; BaP emission rates from unleaded vehicles were similar to the diesel results. During the HWFET, BaP emission rates from both categories of gasoline vehicles were less than the rates from diesels.

Generally, the Ames TA-98 mutagenic activity of the gasoline particulate organics was higher with metabolic activation than without metabolic activation. Ames TA-98 revertant per mile levels were substantially higher for the leaded gasoline vehicles than for the unleaded gasoline vehicles under both FTP and HWFET conditions.

The higher total particulate and particulate organic emission rates of diesels are compensated somewhat by lower Ames TA-98 mutagenic activities when compared to gasoline vehicles. Relative activities (without metabolic activation) were as follows:
(FTP) diesel ~ 0.6 x leaded activity; 0.5 x unleaded activity.
(HWFET) diesel ~ 0.4 x leaded activity; 0.4 x unleaded activity.

Fleet average Ames TA-98 revertant per mile levels (without metabolic activation) for FTP and HWFET conditions compared with light-duty diesels as follows:
(FTP) diesel ~ 3.4 x leaded rate; 12.1 x unleaded rate.
(HWFET) diesel ~ 1.5 x leaded rate; 9.1 x leaded rate.

In conclusion, replacing the gasoline passenger cars represented by the test fleet of this program with diesel passenger cars would decrease HC, CO, and NO_x population exposures, and increase total particulate and mutagenic particulate organic exposures (as indicated by Ames Salmonella strain TA-98).

Table 1. Characterization of Particulate Emissions,
Diesel versus Gasoline

	Diesel *		Leaded		Unleaded	
	FTP	HWFET	FTP	HWFET	FTP	HWFET
Total particulate, mg/mi.	607(1)	345(1)	103	276	31.7	25.6
Dichloromethane soluble organics, mg/mi.	124(1)	79.7(1)	21.1	23.5	14.4	11.5
Benzo-a-pyrene, ug/mi.	4.5(3-7)	2.7(3-7)	14.5	0.89	3.3	0.61
Nitro-pyrene, ug/mi.	7.4(8)	6.8(8)	0.20	0.39	0.24	0.16
TA-98,-S9, rev/mg	4.1(1)	3.0	7.31	8.55	7.57	7.39
TA-98,+S9, rev/ug	**	**	12.5	10.6	13.4	7.43
TA-98,-S9, rev/mi.(x 10^{-3})	509(1)	239	152	163	42.1	26.4
TA-98,+S9, rev/mi.(x 10^{-3})	**	**	258	232	79.3	25.2

* Number in parenthesis indicates reference.
** Information unavailable at this time.

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SURFACE REACTIVITY OF DIESEL PARTICLE AEROSOLS

by

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INTRODUCTION

In 1979 a research project, concerning exhaust emissions from passenger cars, was initiated by Volvo Car Corporation in collaboration with the University of Gothenburg and Chalmers University of Technology. The first report (1), which appeared in September 1979, comprised of investigations of exhaust particulates both from gasoline powered and from diesel powered passenger cars, as well as chemical analyses by several methods, of gaseous and particulate matter in exhaust samples from Volvo cars.

The present work deals with the influence of diesel particles on the oxidation of nitric oxide to nitrogen dioxide at different conditions of load, temperature and dilution. Secondly, spectroscopic investigations of how the chemical composition of diesel particle surfaces may be modified in the atmosphere have been performed. A full report (2) will be published in October 1981.

EXPERIMENTAL

Determination of Conversion Rates

To determine the effect of diesel particles on the conversion $NO \rightarrow NO_2$, two series, each comprising of eight rate constant determinations were made in bag samples of exhausts from a Volvo passenger diesel. The samples were

analysed for nitrogen oxides concentrations at intervals during ~ 24 h after sample collection. A Monitor Labs. 8440 Nitrogen Oxides Analyser was used.

For the first series of samples the engine of the car was run at approximately 1500 rpm idle, while for the second series the car was driven at 40 km/h with a road load of 12.5 horse powers in a chassis dynamometer. The parameters temperature ($0°$ or $23°$ C), dilution rate ($\sim 1/60$ or $\sim 1/120$) and presence/absence of diesel particles were varied. The samples were collected in Tedlar bags contained in an aluminum barrel, which could be evacuated to make the bag extract the appropriate volume of exhaust gases. Dry air from a gas cylinder was used for dilution. Diesel particles were removed by a filter for the particle-free samples.

Spectroscopic Methods

For infrared spectroscopy, samples were collected with an Electrical Corona Sampler (3) on gold covered metal plates and analysed by a reflection-IR method, with a Nicolet MX-1 FTIR instrument.

ESCA samples were collected on Millipore filters and the electron spectra were recorded with a Hewlett-Packard 5960 A electron spectrometer.

RESULTS AND DISCUSSION

Conversion Rates

The formation of NO_2 from NO obeys the relationship:- $\dfrac{d[NO_2]}{dt} = k [NO]^2 [O_2]$. The rate constant k is commonly given in either of the dimensions $1/[M]^2$ per second or $1/ppm$ per minute.

The values for k ($[M]^{-2}$ x sec.$^{-1}$), calculated for the respective 16 experiments, are summarized in Table 1. The rate constant has a negative temperature dependence, especially at higher dilution rates. The reaction is enhanced by the presence of diesel particles. The latter effect, though, is not as strong as the catalytic effect of street and wall surfaces, reported by Lindqvist et al. (4).

Spectroscopic Results

ESCA measurements were made on three kinds of samples of diesel particles, namely unexposed samples, samples which had been exposed to 2 ppm NO_2 for 48 h and finally samples which had been exposed simultaneously to NO_2 and UV-light for 6 h. Nitrogen (1s) responses were obtained only from the latter kind of sample. Signals at 400 eV and at 402 eV were assigned to emanate respectively from N_x and NH_4 by comparison with the results of Chang & Novakov (5).

The infrared spectra obtained by reflection of IR-light through diesel particles precipitated on a gold film showed absorption peaks at 1290/cm and at 860/cm, corresponding respectively to C - N stretch in primary

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aromatic amines and to N - O stretch in aromatic nitro compounds. Unexposed samples gave the same kind of spectra as samples which had been exposed to NO_2 and UV-light.

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Table 1. Calculated Rate Constants. k is the slope of the function $1/[NO]_t - 1/[NO]_0 = kt$, calculated from measurements of $[NO]$ at intervals after the start of an experiment at $t = 0$. The values have been multiplied by 10^{-4}.

				1500 rpm idle			
	Particles				No particles		
No.	Temp.(°C)	Dil. rate	k	No.	Temp.(°C)	Dil. rate	k
1	0	1/60	2.11	5	0	1/60	1.74
2	0	1/120	2.39	6	0	1/120	2.04
3	23	1/60	1.81	7	23	1/60	1.56
4	23	1/120	1.82	8	23	1/120	1.57
				40 km/h. Road load: 12.5 hp			
	Particles				No particles		
No.	Temp.(°C)	Dil. rate	k	No.	Temp.(°C)	Dil. rate	k
9	0	1/60	2.18	13	0	1/60	1.75
10	0	1/120	2.43	14	0	1/120	1.99
11	23	1/60	1.97	15	23	1/60	1.59
12	23	1/120	1.92	16	23	1/120	1.53

EFFECTS OF OZONE AND NITROGEN DIOXIDE PRESENT DURING SAMPLING OF GENUINE PARTICULATE MATTER AS DETECTED BY TWO BIOLOGICAL TEST SYSTEMS AND ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS

by

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Urban particulate matter was collected in wintertime at -5 - 0 ^{o}C during 24 h periods on glass fiber filter with two simultaneously operating high volume samplers. One of the samplers was equipped with an ozone or nitrogen dioxide dosage system enhancing the concentration with about 200 ppb ozone or 960 ppb nitrogen dioxide. Filters were Soxhlet-extracted with acetone and the extracts analyzed with respect to eight polycyclic aromatic hydrocarbons (PAH), mutagenicity in the Salmonella/microsome assay and ability to displace 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) from the rat liver receptor protein.

Polycyclic Aromatic Hydrocarbons

A part of or the whole acetone extract of each filter was subjected to a clean up procedure with respect to PAH and eight components were quantified by gas chromatography on a Carlo Erba equipment with a SE-54 glass capillary column. The range of concentrations detected is given in Table 1.

Exposure to ozone had very little effect on the concentrations of the PAH. Significant degradation occurred only in one of the experiments. At this occasion the concentrations of nitric oxide and nitrogen dioxide in ambient air were rather high and it seems likely that the degradation may have been caused by nitrogen oxides formed by ozone oxidation of nitric oxide.

Statistical analysis between the nitrogen dioxide exposed samples and the simultaneously collected non-exposed samples showed a significant degradation for pyrene, benz(a)anthracene and benzo(a)pyrene being on the average about 20, 40 and 60 %, respectively.

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Salmonella Mutagenicity

Portions of the acetone extracts were reduced in volume, but not to dryness, at < 40 °C and then diluted with dimethyl sulfoxide (DMSO). Each of the DMSO samples was assayed at at least three different occasions for mutagenicity by the Salmonella plate incorporation method with bacterial cultures fully grown overnight. Tests were performed with the strains TA 98 and TA 100 in the absence and presence of the microsome containing liver supernatant from Aroclor 1254-induced male rats (S9) and with the nitroreductase deficient strains TA 98 NR, TA 98/1,8 DNP_6 and TA 100 NR in the absence of S9. The S9 was used at a level of 20 μl per plate and was added together with necessary co-factors.

The response expressed as revertants per cu.m was calculated from the linear or approximately linear dose response curves. The results for assays in the absence of S9 are given in Table 2. The addition of S9 either decreased or did not change the mutagenic response.

Exposure to ozone did not generally alter the mutagenic response except for an increase in the experiment which also resulted in degradation of PAH.

Exposure to nitrogen dioxide increased the mutagenic response in nitroreductase proficient as well as nitroreductase deficient strains. The average enhancement found in the three investigated experiments was a 3 - 4-fold increase.

Affinity to the Rat Liver TCDD-Receptor Protein

Assays for the ability to displace TCDD from the rat liver receptor protein were performed by adding different amounts of the DMSO samples to one ml of rat liver cytosol containing tritium-labeled TCDD and determining the amount of TCDD which remained bound to the receptor. After correction for non-specific binding, the relative specific binding was calculated from log-logit plots and was expressed as the amount that competes for 50 % of the specific TCDD-binding, EC_{50}, cu.m per ml cytosol.

Two experiments from the nitrogen dioxide and one from the ozone exposure were analyzed and the results are given in Table 2. Neither nitrogen dioxide nor ozone altered the affinity.

Conclusions

High volume sampling of ambient particulate matter on glass fiber filter in the presence of a high level of ozone does not cause a significant degradation of PAH, alter the mutagenic response which is detected by the Salmonella assay or change the affinity to the TCDD-receptor protein.

Sampling in the presence of a high level of nitrogen dioxide causes a significant degradation of reactive PAH and increases the mutagenic response which is detected by the Salmonella assay, but does not change the affinity to the TCDD-receptor protein.

Simultaneous sampling of genuine particulate matter without and with enhanced concentrations of reactive gases may be the best method for studying artifact reactions during sampling. Further studies are in progress with nitrogen dioxide, nitrous acid and nitric acid.

Table 1. Concentration ranges of analyzed PAH components; ng / m^3.

PAH component	8 samples non-exposed	3 samples O_3-exposure 200 ppb	5 samples NO_2-exposure 960 ppb
Phenanthrene	0.42 - 2.2	0.66 - 2.0	0.39 - 1.9
Fluoranthene	0.64 - 22	1.7 - 3.6	0.81 - 4.5
Pyrene	1.1 - 8.0	1.5 - 3.6	0.81 - 5.8
Benz(a)anthracene	1.1 - 5.2	0.93 - 2.0	0.82 - 3.8
Chrysene / Triphenylene	0.8 - 4.0	1.1 - 2.1	1.1 - 4.0
Benzo(b&k)fluoranthenes	1.6 - 7.8	2.0 - 3.5	2.2 - 7.6
Benzo(e)pyrene	0.39 - 3.6	0.48 - 1.0	0.36 - 2.2
Benzo(a)pyrene	0.17 - 2.4	0.16 - 0.66	0.07 - 1.0

Table 2. Mutagenic response in the absence of mammalian metabolic activation and affinity to the TCDD-receptor protein of extracts of particulate matter collected without and with enhanced concentrations of nitrogen dioxide or ozone; n.d. not determined.

Sample		Revertants per m^3					Receptor affinity $EC_{50} \pm s.d.$ (n = 4) m^3 / ml
		TA 98	TA 98 NR	TA 98/ 1,8 DNP$_6$	TA 100	TA 100 NR	
800225	AMB	62	37	28	76	36	n.d.
	NO_2	87	48	32	142	62	n.d.
800226	AMB	14	8	3.2	14	5	0.17 ± 0.04
	NO_2	37	24	6.5	41	20	0.09 ± 0.04
800228	AMB	11	6	5	20	5	0.07 ± 0.02
	NO_2	76	35	20	110	41	0.10 ± 0.03
800326	AMB	4.3	n.d.	n.d.	2.9	n.d.	n.d.
	O_3	4.6	n.d.	n.d.	3.3	n.d.	n.d.
800327	AMB	28	11	4.5	21	5	0.08 ± 0.02
	O_3	31	12	4.4	25	7	0.06 ± 0.04
800331	AMB	10	n.d.	n.d.	8.4	n.d.	n.d.
	O_3	16	n.d.	n.d.	14	n.d.	n.d.

ALUMINA COATED METAL WOOL AS A
PARTICULATE FILTER FOR DIESEL POWERED VEHICLES

by

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Pending federal regulations will probably require 1985 and later diesel powered vehicles to be equipped with aftertreatment devices to control particulate emissions. Filtering devices which employ alumina coated steel wool as the filter matrix show promise of being a practical means for removing submicron size particles from diesel exhaust. Our diesel exhaust filters (DEF's) consist of two alumina coated stainless-steel-wool filled cartridges inserted into an outer housing about the same size as a conventional muffler (Figure 1). The alumina coating, which is applied to the steel wool by a proprietary process, characteristically retains soot particles which come in contact with it.

In our design, metal wool packing density, alumina coating thickness and the physical dimensions are the dominant performance determinants for the collection efficiency and exhaust back pressure. The surface area of the alumina in the DEF, which is a function of all of these, correlates well with collection efficiency.

Our tests have demonstrated that particulate removal in the 70% range in FTP testing can be achieved at acceptable backpressures. Measurements also show that trapping efficiency is constant over a gas flow range of 0.7 to 20 ft/sec. The trapping efficiency of a given size filter can be increased above 80% but only at an increased backpressure penalty. Satisfactory filters have been made for diesel engines varying in size from 2 to 14 litres.

Generally, filters capable of trapping soot with efficiencies in excess of 70% have a pressure drop of 3 to 4 inches of water per inch of filter at gas velocities of 15 ft/sec. For example, a filter mounted on a vehicle equipped with a 1980 Oldsmobile engine, having a trapping efficiency in this range demonstrated a pressure drop of 31 inches of water at 40 mph level road conditions.

In addition to removing soot from diesel exhaust, alumina coated steel wool filters remove significant amounts of hydrocarbons and sulfates. Importantly, a significant percentage (50%) of the polynuclear aromatics, as indicated by benzo (a) pyrene measurements, is removed. Noise is attenuated to levels equivalent to those observed using conventional mufflers.

Since the volume of soot generated by current diesel engines is quite large, soot collected on DEF's must be periodically removed for the filter to trap effectively for extended mileage. Burning at temperatures above 1000°F is the most feasible method for doing this. Since diesel exhaust temperatures at moderate loads are generally below 1000°F, consideration is being given to mounting torches in the exhaust system to increase exhaust gas temperature when regeneration is required. In that conventional torches present difficult operating problems when employed in exhaust systems, a catalytic torch is being developed in our laboratories. With this kind of torch, hydrocarbons injected into the exhaust are catalytically oxidized to increase the exhaust gas to regeneration temperature. The catalytic converter used was made by applying a noble metal catalyst to alumina coated steel wool. Although the torch performed satisfactorily using either propane or diesel fuel, propane was used in most of the development work because of experimental convenience.

To date, about 3,000 miles have been accumulated on a 1980 Oldsmobile vehicle equipped with a diesel exhaust filter. During this test, the filter was regenerated at 150-200 mile intervals with our catalytic torch and a trapping efficiency of greater than 65% was maintained throughout the entire test. Since the amount of heat released from burning soot in the limited DEF volume can be large, the regeneration interval is determined by this factor rather than by the potential for high backpressure due to filter loading.

In addition to the durability experience achieved with diesel engines, it is worth noting that alumina coated mesh filters have undergone millions of miles of durability testing as a lead particulate filter in the exhaust of a variety of gasoline engines.
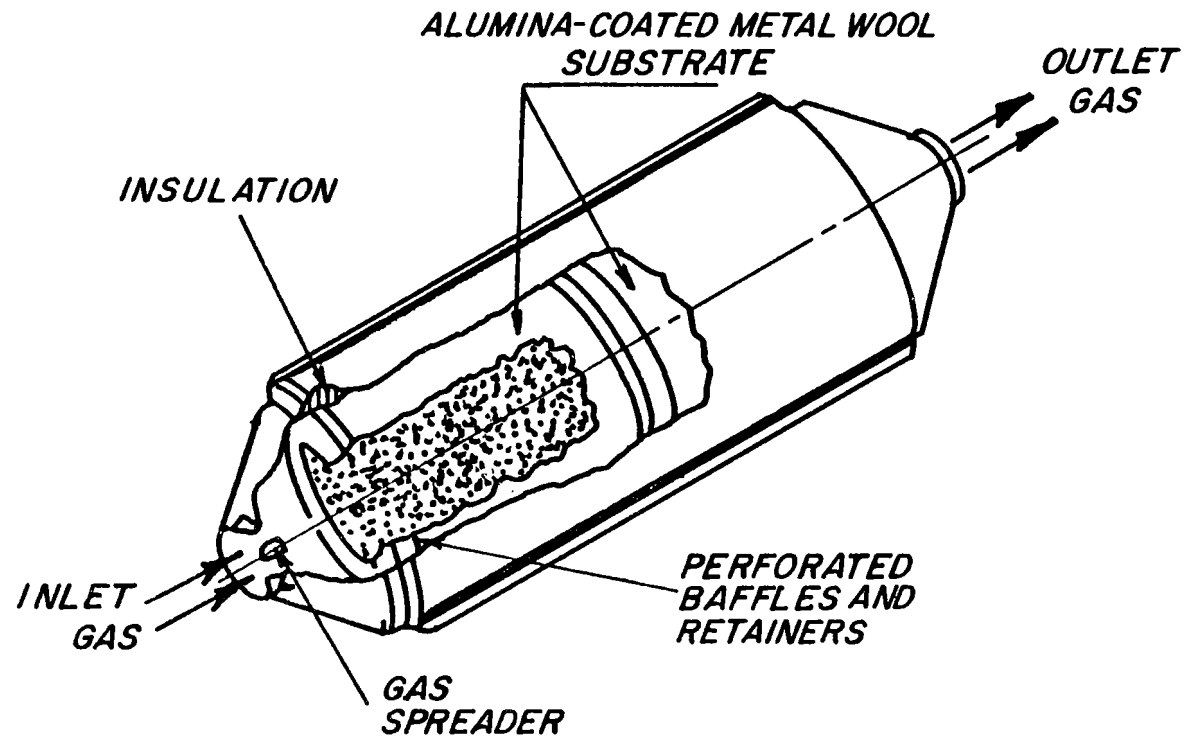
ALUMINA-COATED METAL WOOL
SUBSTRATE

OUTLET
GAS

INSULATION

INLET
GAS

GAS
SPREADER

PERFORATED
BAFFLES AND
RETAINERS

FIGURE 1 - Typical Texaco Diesel Exhaust Filter Design

ISOLATION AND IDENTIFICATION OF MUTAGENIC NITROARENES IN DIESEL-EXHAUST PARTICULATES

by

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Particulate matter emitted from diesel engines contains chemicals which are active in the Ames Salmonella typhimurium assay. The major portion of this mutagenic activity is liver enzyme independent and thus indicates that diesel exhaust contains a different class of mutagens than unsubstituted polynuclear aromatic hydrocarbons, which are liver enzyme dependent.

Diesel exhaust particulates were collected on glass fiber filters from heavy-duty engine test apparatus. A total of 14 filters were extracted for 24 hours with 6 L dichloromethane in a Soxhlet apparatus, the extract filtered and concentrated by rotary evaporator. The combined extracts contained approximately 225 g of organic matter which yielded 0.46 net TA98 revertants/μg (1.0×10^8 net TA98 revertants).

The CH_2Cl_2 extract was further fractionated on a preparative silica column with successively increasing solvent strength: hexane, chloroform, and methanol. The intermediate polarity fraction had the highest specific mutagenic activity and was further separated on gel permeation, high performance normal and reverse phase chromatography. Mutagenic activity was detected in virtually all fractions, so that fractions containing the highest specific activity were selected for further analysis.

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High resolution mass spectrometry (HRMS) was performed on selected subfractions from the reverse phase separation. Each sample was evaporated under N_2 in the probe which was inserted into the electron impact source. Identification of nitroarenes was based upon accurate mass determinations ($\pm$ 15 ppm at a resolution of 9000) of the molecular ion and of fragment ions corresponding to losses of the neutral fragments NO and/or NO_2. Some nitroarenes tentatively identified are listed in Table 1. Those with an asterisk (*) were found to have the same HRMS spectrum and high pressure liquid chromatography (HPLC) retention volume as their corresponding standard.

In this study, about 50 nitroarenes have been tentatively identified by HRMS. The variety of nitroarenes in diesel exhausts is extensive and, because only a few of the mutagenic fractions were examined, it is likely that more nitroarenes will be characterized. This complexity is not surprising if one considers the numerous PAH substrates available for aromatic ring nitration: a chemical process which will readily occur in the presence of even low concentrations of nitrogen oxides. Positive identification of each nitroarene is made difficult by the small quantity of nitroarenes relative to co-eluting oxygenated and some sulfur-containing PAH and also by the absence of synthetic standards. Comparison of mass spectra and retention volumes with available standards, however, support the identifications which have been suggested.

A number of nitroarenes are potent mutagens in the Ames Salmonella assay, because nitroreductases in the tester strains facilitate reduction of nitroarenes of electrophilic intermediates which, in turn, react with nucleic acids. This raises the question of whether nitroarenes in diesel exhaust or any other environmental source pose a significant human health hazard. From a qualitative viewpoint, nitroarenes are hazardous because compounds such as 2-nitronaphtalene and 2-nitrofluorene are carcinogenic to animals. However, from a quantitative viewpoint, issues of the human dose and the biologic potency of nitroarenes have not yet been established. It is also not known if nitroarene emissions are independent of or bear a reciprocal relationship to the amount of unsubstituted PAH that is emitted. The development of convenient techniques to separate and quantify nitroarenes and metabolites may expedite the acquisition of data to answer these questions.

TABLE 1

Mass	Possible Compound
161.048	Nitroindene
197.048	Nitroacenaphthylene
199.063	Nitrobiphenyl*
211.063	Nitrofluorene*
213.079	Nitro-methylbiphenyl
223.063	Nitroanthracene*
225.079	Nitro-methylfluorene
247.063	Nitropyrene*
261.079	Nitro-methylpyrene
287.095	Nitro-methylchrysene
225.043	Nitro-fluorenone*
241.074	Nitro-hydroxymethylfluorene
253.038	Nitro-anthraquinone
256.048	Dinitrofluorene*
340.143	Dinitro-(C_6)alkylfluorene
348.111	Dinitro-(C_4)alkylpyrene
371.112	Trinitro-(C_5)alkylfluorene

COMPARISON OF NITRO-PNA CONTENT AND MUTAGENICITY OF DIESEL EMISSIONS

by

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INTRODUCTION

The increasing number of automobiles and light-duty trucks powered by diesel engines has generated concern over the emissions associated with these engines. The diesel engines have a higher particulate emission rate than gasoline catalyst engines. The extractable organics from both diesel and gasoline particle emissions have been found to be mutagenic and carcinogenic (1). Recently, nitro-substituted polynuclear aromatic hydrocarbons (nitro-PNA) have been identified in diesel particle extracts (2). Several of these nitro-PNAs are very potent bacterial mutagens (3).

A study was carried out to identify and quantitate nitroaromatic compounds in the extract of particulate material from three different diesel engines (Datsun Nissan diesel 220C, Oldsmobile diesel 350, and VW Rabbit diesel) and one gasoline engine (Ford Mustang II). The operating and sampling conditions have been described elsewhere (4). Mutagenic assay data was also collected on these extracts using the Salmonella typhimurium TA98 bioassay. The results of these two studies were compared to determine whether the amount of nitroaromatics detected can fully account for the direct-acting mutagenic activity indicated by the bioassay data.

METHODS AND RESULTS

Chemical

Two separate methods were developed to analyze the emission extracts: 1) combined high performance liquid chromatography/mass spectrometry (HPLC/MS) with positive chemical ionization (PCI); and 2) on-column injection high resolution gas chromatography/mass spectrometry (HRGC/MS) with negative

chemical ionization (NCI). The total dichloromethane extract was screened first by the PCI HPLC/MS method, which consisted of a Supelco normal phase HPLC column (Supelcosil LC-Si, 5 µm) interfaced to a Finnigan 4000 mass spectrometer via a microbore capillary and a polyimide moving belt. Nitropyrene and nitrophenanthrene/anthracene were detected by this method in all of the diesel engine extracts, but not in the gasoline engine extract.

The extracts were fractionated on silica gel by open-bed liquid chromatography into four compound class fractions: 1) hexane - aliphatic hydrocarbons, 2) hexane:benzene - polycyclic aromatic hydrocarbons and mononitroaromatics, 3) methylene chloride - moderately polar neutrals, including di- and trinitroaromatics, and 4) methanol-highly polar neutrals, primarily oxygenated compounds. Over 94% of the total extract was recovered for all four engines.

The three fractions expected to contain nitro-PNA (2,3,and 4) were analyzed by the NCI HRGC/MS system, which consisted of a J&W DB-5 bonded fused silica capillary column interfaced to a Finnigan 4000 mass spectrometer. The quantitation of nitroaromatics was based on response factors for eight standard nitroaromatics relative to the internal standard d_7-nitronaphthalene calculated over a concentration range of 100. More than twenty nitroaromatics were detected in the diesel engine extracts but only 1-nitropyrene was detected in the gasoline engine extract. In all cases the 1-nitropyrene was the nitroaromatic detected in greatest quantity and its concentration in the extracts is shown in Table 1. Quantitative data on the other nitroaromatics will be presented.

At masses higher than nitropyrene, the mono nitro derivatives of the molecular weight 228 (benz[a]anthracene, chrysene) and molecular weight 252 (B[a]P, B[e]P, perylene, benz[o]fluoranthenes) PNAs were also detected. Two nitropyrenone isomers were tentatively identified in the Nissan and Oldsmobile samples and three dinitropyrene isomers were identified in the VW sample.

Most of the compounds detected in the methanol fraction of the Nissan and VW samples were quinones. The methanol fraction represented 30% and 17% of the total organic mass for the Nissan and VW extracts, respectively.

Biological

The total dichloromethane extractable organics from each of the emission samples were bioassayed in the Salmonella typhimurium plate incorporation assay with minor modifications as reported elsewhere (5). The slope of the dose-response curve (rev/µg) for each of these samples with and without S9 activation was determined using a non-linear model (6) and is shown in Table 1. The emissions from the gasoline catalyst (Mustang II) differ from the diesel emissions in that these organics are significantly more mutagenic in the presence of the S9 activation system.

DISCUSSION

Both the PCI HPLC/MS and NCI HRGC/MS methods are capable of detecting nitroaromatic compounds. However, the greater chromatographic resolution and lower detection limit of the NCI HRGC/MS method favor the use of this method over the PCI HPLC/MS method. The limit of detection by NCI HRGC/MS is approximately 0.05 ng for the mononitroaromatics, but only about 100 ng for nitropyrene by the PCI HPLC/MS method.

The concentration of 1-nitropyrene detected in each of the samples in not highly correlated with the direct-acting mutagenic activity. The total nitroaromatic content does account for a substantive portion of the direct-acting mutagenicity of the Olds diesel, VW Rabbit diesel, and Mustang II gasoline vehicles. However, the nitroaromatics detected cannot account for the significantly higher mutagenic activity associated with the Nissan diesel extract.

It is possible that the quinones detected in the Nissan and VW extracts are the oxidation productions of nitroaromatics in the stored extracts, as similar extracts were shown to increase in toxicity with time (6). A greater concentration of quinones in the Nissan sample may indicate that a greater concentration of nitroaromatics may have been originally present in the extract. This is consistent with the fact that the Nissan extract originally had higher mutagenic activity than the VW extract.

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Table 1. Quantification of 1-Nitropyrene in Engine Exhaust Extracts and Mutagenic Activity of the Extracts

Sample	Conc. 1-Nitropyrene wt ppm	Mutagenic Activity, rev/μg	
		TA98/-S9	TA98/+S9
Nissan diesel	407	20.8	15.1
Olds diesel	107	2.1	1.4
VW Rabbit diesel	589	5.2	6.1
Mustang II gasoline	2.5	2.1	8.6

1-NITROPYRENE EMISSIONS FROM FIVE PRODUCTION MODEL DIESEL VEHICLES AND THE EFFECT OF DAMPING VALVE ON THE EMISSION

OCTOBER 1981

NISSAN MOTOR CO., LTD.

Background

At the EPA Symposium on Health Effects of Diesel Engine held in Cincinnati in December 1979, EPA and other organizations reported that a particulate exhaust sample obtained from a Nissan car had a very high BaP concentration. The cause of this high BaP level was investigated by Nissan and reported to EPA. (A copy of the report is attached to this handout.)

In March 1981, at CRC meeting, General Motors reported a very high 1-nitropyrene concentration in a sample from a Nissan diesel car. This sample was collected by EPA and its extract was distributed to various laboratories. The sample is sometimes called NI or NI-1 and is believed to be the same sample that was discussed in the last Symposium.

This is a preliminary report on 1-nitropyrene emissions from Nissan and other manufacturers' recent production vehicles. Possible cause of high 1-nitropyrene emission is also investigated.

Experimental Method

Exhaust particulate samples from five diesel vehicles were collected using a chassis dynamometer and a dilution tunnel on 20" x 20" Pallflex T60A20 filters. Samples were extracted with dichloromethane.

The extract was treated with a reducing agent to convert nitropyrene to aminopyrene. Liquid chromatograph separation and fluorescence detection and measurement of the aminopyrene were performed by comparison with standard solutions.

Results

Figure 1 shows 1-nitropyrene emissions of five test vehicles under Highway Fuel Economy Test cycle. Datsun 810 and Datsun Pickup are 1981.5 MY production models for the U.S. Federal market. Car A is a 1979 Federal production model. Cars B and C are 1979 production models for Japanese market. Cars A,B and C are not Nissan's product. Datsun Pickup was also tested by the Southwest Research Institute and the data is included in the figure.

Sample NI-1 was collected by EPA from a Nissan prototype car equipped with a 2.2 ℓ engine. The car was sent to EPA in 1973 and the sample was collected sometime in 1979. This sample was analyzed by GM and the result was reported in the CRC Diesel Exhaust Emission Workshop in March 1981. The same sample was also measured by Nissan.

Sample NI-1 shows a very high 1-nitropyrene concentration by both GM and Nissan measurement. Compared to NI-1, samples from five production models show lower levels. Although Datsun Pickup

is equipped with the same type engine (SD-22) as for the proto-type car provided to EPA, its 1-nitropyrene emission is the lowest among five vehicles.

As in the case of BaP, secondary injection was suspected to be a cause of high 1-nitropyrene emission. Secondary injection or injector "bounce" makes extra fuel injected late in the com-bustion process and results in high hydrocarbon emission. Damping valves installed in the fuel line are known to eliminate the sec-ondary injection.

Figure 2 shows the effect of damping valve on 1-nitropyrene, BaP and HC emissions. As expected, all three emissions decreases both in FTP and HFET when damping valves are installed. As shown in Figure 1, Datsun 810 and Pickup are equipped with damping valves whereas the prototype car provided to EPA had no damping valves. Car C which has damping valves shows low emission. Car A which has no damping valves gives highest 1-nitropyrene emission. How-ever, it is too early to say that damping valves are indispens-able to reduce 1-nitropyrene because Car B shows a modest level without damping valves. And when damping valves were taken off from the Datsun Pickup, 1-nitropyrene emission did not increased to the level of sample NI-1.

Other factors to increase secondary injection such as larger diameter injection tube and fuel injectors with carbon deposit are worth examining to reproduce the 1-nitropyrene level of NI-1. At the same time, sampling conditions such as gas temperature, NO_2 concentration and total gas volume passing through the filter should be investigated.

Summary

1.   1-nitropyrene emissions from five diesel powered production vehicles were measured and compared with sample NI-1 obtained from EPA. Samples taken from all five vehicles show consider-ably lower 1-nitropyrene concentration than NI-1. From these data NI-1 is not believed to be a proper sample to represent the production model diesel vehicles, even less it represents Nissan's current products.

2.   Damping valves installed in a fuel line have an effect to reduce 1-nitropyrene emission with a certain model of engine. Further investigation is necessary to clarify the cause of unusually high 1-nitropyrene concentration in EPA sample NI-1.

Figure 1

1-Nitropyrene Emission from Diesel-Powered Vehicles

HFET

Vehicle	Eng.	I.T.	EGR	※ D.V.	I.W.
Datsun 810	2.8 ℓ	7°BTDC	W	W	3,500lbs
Datsun Pickup	2.2	20°BTDC	W/O	W	3,000
A	1.5	3°ATDC	W/O	W/O	2,250
B	2.2	0°BTDC	W/O	W/O	2,750
C	1.8	7°BTDC	W/O	W	2,750

Test Fuel : Type 2D
(C.I.45)

※ D.V. : Damping Valve

SwRI Data
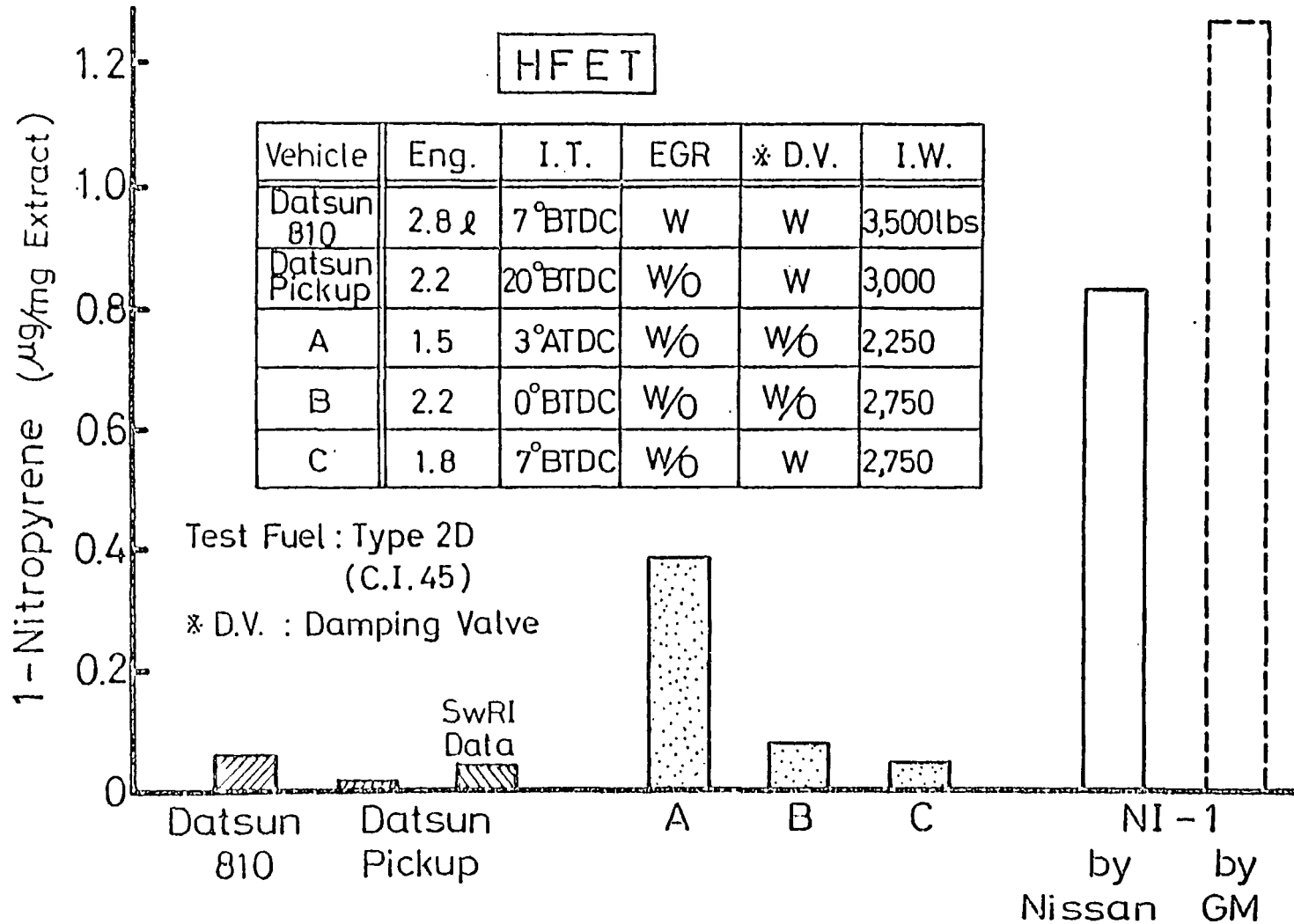
Figure 2

Effect of Damping Valve on 1-Nitropyrene, BaP & HC
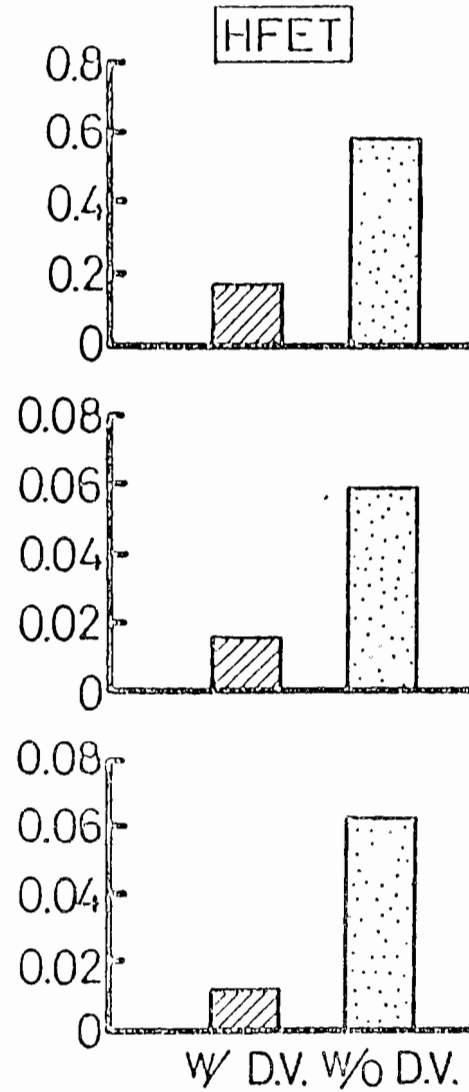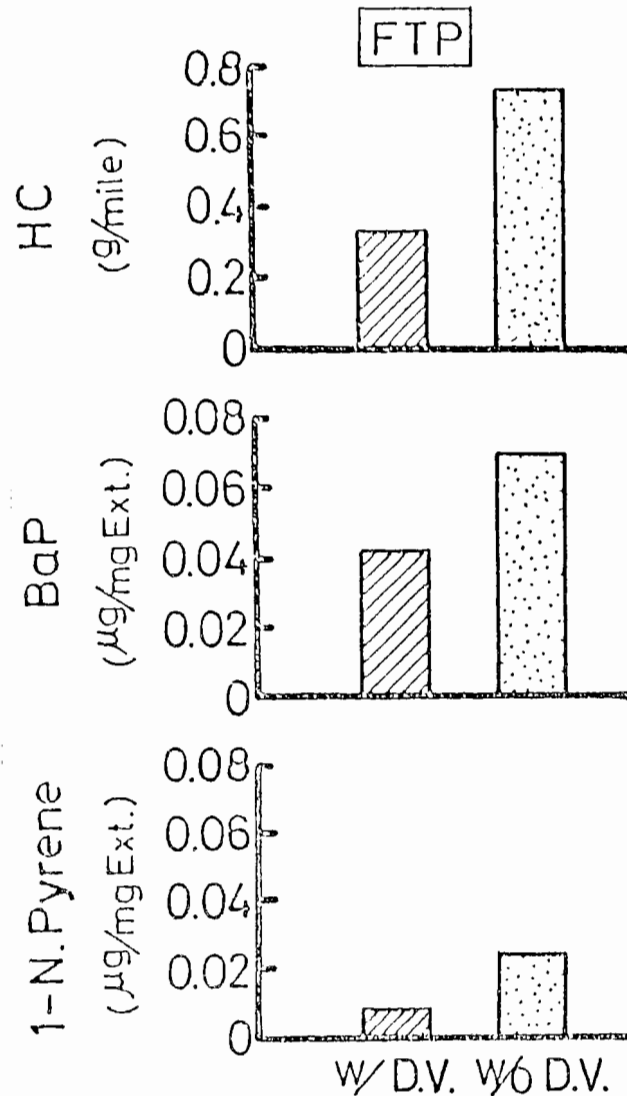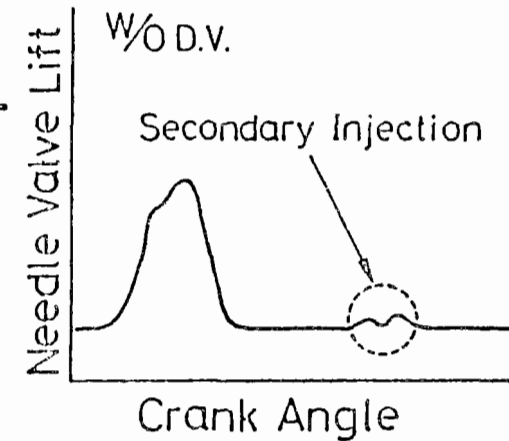
ANALYSIS OF THE FACTORS AFFECTING UNUSUALLY
HIGH BaP EMISSION FROM A NISSAN SD-22 DIESEL
ENGINE VEHICLE OBSERVED AT EPA

OCTOBER 1981

NISSAN MOTOR CO., LTD.
NISSAN DIESEL MOTOR CO., LTD.

Background

At the EPA Symposium on Health Effects of Diesel Engine held in Cincinnati in December 1979, it was reported that Nissan's Experimental Diesel Vehicle mounted with a SD-22, 4 cylinder, 2.2 liter engine showed the highest levels on the bioassay tests among the cars tested by the EPA. Additionally, it was found that benzo(a)pyrene (BaP) in the particulate extracts also scored exceptionally high level.

This experimental vehicle was sent to the EPA in reply to the request by Mr. E. Stork in 1973. The vehicle was equipped with an engine which had the fuel injection system modified from the 1973 Japanese domestic specifications.

We were very concerned about the EPA test results, and Nissan engineers visited the EPA ESRL at Research Triangle Park shortly after the Symposium in order to study the vehicle. This vehicle was sent back to Nissan, and the causes of these problems were investegated by our laboratory in Japan.

Objective

The objective of our study was to determine the causes of the high BaP emission level.

Experimental Results

The study described in this paper was done using the 2-D fuel made by Nippon Sekiyu. Table 1 shows the specifications of the test fuel in comparison with those of the EPA requirement and the EPA test fuel made by Union Oil.

BaP analysis was performed using the High Performance Liquid Chromatography (HPLC; Hitachi Model 635).

(1) Emission Confirmatory Tests

After the SD-22 vehicle was returned to Nissan, emission confirmatory tests were conducted as received condition.

As shown in Table 2, the test results indicated that:

- BaP and HC emissions at Nissan showed a large difference from the results of EPA test.

- The BaP level at Nissan was within the range of other companys' vehicles presented at the EPA Symposium in Cincinnati.

(2) Relationship between BaP and HC emissions

The tests were conducted with parameters of new and aged injectors and with and without damping valve.

It can be said that there is a direct proportional relationship between BaP and HC emissions, as shown in Figure 1.

(3) Factors Influencing on BaP Emission

(i) Effect of Injection Timing

The injection timing of the vehicle as received was 18° BTDC against the original setting of 20°. Our test results showed there was no significant difference in BaP emission within the range of 20 ± 2° BTDC.

(ii) Effect of Aged Injector

A comparison study of new and aged injectors was conducted. HC and BaP emissions with the aged injectors implied the existence of deterioration.

The obtained results are shown in Figure 2. Although particulate emission did not increase by the aged injector, a marked increase in BaP emission was observed.

It is thought that the high BaP emission was caused by deterioration of injector which led to the increase of the secondary injection.

(iii) Effect of Damping Valve

Testing with damping valve resulted in a large scale reduction of both HC and BaP. As shown in Figure 2, the damping valve is more effective to reduce HC and BaP when the aged injector is used. For particulate emission, however, no significant diference was seen by use of the damping valve.

Figure 3 and 4 show the fuel injection rates for without and with damping valve respectively. The large amount of secondary injection was seen when the damping valve was not used. From these figures it is found that the application of damping valve eliminates the secondary injection.

The drawing of damping valve is shown in Figure 5.

(iv) Effect of Injection Tube Inner Diameter

The effect of injection tube inner diameter on the secondary injection rate and emissions was studied using the diameter of 2 mm and 3 mm. The 3 mm tube was equipped in the EPA tested vehicle.

Figure 6 shows the secondary injection rate for the injection tube inner diameter of 3 mm. Extremely high secondary injection rate was observed in entire engine operating conditions. As shown in Figure 7, there was a marked reduction of secondary injection rate in the 2 mm inner diameter tube in comparison with the usage of 3 mm diameter tube.

HC and BaP emissions seen in Figure 2 decreased significantly when the 2 mm diameter tube was used. In addition, it is found that the system with damping valve and 2 mm inner diameter injection tube does eliminate the high BaP problem even when aged injectors are used.

Summary of the Study

- HC and BaP emission levels of the SD-22 vehicle tested in Nissan were much lower than results of EPA testing.

- This BaP level at Nissan was the same level as other company's vehicles presented at the EPA Symposium in Cincinnati.

- The direct proportional relationship between HC and BaP emissions was observed.

- BaP emission increased significantly when clogged injectors were used, due to their secondary injections.

- The system with damping valves and smaller inner diameter injection tubes is effective to reduce the secondary injection and does eliminate the high BaP emission problem even when clogged injectors were used.

BaP Emission from Nissan's Current Diesel Vehicles

(1) Comparison of Fuel Injection System

Table 3 shows the comparison of fuel injection system between the EPA tested vehicle and the improved specification vehicle. This improved specification, which is being used in Nissan's current U.S. models, includes damping valves, new type injectors, and 2 mm diameter injection tubes.

(2) BaP Emission Level

The emission test results of a vehicle with the improved fuel injection system are shown in Table 4.

As a result, the current improved system eliminates the high BaP emission problem, even when aged injectors are used.

Conclusions

It is concluded that the high BaP emission rate of the SD-22 diesel engine is not a problem inherent in all SD-22 diesel engines, but rather is peculiar to this engine alone.

The problem should be considered in light of the following factors;

(1) The vehicle in question was originally sent to the EPA for testing as far back as 1973.

(2) The high BaP emission rate seems to be caused by the secondary injection of the injectors.

(3) The details of the engine maintenance performed by EPA are unknown to us, but the injectors probably clogged up and this promoted the occurance of secondary injection.

(4) The fuel injection system of the 1973 model was not equipped with damping valves, since they were not available at that time.

(5) In view of the high HC and BaP levels, Nissan's current models adopting the improved fuel supply system eliminate them.

Table 1

Test Fuel Specifications

		EPA Requirement	2-D by Union Oil*	2-D by Nippon Sekiyu
Specific Gravity	15/4 °C	0.8393 - 0.8597	0.847	0.845
Viscosity	cst @ 37.8 °C	2.0 - 3.2	-	2.3
Cetane Index		45 - 50	48	44.3
Sulfur	wt %	0.2 - 0.5	0.16	0.25
Distillation	°C			
IBP		171 - 204	182	202
10%		204 - 238	219	224
50%		243 - 282	262	249
90%		288 - 321	316	291
EP		304 - 349	339	322
Aromatics	vol %	> 27	32.5	27
Olefins	vol %	-	1.3	-
Saturates	vol %	-	66.2	-

* used at EPA RTP (SAE Paper 790422)

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Table 2

BaP Emission from a Nissan Diesel Vehicle (Test Results at Nissan)

VEHICLE AS RECEIVED FROM EPA

I. W.   :   3,500 lbs
Engine   :   SD-22 (4 cyl., 2.2 liter)

Mode	Part (gpm)	% Extract	BaP ** (μg/mi)	HC (gpm)	CO (gpm)	NOx (gpm)	mpg
LA-4 Hot	0.25	16.5	2.87	0.22	1.51	1.68	28.6
HFET	0.33	13.9	0.87	0.13	0.80	1.48	30.5
HFET * (EPA Data)	0.33	5.3	20.5	0.85	1.31	1.08	32.7

(n = 2)

*   :   From the data presented at the EPA Symposium and
         BaP emission is calculated by Nissan

** :   Sampled using 8" x 10" Pallflex T60A20 filter and
         analyzed by HPLC (Hitachi 635)

Figure 1

Relationship between HC & BaP

Test Mode : HFET   Test Fuel : Type 2D
                                   (Cetane Index 44.3)
Engine : SD 22   Injector : OSD 211
I.W. : 3,500 lbs   New & Aged(23,500 km)
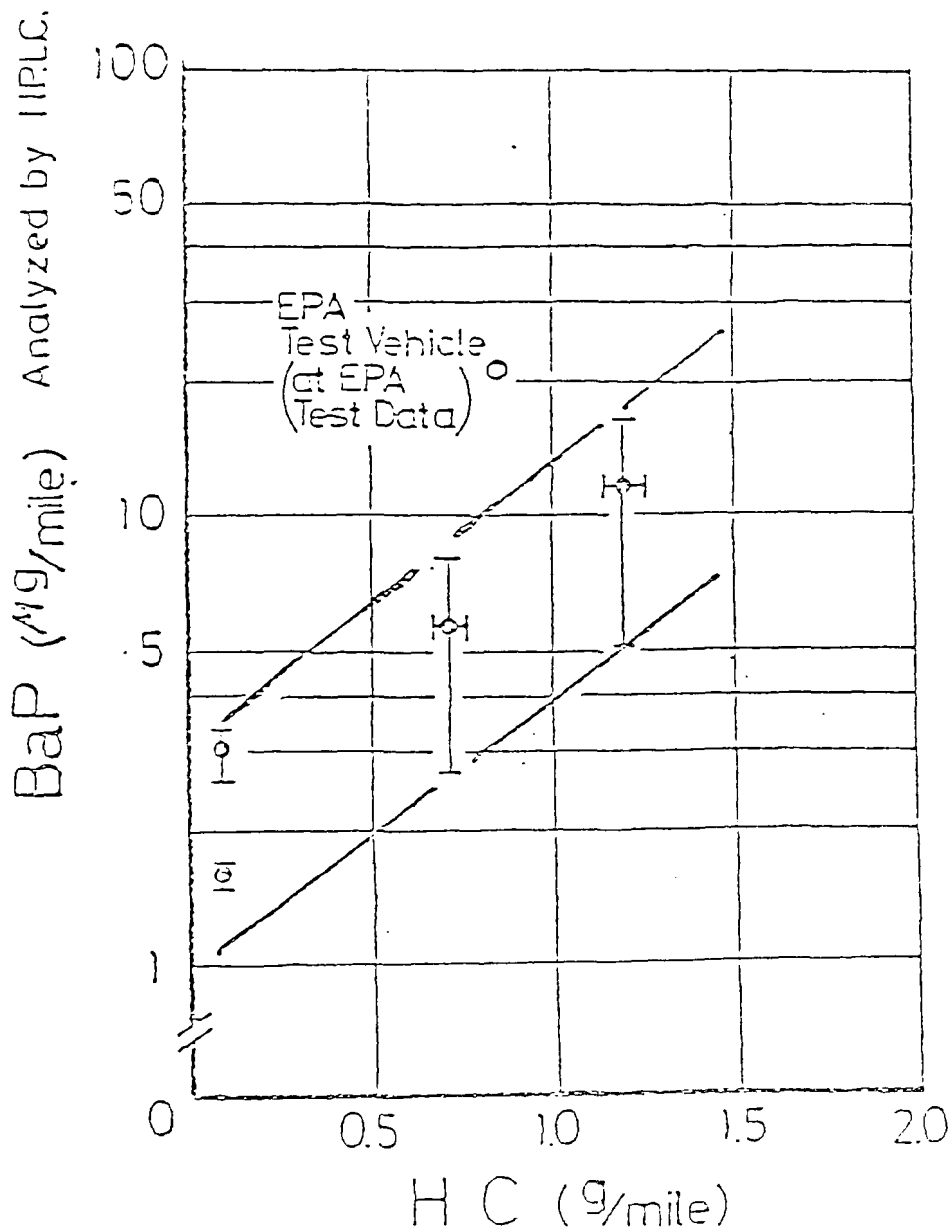   Damping Valve : w/ & w/o

Figure 2

Effect of Mileage Accumulation of Injectors and Damping Valve on HC, Part. & BaP

Test Mode : HFET   Test Fuel : Type 2D
Engine : SD 22   Injector : OSD 211
I.W. : 3,500 lbs   Injection Timing : 20°BTDC

Figure 3

Fuel Injection Rate

2400 rpm
Q = 20 mm³/st.

Secondary Injection

Needle Lift
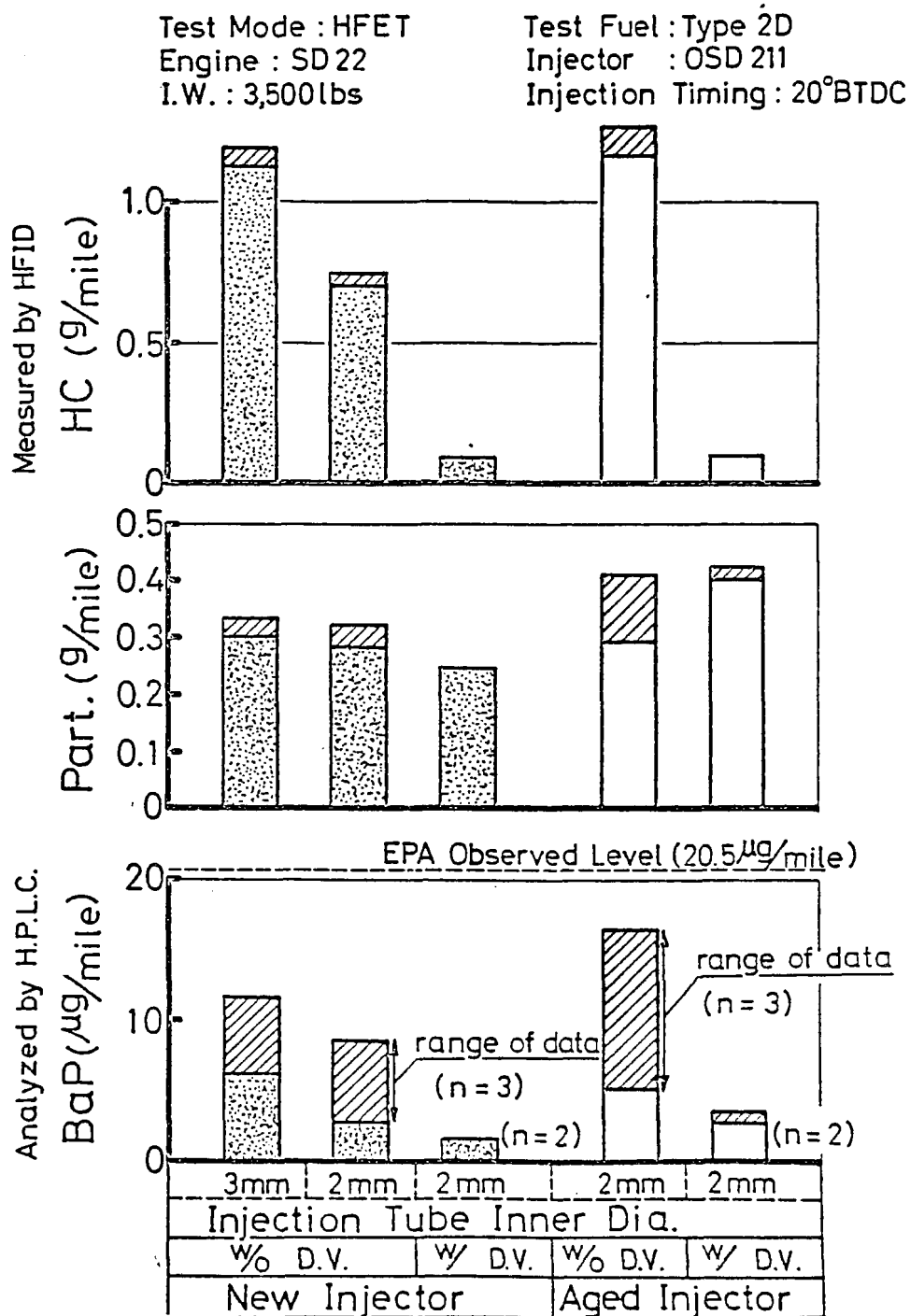
Main Injection

Injector OSD 193
Inj. Tube Dia. 3 mm
Without Damping
Valve

0   .577.   20   30   40   50   60   70   80   90   100 deg.

20   30   40   50   60   70   .   80   90   100 deg.

Figure 4

Fuel Injection Rate

Main Injection

2400 rpm

Q=20 mm³/st.

Needle Lift

0   20   30   40   50   60   70 deg.

Fuel Spray

20   30   40   50   60.   70 deg:
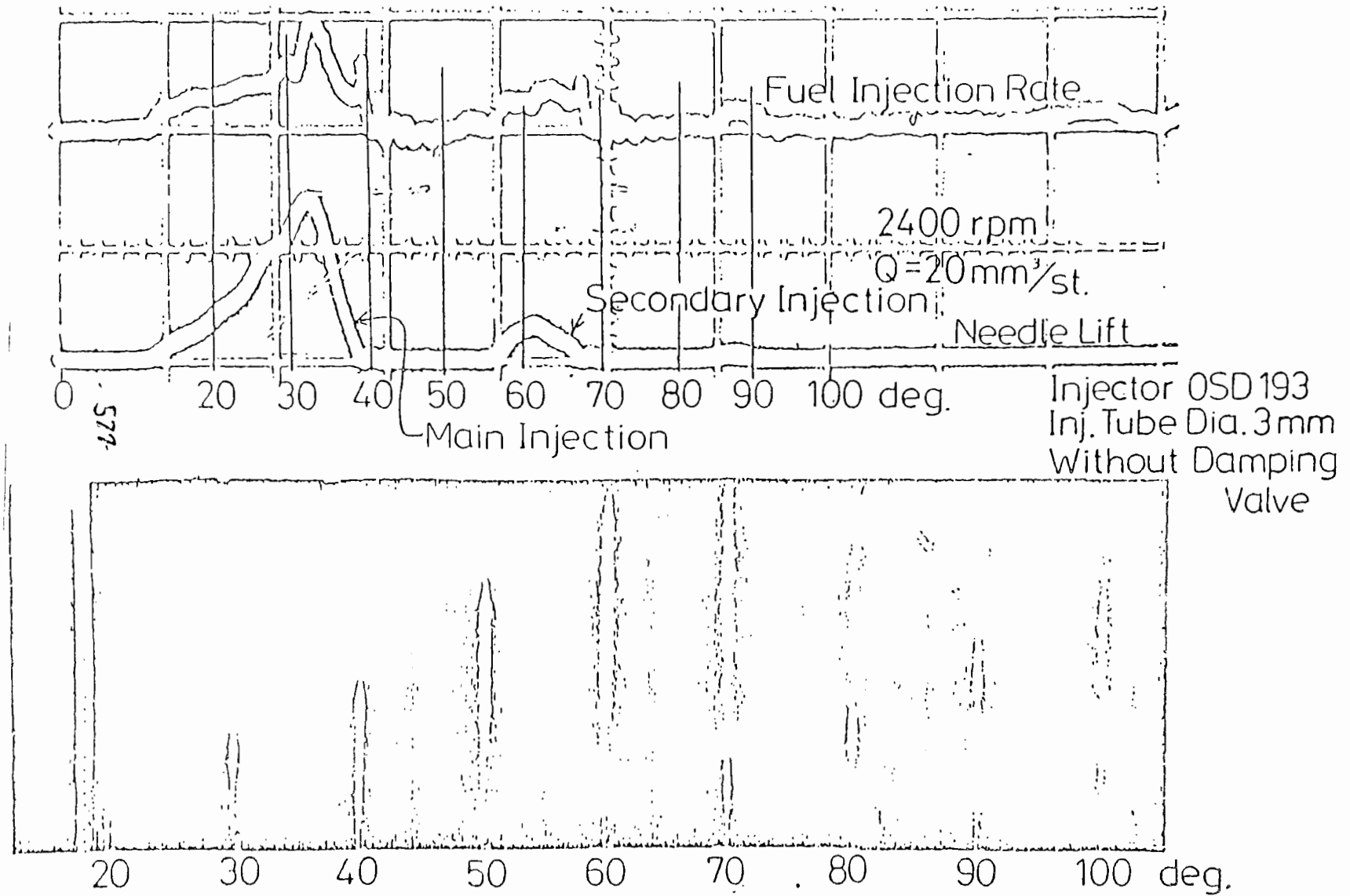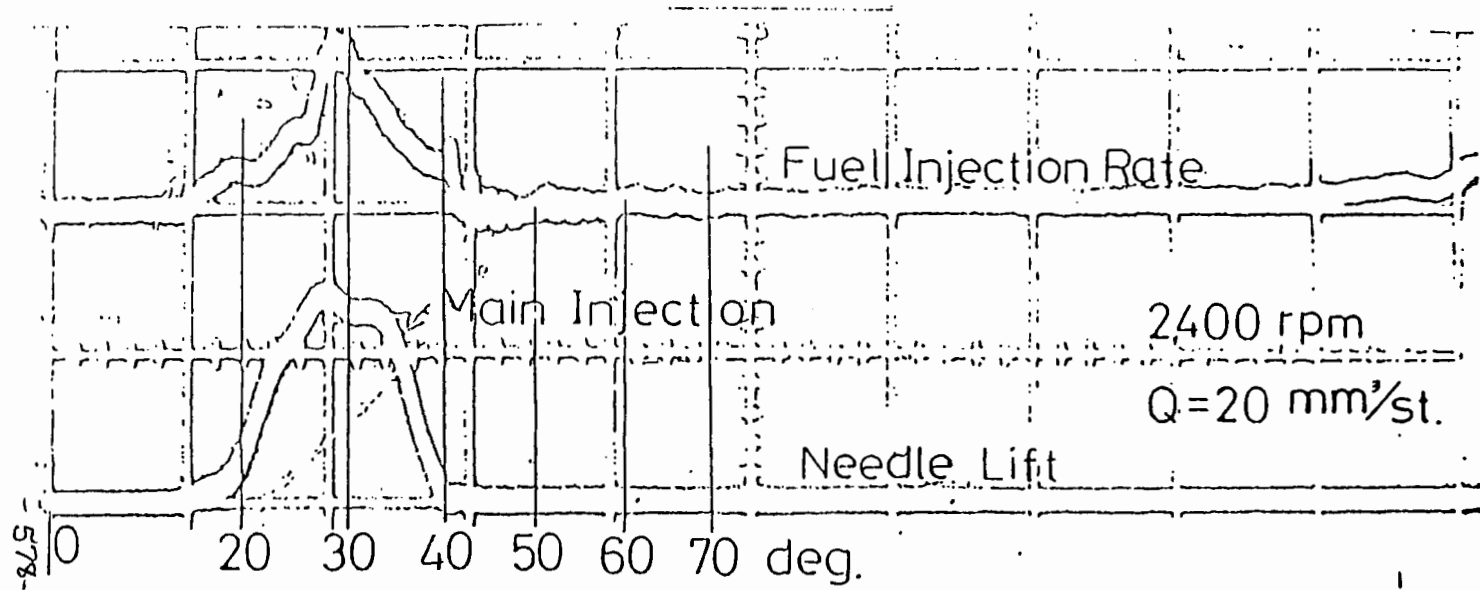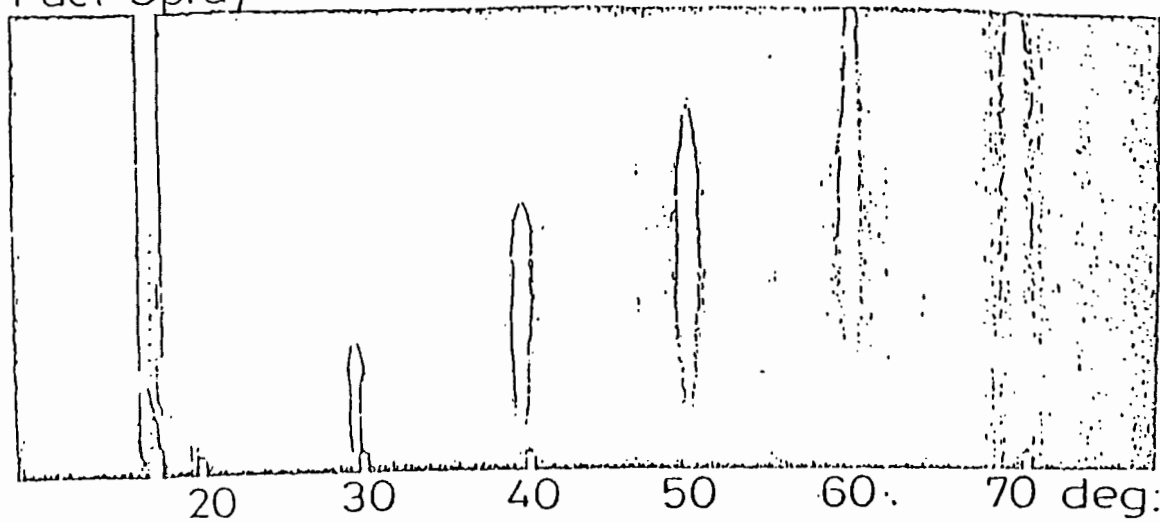
Injector 0SD193
Inj. Tube Dia. 3mm
With Damping Valve

Figure 5

The Structure of Delivery Valve

W/ Damping Valve
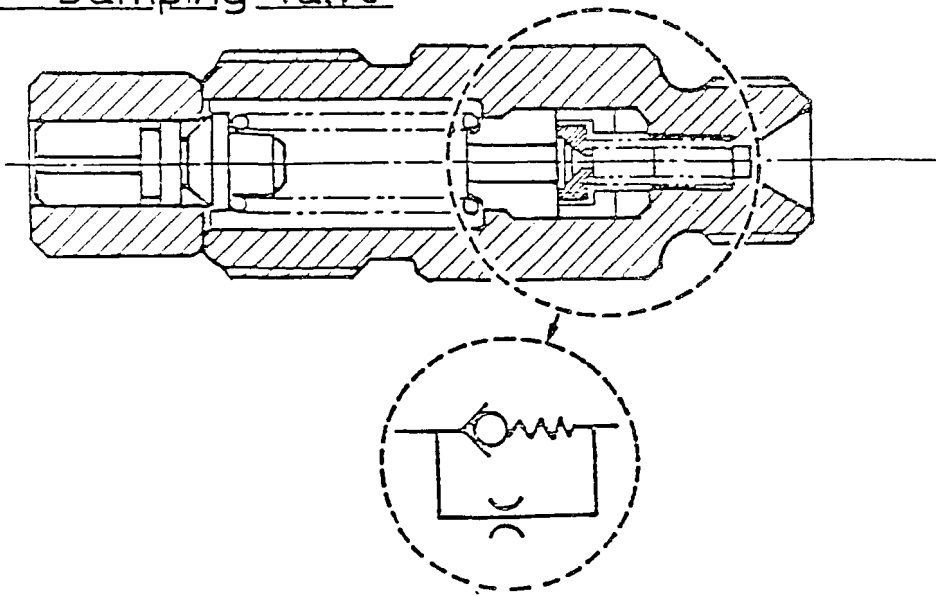
W/o Damping Valve
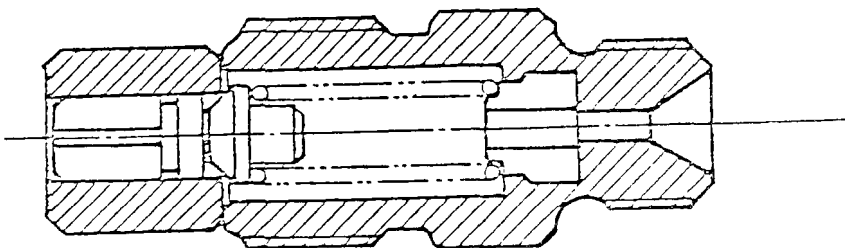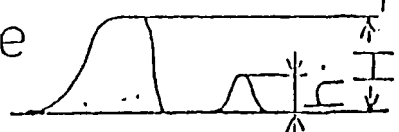
Figure 6

Map of Secondary Injection Rate

Inj. TubeDia. 3mm
Without Damping Valve
Injector : OSD 211

Secondary Inj. Rate = $\dfrac{h}{H}$ ×100%
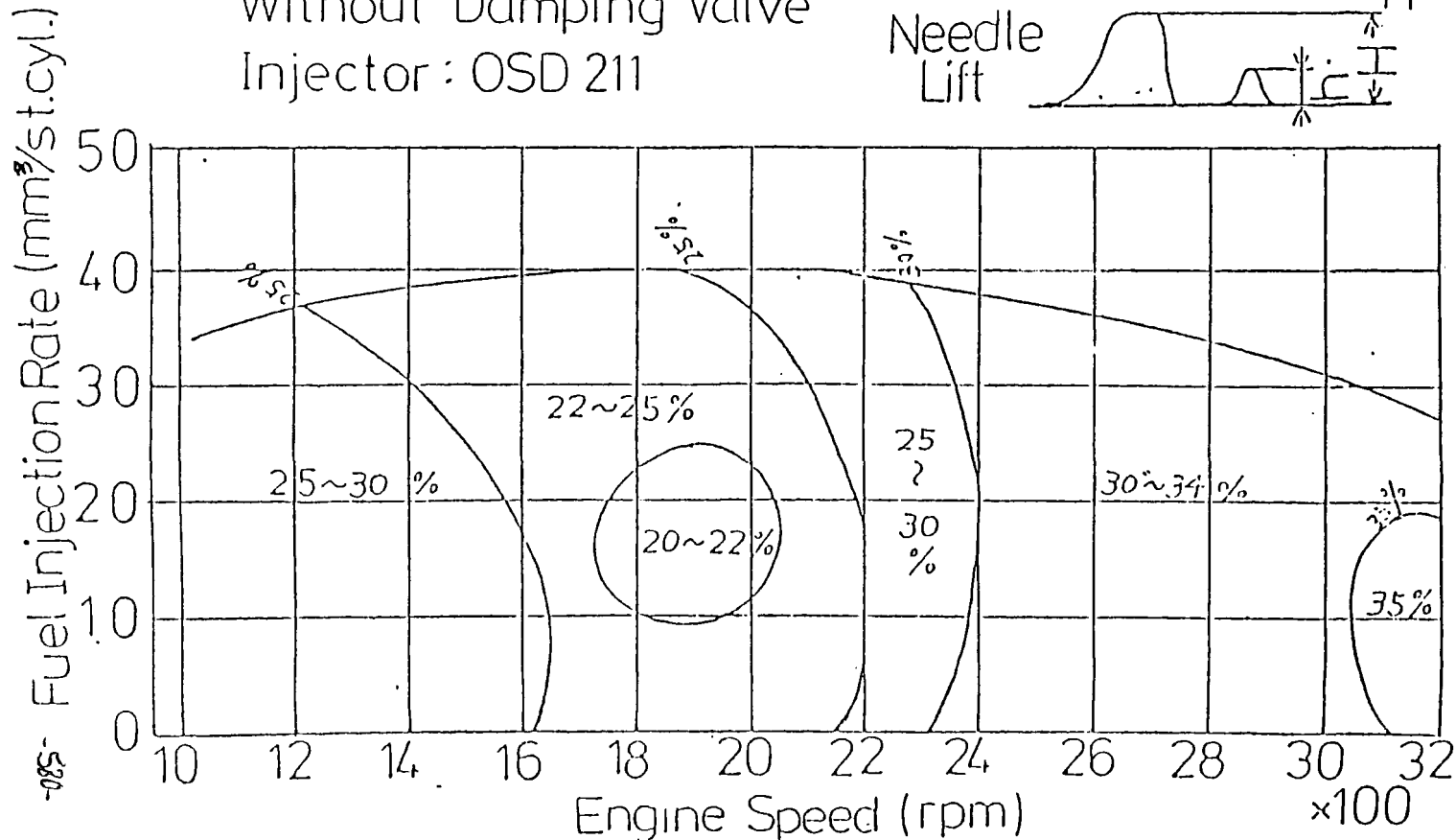
Needle
Lift

Figure 7

Map of Secondary Injection Rate

Inj. TubeDia.: 2mm
Without Damping Valve
Injector : OSD 211

Secondary Inj. Rate = $\dfrac{h}{H}$ ×100%

Needle
Lift
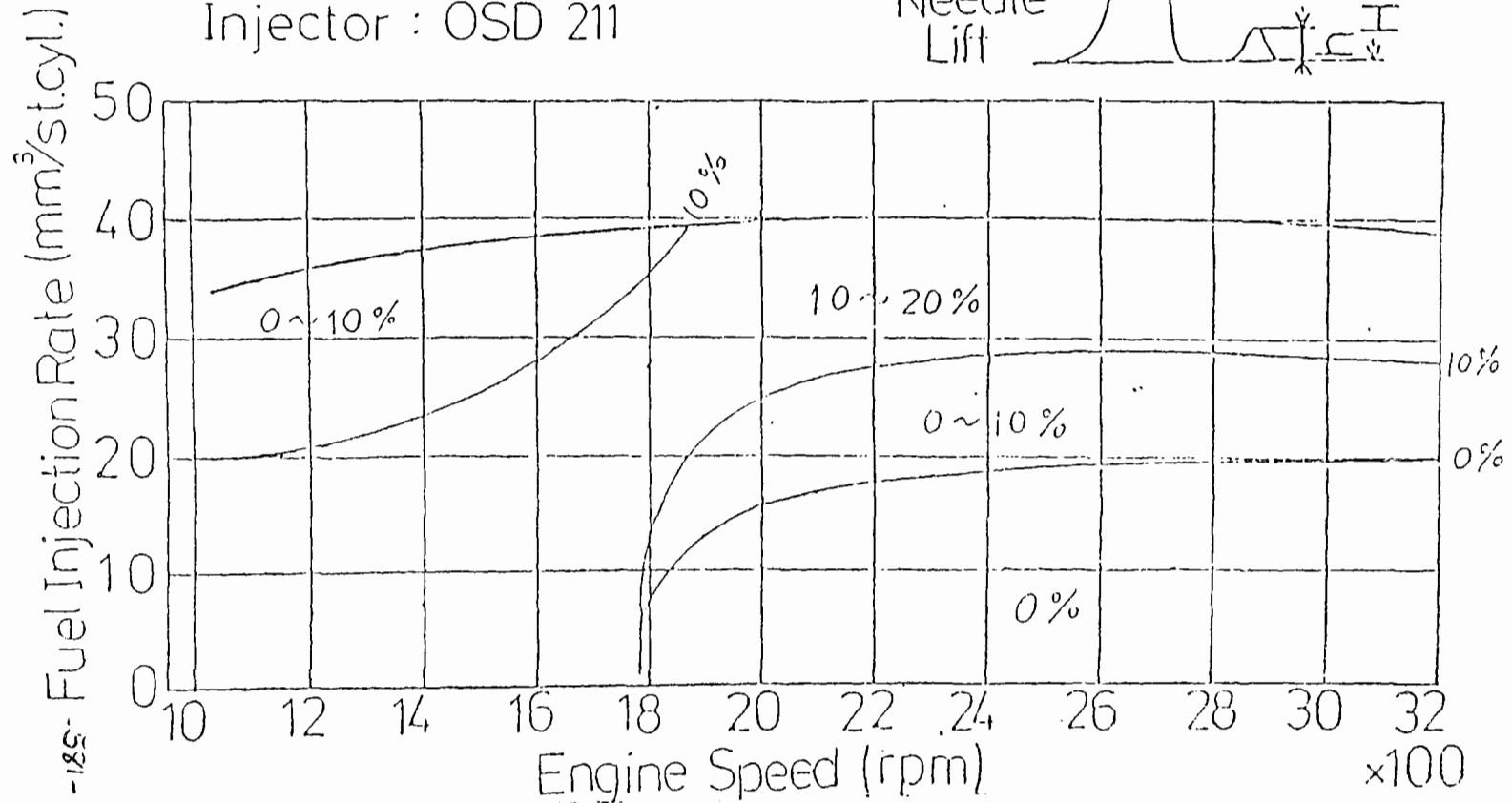
Table 3

Comparison of Fuel Injection System

Item		EPA Tested Vehicle	Improved Spec.
Injection Pump		Without D/V	With D/V
Injector	Type	OSD 211	OSD 193
	Clearance ※ in Dia.	31~41 μ	28~34 μ
Injection Tube Dia.		3 mm	2 mm

Needle

Body

※ Clearance

Carbon Deposit
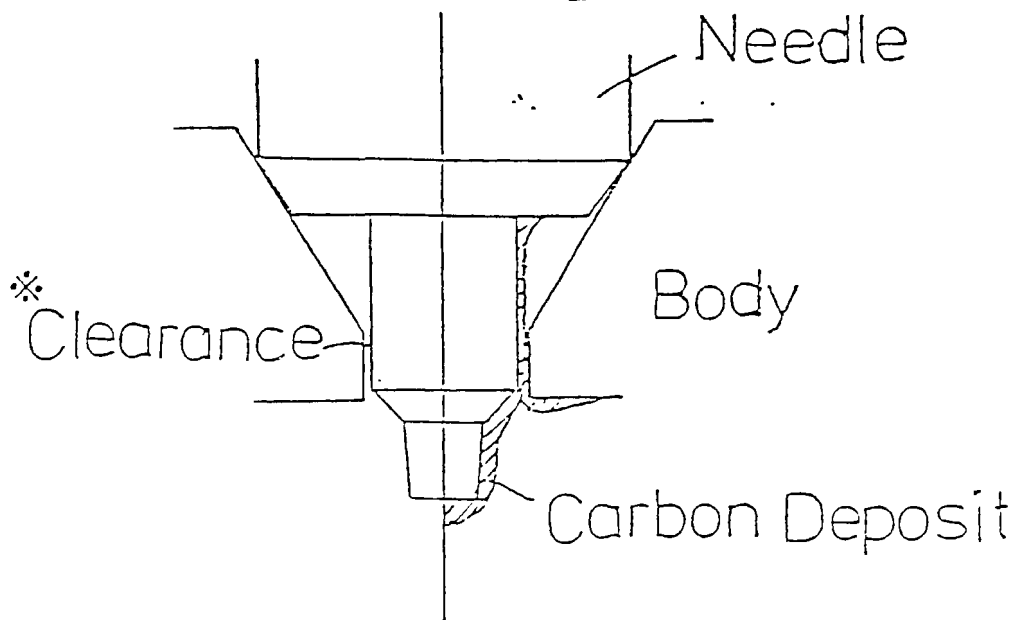
Table 4

Emission Test Results of the Vehicle with Improved Fuel Injection System

TEST MODE : HFET

Injector	Part. (gpm)	BaP (µg/mi)	HC (gpm)	CO (gpm)	NOx (gpm)	mpg
New Injector	0.27	0.73	0.11	0.48	1.54	34.0
	0.23	0.53	0.08	0.47	1.58	32.8
Aged Injector*	0.25	0.44	0.07	0.47	1.52	32.4
	0.24	0.41	0.06	0.46	1.49	32.3

* Aged injector : AMA 50,000 miles

CAPILLARY COLUMN GC/MS CHARACTERIZATION OF DIESEL EXHAUST PARTICULATE EXTRACTS[1]

by

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INTRODUCTION

Previous studies have shown that most of the direct-acting Salmonella typhimurium mutagenic activity (>50%) in diesel exhaust particulate extracts is concentrated in chemical fractions which contain compounds of moderate polarity (2). Analytical-scale normal phase high performance liquid chromatography(NP-HPLC) (3), packed-column GC/MS and high resolution MS analysis were used in that work to determine that these moderately-polar fractions consisted primarily of polynuclear aromatic hydrocarbon (PAH) derivatives. The objective of this study was to extend the previous work by developing preparative scale NP-HPLC fractionation followed by fused silica capillary GC/MS analysis in order to improve component resolution.

EXPERIMENTAL

Light duty diesel exhaust particulate samples were collected on T60A20 Pallaflex filters using a dilution tube and a chassis dynomometer test facility. Filter samples were soxhlet extracted with dichloromethane (DCM).

HPLC analysis was performed on a Varian Model 5600 LC equipped with a 7.8 mm i.d. x 30 cm long Microporasil 10 μ column. The solvent program consisted of 100% hexane for 5 min, then 1% DCM/min for 5 min, followed by a linear gradient to 100% DCM in the next 25 min, 100% DCM for 10 min, then 10% acetonitrile/min for 10 min, and a final 10 min flush with 100% acetonitrile. The chromatographic separation was monitored by UV at 254 nm and by fluorescence at 254/320 nm. Further details of this technique are presented elsewhere (3).

GC/MS analyses were performed on a VGMM ZAB-2F mass spectrometer equipped with a 30 m long x 0.25 mm i.d. SE54 fused silica capillary column interfaced directly to the mass spectrometer source. Samples were injected directly on-column and temperature programming was 80° to 270° at 4°/min. Electron impact ionization techniques were used.

RESULTS AND DISCUSSION

This investigation emphasized the analysis of HPLC fractions containing nonpolar and moderately polar PAH derivatives. Nonpolar aliphatic and highly polar HPLC fractions were not characterized by GC/MS. The nonpolar and moderately polar PAH derivatives which were identified are listed respectively in Tables I and II. Many of the compounds identified have a large number of isomers as indicated by the parentheses in the tables. This is illustrated by the mass chromatograms in Fig. 1 which show the increasing complexity of the isomer series as methyl substituents are added to anthracene and phenanthrene. There are probably more isomers for the methylated anthracene and phenanthrenes than we were able to resolve even with the high-resolution fused-silica capillary column. It would be difficult and of limited utility to identify every specific isomer present in these fractions. For this reason, synthesis of standards and identification of isomers are only being undertaken on those groups of PAH and PAH-derivatives which yield a relatively high level of direct- or indirect-acting Ames activity. This has been found to be the case for the nitrated-PAH derivatives which tend to show high levels of direct-acting mutagenicity compared to other PAH and PAH derivatives in these samples.

The combination of normal phase HPLC fractionation followed by capillary GC/MS analysis proved to be a very useful approach to the qualitative characterization of diesel exhaust particulate extracts. The quantitative analysis of these diesel extracts by capillary GC/MS is complicated by the labile nature of some of the PAH derivatives. The quantitation technique found to be most accurate and presently being used in our laboratory employs the deuterated analog of the PAH derivative of interest which is added to filter samples prior to extraction. The deuterated standard exhibits the same chemical characteristics as the native compound, but it can be distinguished mass spectrometrically. Therefore, losses which occur during sample workup and analysis can be accounted for.

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4. Levine, S. P., and L. Skewes. 1981. High performance semi-preparative liquid chromatography of diesel engine emission particulate extracts. J. Chromatogr. In preparation.

Table I. Nonpolar PAH Identified in Diesel Exhaust Particulate Extract

dibenzothiophene
anthracene and phenanthrene
methyl dibenzothiophene isomers(3)
methyl (phenanthrene and anthracene) isomers(4)
dimethyl dibenzothiophene isomers(7)
dimethyl (phenanthrene and anthracene) isomers(13)
fluoranthene and pyrene
trimethyl dibenzothiophene isomers(9)
BaP, BeP, perylene,and isomers(3)
trimethyl (phenanthrene and anthracene) isomers(15)
tetramethyl dibenzothiophene isomers(12)
tetramethyl (phenanthrene and anthracene) isomers(16)
benzo(g,h,i) fluoranthene
benz(a)anthracene, chrysene, benzo(c)phenanthrene,
  triphenylene isomers(2)
methyl benz(a)anthracene isomers(4)
pentamethyl dibenzothiophene isomers(4)
dimethyl benz(a)anthracene isomers(2)
methyl (fluoranthene and pyrene)isomers(7)

Table II. Moderately Polar PAH Derivatives Identified in Diesel Exhaust
                Particulate Extract

benz(a)anthracenedione
methyl (anthrone and phenanthrone) isomers
thiozanthone isomer
dimethyl (anthrone and phenanthrone) isomers
pyrenone
trimethyl (anthrone and phenanthrone) isomers
methyl thioxanthone
dimethyl thioxanthone isomers(2)
benz(d,e)anthrone and isomers(3)
1-nitropyrene
1,1' biphenyl-ol
9-fluorenone
(pyrene or fluoranthene) carboxaldehyde
dibenzofuran carboxaldehyde
phenanthrone
anthrone isomer
9-xanthone
xanthene carboxaldehyde
(anthracene or phenanthrene)dione
dibenzothiophene carboxaldehyde
methyl (anthracene or phenanthrene)dione
phenanthrene carboxaldehyde
anthracene carboxaldehyde
methyl (anthracene and phenanthrene)
  carboxaldehyde isomers(9)
dimethyl (anthracene and phenanthrene)
  carboxaldehyde isomers(8)

Figure 1. Mass chromatograms of phenanthrene(P) and anthracene(A) (178), methyl-A and -P (192), dimethyl-A and -P (206), and trimethyl-A and -P (220)
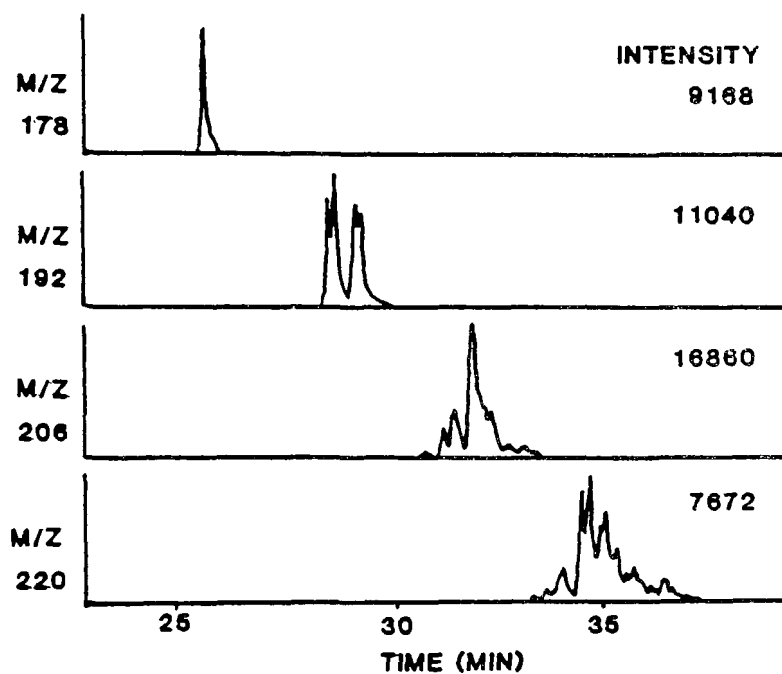
RESPIRATORY HEALTH EFFECTS OF EXPOSURE TO DIESEL EXHAUST EMISSIONS
(Bus Garage Mechanics; Salt, Potash, Metal, and Coal Miners)

by

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A comprehensive research program has been mounted relating to chronic and acute respiratory health effects of diesel emissions exposure. Special attention has focused on occupational groups exposed in enclosed spaces. This study involves over 5,000 workers engaged in various types of mining occupations as well as bus garage mechanics. These subjects were given chest radiographs, asked questions on respiratory symptoms, smoking and occupational histories, and given spirometric tests. These data have been coupled with industrial hygiene information to evaluate relationships between selected health parameters and component measures of diesel exhaust emissions. This paper reports the results for each group of workers separately.

PHYSICO-CHEMICAL PROPERTIES OF DIESEL PARTICULATE MATTER

by

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Numerous studies have dealt with the identification and quantification of the compounds sorbed onto Diesel particulate matter. The ultimate environmental significance of these sorbed species depends upon the relative bioavailabilities which, in turn, depend upon the nature and strength of interactions prevailing at the gas-solid interface. This research focused on the fundamental adsorptive properties and surface characteristics of Diesel particulate matter. In addition, the free radical nature of the particles and the associated reactivity with selected stimuli was investigated.

This study was carried out using a graphitized carbon black, Spheron 6, as a "reference" solid. Two Diesel particulate samples were used. The first, DPM-PSU, was collected from a single-cylinder engine operated with a prototype fuel and a lubricant free of trace inorganic compounds. The second, DPM-EPA, was collected from an Oldsmobile 350 engine operated with a commercial fuel and lubricant at the US E.P.A.

Electron micrographs and elemental compositions revealed the common spherical carbon particle structure of all the samples. Yet, the Diesel samples had lower bulk densities and higher external surface areas, as calculated from mean particle diameters. Nitrogen B.E.T. surface areas

were measured in order to determine internal plus external surface areas. The surface area of DPM-EPA was found to be dependent on outgassing pretreatment. The surface area accessible to nitrogen increased from 41 M^2/g at 50°C degassing to 112 M^2/g at 400°C degassing. DPM-PSU was measured to have a surface area of approximately 104 M^2/g and showed little change upon increasing activation temperature. Nitrogen porosity experiments revealed the existence of pores of 100-200Å in diameter in both Diesel samples.

Isosteric heats of adsorption of a variety of organic compounds on the particulate samples were measured with gas-solid chromatography. The heats on DPM-PSU were consistently greater than those on graphitized carbon blacks (gcb). The variations of adsorption energies with adsorbate surface coverage were determined by measurement of adsorption isotherms at different temperatures. DPM-PSU exhibited adsorption characteristics similar to those of gcb and the few differences are attributed to a more polar and energetically heterogeneous DPM-PSU surface. DPM-EPA was determined to have markedly different properties due to the increased quantity of presorbed material. The DPM-EPA surface was found to be relatively non-polar and homogenous. Removal of the presorbed species caused the surface to become more active and similar to that of DPM-PSU. The significance of these results is that compounds close to the carbon surface will be more difficult to remove than those adhering to presorbed layers. Sorbed compound bioavailability and surface properties of Diesel particulate matter are dependent upon the nature and amount of presorbed material.

A related study of the reactivity of Diesel particulate matter with respect to atmospheric stimuli was performed using electron paramagnetic resonance (EPR) spectrometry. The EPR signals of the three samples were monitored after selected heat and evacuation treatments, gas (O_2, NO, NO_2) exposures, and ultraviolet/visible irradiation. The sample signals differed with respect to line widths but all signals narrowed upon sample evacuation and heat treatment and broadened upon exposure to oxygen and nitric oxide. The Diesel particulate sample signals were extremely sensitive to nitrogen dioxide and irradiation. Exposure of NO_2 caused an increase in the free radical concentration in the Diesel samples. Irradiation effects were varied depending on sample conditions but the greatest signal increase occurred with evacuated Diesel particles. With EPR, the existence of free radicals in Diesel particulate matter and the reactivity of these species with respect to selected treatments were demonstrated. The results provide evidence of potential photochemical reactivity of airborne particulate matter.

SOME FACTORS AFFECTING THE QUANTITATION OF AMES ASSAYS

by

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The simple theory of bacterial mutation experiments of Luria and Delbrück (1) starts with the assumption

$$\frac{dm}{dt} = \mu n(t) \tag{1}$$

where m is the number of mutants, n is the number of wild type, and μ is the mutation rate coefficient. We have shown (2) that, in the absence of cell killing, this equation predicts for the Ames assay

$$M = \alpha CN \tag{2}$$

where M is the number of revertants per plate, α is the mutation rate per concentration, C, of mutagen and N is the total number of histidine auxotrophs in the background lawn. Thus the dose-response function is linear, but the slope is proportional to N. In the Ames assay, $N \approx n_0 P$, where n_0 is the initial inoculum and P is the average number of bacteria per background colony of histidine auxotrophs.

In a series of experiments we determined dose-response curves as a function of n_0; estimated n_0 by counting background colonies in photomicrographs (100X) of the background lawn; and estimated the volume of individual background colonies which is proportional to P. We found that P depends nonlinearily on n_0; P is much larger at low n_0 than it is at high n_0 presumably because at lower n_0 there is less competition among the colonies for the trace histidine. We observe that N decreases by about 1/3 when n_0 is decreased from 10^8 to 10^7 bacteria/plate. N is roughly independent of n_0 when n_0 is less than about 5×10^6 bacteria/plate. For merely detecting mutagens, neglect of the dependence of slopes on N, while conceptually incorrect, may have no serious effect. For quantitative experiments, such as determining the contribution of a compound to the mutagenicity of a mixture, failure to take into account the dependence of the slopes on N may

cause errors by factors of 2 to 3.

When the test compound causes killing, then equation (1) becomes:

$$\frac{dm}{dt} = \mu n(t) - k_b m \qquad (3)$$

where k_b is the killing rate coefficient for mutants and, now, n(t) must include a specific killing term for the histidine auxotrophs. If we assume that

$$n(t) = n_0 \exp[(\gamma - k_a)t] \qquad (4)$$

where γ is the growth rate coefficient and k_a is the killing coefficient for histidine auxotrophs, and if we also assume that killing and mutations are independent events and that the killing coefficients of auxotrophs and revertants are equal and proportional to the concentration of mutagen, i.e., $k_a = k_b = kC$, then the number of revertants per plate is of the general functional form

$$M \sim \alpha CN' \exp(-kC) \qquad (5)$$

where N' is the total number of histidine auxotrophs in the background population when kC<<1. The concentration which yields the maximum in the dose-response function is $C_m = 1/k$.

We have obtained dose-response functions using diesel particulate extract which have a maximum followed by a monotonic decrease to zero. We determined the killing coefficient k in three ways: (1) from the value of the concentration corresponding to the maximum of the dose-response function; (2) from a classical dilution-plating killing curve; and (3) from a killing curve developed from counting colonies in photomicrographs of the background lawn. The killing rates determined from these 3 methods agree to within about 15%, suggesting that this simple model is a good approximation to the mutation-killing kinetics, at least for these samples.

This model is free of adjustable parameters in the sense that the two parameters can be determined directly from the data. Functional forms similar to equation (5) have sometimes been used ad hoc in statistical curve fitting routines to describe Ames assay data; the above derivation provides theoretical support for use of this function. We will show data to illustrate that the apparent slope of the initial approximately linear portion of non-linear dose-response functions obtained with several diesel-particulate extracts can over estimate, by as much as a factor of 2, the actual mutation frequency in the Ames assay.

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CHEMICAL AND MUTAGENIC CHARACTERISTICS OF DIESEL EXHAUST
PARTICLES FROM DIFFERENT DIESEL FUELS

by

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A potential for increased use of a wide variety of diesel fuels has in-
creased the importance of studies to determine whether the nitrogen content
of different fuels affects the chemical and mutagenic characteristics of the
particles produced during combustion. In this study, the exhaust particles
from five diesel fuels with various nitrogen and aromatic hydrocarbon contents
were examined. The fuels included minimum quality #2 (Min. Qual.), jet fuel
(JP-7), shale diesel fuel-marine (DFM), a base fuel plus heavy aromatics and
hexylnitrate (BF+HAN+HN), and the base fuel plus isoquinoline (BF+IQ). Table 1
indicates the aromatic and nitrogen contents of these fuels. Particles used
in the study were generated by Southwest Research Institute for EPA using a
Mercedes 240D vehicle driven through five consecutive Highway Fuel Economy
Tests.

Experimental

The particle samples were soxhlet extracted with methylene chloride and
these extracts and the fuel samples were fractionated by acid-base and silica
gel column chromatography methods. Six fractions resulted from each material:
base, acid, aliphatic hydrocarbon, aromatic HC, moderately polar neutral, and
highly polar neutral. Several fractions were analyzed by selective detector
gas chromatography and by gas chromatography-mass spectrometry. A 30m fused
silica Carbowax column was used for the moderately polar and highly polar
fractions, and a 30m SE-52 column was used for the aromatic hydrocarbon frac-
tion.

All fractions were assayed for mutagenicity in the Ames histidine rever-
sion test with Salmonella typhimurium TA98 as the target cell. All samples were

tested with and without metabolic activation with Aroclor induced rat liver (S9) homogenates.

Because of the current interest in nitroaromatics in diesel particles (1,2,3), an experiment was done to estimate the recovery of smaller ring nitroaromatics from filters. 2-Nitrofluorene was added to one of the filter samples (BF+HAN+HN) and this "spiked" filter as well as a control filter of the same fuel were extracted, fractionated, and assayed in the Ames test with TA98 minus S9.

Results and Discussion

The amount of material extracted with DCM from the diesel particles ranged from 9% to 16%. Recovery of material in the fractionation procedure ranged from 82% to 105%. The weight distribution in the six chemical class fractions did not appear to correlate with either the aromatic or nitrogen content of the parent (uncombusted) fuel. Nitroaromatic standards distributed between the aromatic hydrocarbon and moderately polar neutral fractions.

Gas chromatography with a nitrogen selective detector was used to compare the nitrogen-containing components from the five particle samples. The moderately polar fractions showed similar patterns in all five samples, as did the highly polar fractions (Fig. 1). However, particles from the diesel fuel marine and minimum quality fuels appeared to contain more nitrogen compounds than did the other fuels. There did not appear to be a correlation between the major nitrogen-containing peaks in the fractions and the nitrogen content of the fuel.

The aromatic hydrocarbon fraction of the shale diesel fuel-marine particles was analyzed by GC-MS. It contained numerous polycyclic aromatic hydrocarbons including phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzofluoranthenes, and benzpyrenes. The moderately polar fraction of the particles from minimum quality #2 contained a number of carboxylic acids, carboxylic acid methyl esters, phenanthrenequinone, alkylated phenols, fluorenone, alkylated fluorenones, and possible alkanones. Compounds tentatively identified in the highly polar fraction of particles from the base fuel plus isoquinoline include a number of phenols, carboxylic acids, benzoic acid, quinoline, xanthenone, and benzofuranone. Another peak with a probable molecular weight of 179 is tentatively identified as an anhydride, possibly, a nitrogen-containing phthalic anhydride.

The fractions from the parent fuels showed no mutagenic activity in the Ames assay. The mutagenic activity of the fractions from the diesel particles is shown in Figure 2. After fractionation mutagens were recovered in four fractions: acid, moderately polar neutrals, strongly polar neutrals, and aromatic hydrocarbons. Recoveries of mutagenic activity in the diesel fractions were low; however, standard reference mutagens, both direct and indirect, did not appear to be destroyed by the fractionation procedure. In particular, in the experiment with the 2-nitrofluorene spike, much of the mutagenic activity was recovered in the aromatic hydrocarbon and moderately polar neutral fractions. It is of interest to note that ~50% of the recovered mutagenic

activity from the samples is not in the fractions in which the 2-nitrofluorene was concentrated but instead is in the highly polar neutral fraction.

The three most mutagenically active crude DCM extracts were derived from the diesel fuels that contained relatively high concentrations of aromatic compounds. No correlation was observed between increased mutagenicity and nitrogen content of the parent diesel fuels.

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Table 1. Aromatic and Nitrogen Content of Diesel Fuels

	Aromatics (vol %)	Nitrogen (ppm)
DFM	29.9	5
Min. Qual.	34.6	240
BF+HAN+HN	30.8	718
BF+IQ	6.6	930
JP-7	2.7	<1

DIESEL FILTERS
MODERATELY POLAR NEUTRAL FRACTIONS
NPD
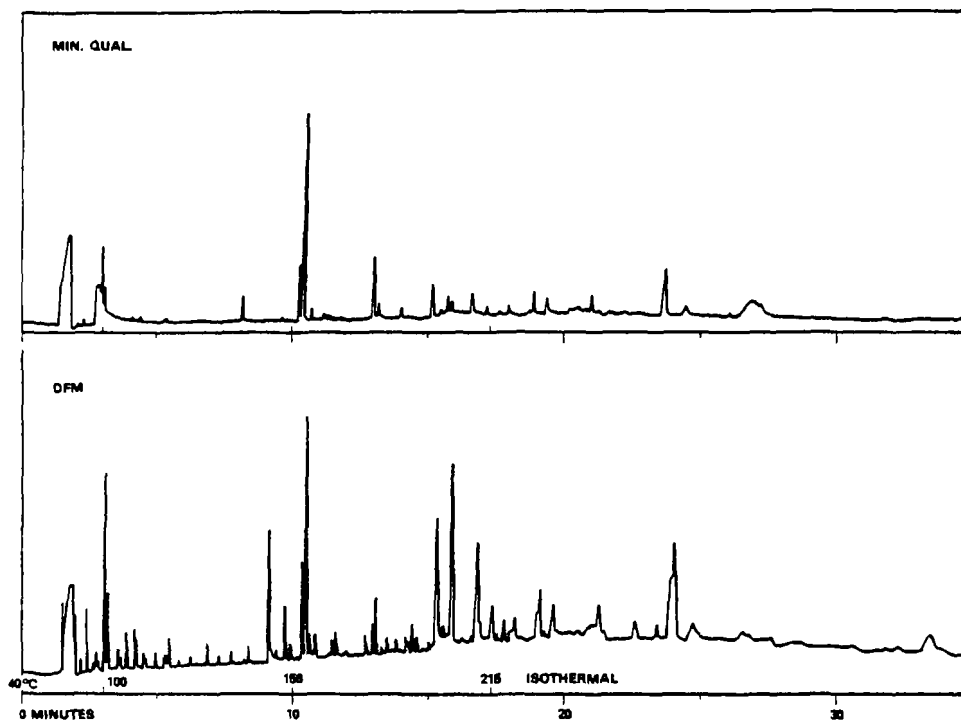
DIESEL FILTERS
HIGHLY POLAR NEUTRAL FRACTIONS
NPD

Fig. 1.   Gas chromatogram with nitrogen selective detector of the moderately polar and highly polar fractions from two diesel particulate samples.
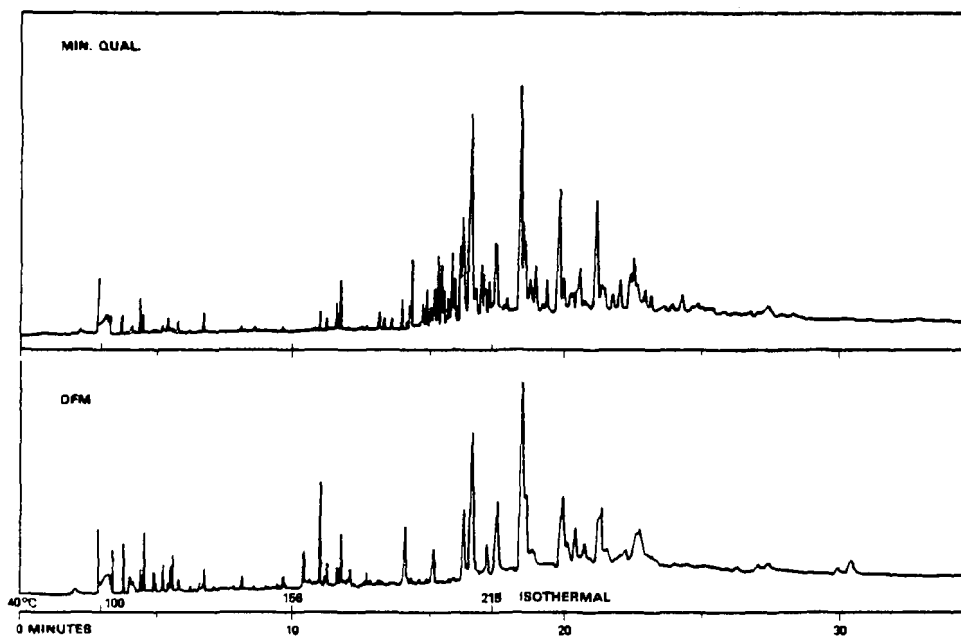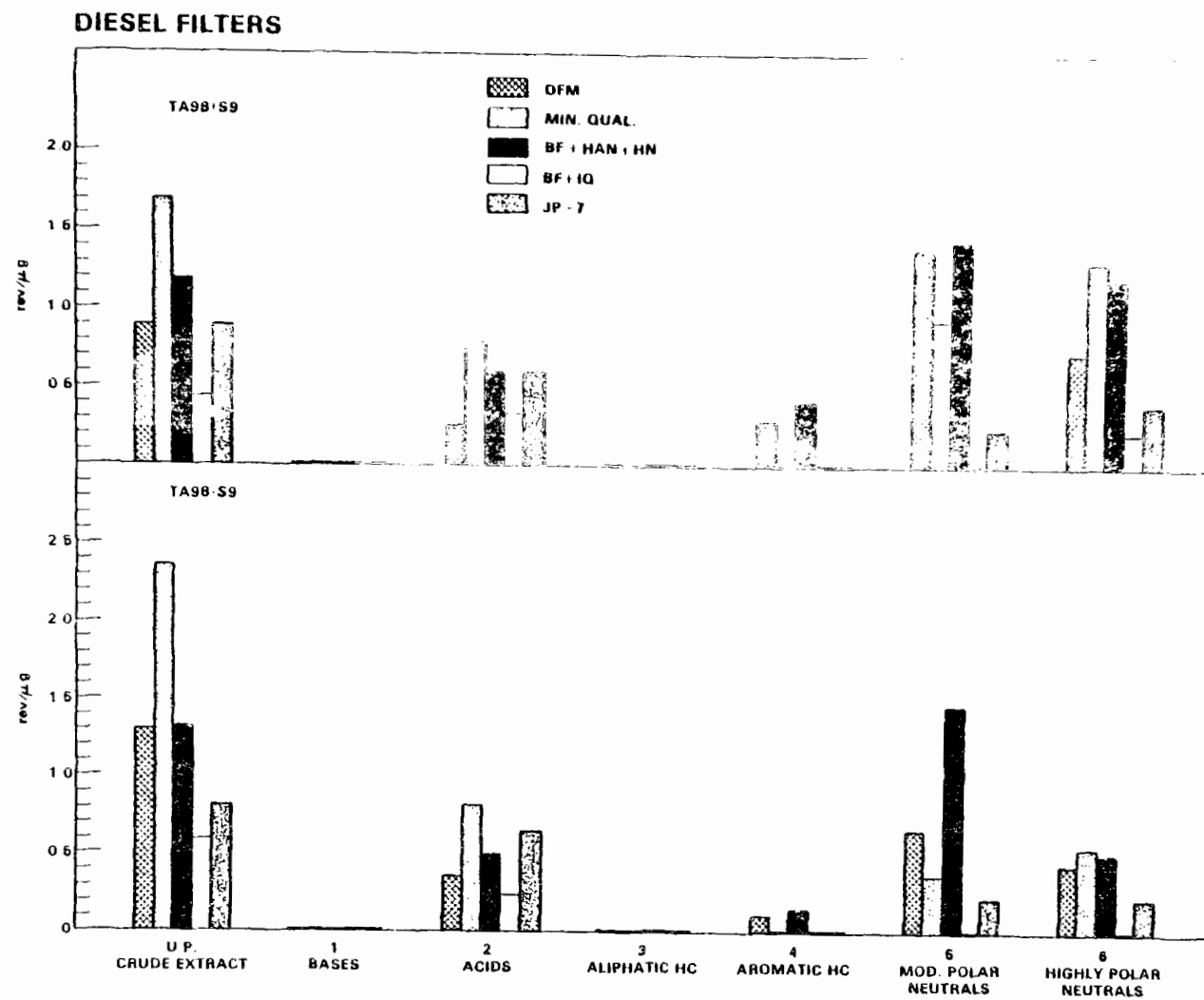
Fig. 2.   Ames mutagenicity data of fractions from five diesel filters.

FRACTIONATION AND CHARACTERIZATION OF THE ORGANICS
FROM DIESEL AND COMPARATIVE EMISSIONS

by

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Semi-volatile materials were analyzed from various media including diesel soot, tar from coke oven residues, roofing tar and cigarette smoke condensate (CSC).

The diesel exhaust particles were collected from a 1978 Oldsmobile 350 diesel vehicle operated on the Highway Fuel Economy Test Cycle (HWFET) with No. 2 diesel (Union 76) fuel. The particles were collected on Pallflex T60A20 filters and the organics were removed by Soxhlet extraction with methylene chloride as previously described (1).

The 2RI Kentucky Reference cigarette smoke condensate was generated according to the method of Patel (1977) at Oak Ridge National Laboratory (1).

The coke oven main sample was collected from a separator located between the gas collector main and the primary coolers within a coke oven battery at Republic Steel in Gadsden, Alabama, about 60 miles northeast of Birmingham.

The roofing-tar sample was generated and collected using a conventional tar pot containing pitch-based tar, enclosed within a chamber and heated to 360°-380°F, a normal temperature for commercial use. The evaporative emissions were collected using a small bag house fitted with Teflon filter bags (1).

The solvents used to extract or condense the organics from each of these samples was removed by evaporation under nitrogen.

The nature and number of organic compounds associated with samples of the type addressed in this program render them among the most complex of environmental samples. At the present time no direct determinant approach, regardless of the resolving power, is available for routine characerization. The analytical problems can be minimized by pretreatment of the sample in order to distribute the sample compounds into fractions of similar chemical or physical properties. This class division provides useful information regarding the sample's overall make-up, yields convenient and meaningful subfractions for biotesting purposes, and lessens the analytical burden for ultimate characterization. This procedure is

based on work by Novotny et al., (2) which, after significant alteration, was used for this study. The entire scheme is depicted in Figure 1.

Each sample was subjected to this fractionation procedure to yield six fractions of various chemical properties and polarities. The acid fraction contains both weak (e.g., phenols) and strong (e.g., carboxylic acids) acids. The base fraction contains organic, Bronsted bases (e.g., amines). The neutral fraction is subdivided into 3 main fractions based on compound polarity. The non-polar neutral (NPN) fraction is comprised of compounds less polar than $\sim$ naphthalene. Paraffinic materials are characteristic of this fraction. The PNA fraction contains compounds of intermediate polarity, and is selective for condensed ring aromatics. All neutral materials with polarities greater than PNA hydrocarbons are found in the polar neutral (PN) fraction. Prior to the subfractionation of the neutral fraction, the latter must be dissolved in cyclohexane. All components are not soluble in this solvent. The insoluble material is collected as a separate fraction (CI), and is comprised of intermediate and highly polar compounds.

Spillover of various compounds into all fractions is a natural feature of solvent partitioning processes. Polar neutral material was removed from the PNA fraction by silica gel chromatography. The PNAs were chromatographed using gradient elution such that a fraction containing only PNA hydrocarbons was obtained (PNA-1). Other fractions (PNA 2-4) were collected that contained compounds of intermediate to high polarity.

Most fractions were directly analyzed by capillary GC/MS. The fractions enriched in polynuclear aromatic hydrocarbons (PNAs) were further purified by column chromatography, and the collected subfractions were analyzed by GC/MS. A large portion of each sample, after fractionation, was submitted to the EPA for biotesting. The remainder of each sample was used for all analytical work.

Approximately 1 g of each sample was partitioned. Recovery of material after application of the fractionation scheme was generally ca. 80%. Overall mass balances are shown in Table 1. The recovery for CSC was uncharacteristically low (47.6%). Extensive emulsions were not formed during partition; the formation of insoluble material upon dissolution in methylene chloride prior to fractionation may account for the low figure.

The mass distribution for each sample is shown in Table 2. These results represent approximate quantities since any solvent partition process is a rough separation method. The cyclohexane insoluble (CI) fraction contains significant proportions of material in some samples. This fraction is a measure of the amount of sample, after acid/base removal, that is not soluble in cyclohexane, and is therefore presumably polar neutral material. The CSC and coke oven samples both contain major quantities of such material.

The CSC sample contained a significant amount of organic bases, while the acid fraction was more important for the diesel soot extract. The large amount of non-polar neutral material associated with the diesel soot is not unexpected; aliphatic hydrocarbons are known to constitute a major proportion of such mixtures (3). The roofing tar sample is notable for the size of the PNA fraction.

The partition scheme proved effective in providing some fractions that were amenable to direct GC/MS analysis. Acid fractions require derivatization before comprehensive GC/MS analysis can be carried out. The bases, NPN, PNA and CI fractions can be successfully approached via GC/MS, although clean-up is required for some fractions. The PN fraction represents a very difficult analytical problem that probably requires LC/MS, derivatization, further fractionation, etc. The actual extent to which any fraction can be comprehensively analyzed by any GC technique is unknown. More work is clearly required in this area.

The partition scheme fractions showed, after GC/MS analysis, more or less the expected compositions. The acid fractions were shown to contain phenolic materials; carboxylic acids such as fatty acids were detected infrequently (derivatization required). The non-polar neutral fraction was, for all sample types, highly enriched in saturated and unsaturated aliphatic hydrocarbons. The compounds covered a molecular weight range corresponding to ca. C_{10}-C_{24} paraffins. The CSC fractions contained several plant natural products. The PNA fraction from CSC was too small to permit both adequate bioassay and complete analysis. The PNA fraction from all other samples showed the presence of PNAs and methylated PNAs containing 2-5 rings. Other compounds found were dibenzo-thiophene, dibenzofuran and oxygenated fluorenes.

The preparative chromatography of the PNA fraction provided separation of PNA hydrocarbons from the more polar contaminants of that fraction. The amount of material recovered in the chromatographic fractions (PNA 2-4) was usually quite small; only phthalate esters and unknown species were indicated. Based on similar chromatographic schemes (4), nitro-arenes, oxygenated PNAs and non-basic nitrogen containing PNAs (e.g., carbazoles) would be expected constituents of these fractions.

The polar neutral fractions showed, for all samples, significant spillover of PNAs. Some oxygenated PNAs (e.g., benzanthrone, anthraquinones, hydroxyaromatics) were also identified in this fraction.

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Table 1. Mass Balance Results from Fractionation of Comparative Samples

	Diesel Vehicle	Cigarette Smoke	Coke Oven	Roofing Tar
Amount fractionated (mg)	803.0	913.8	936.8	1071.2
Fractional totals (mg)	696.9	435.0	783.1	895.9
Mass balance (%)	86.8	47.6	83.6	83.6

Table 2. Percent of Total Mass Recovered Upon Fractionation of Comparative Samples

Fractions	Diesel Vehicle	Cigarette Smoke	Coke Oven	Roofing Tar
Acid	3.6	1.0	0.4	2.3
Base	1.0	12.1	4.3	2.9
PN	7.6	11.5	7.1	13.1
NPN	74.2	0.6	15.2	40.4
PNA1	1.4	0.02	4.9	31.8
PNA2	1.7	0.0	0.0	1.2
PNA3	0.9	0.1	4.0	0.02
PNA4	1.6	0.9	0.7	0.3
CI	7.8	73.7	63.3	8.0

Figure 1.   SOLVENT PARTITIONING FRACTIONATION SCHEME

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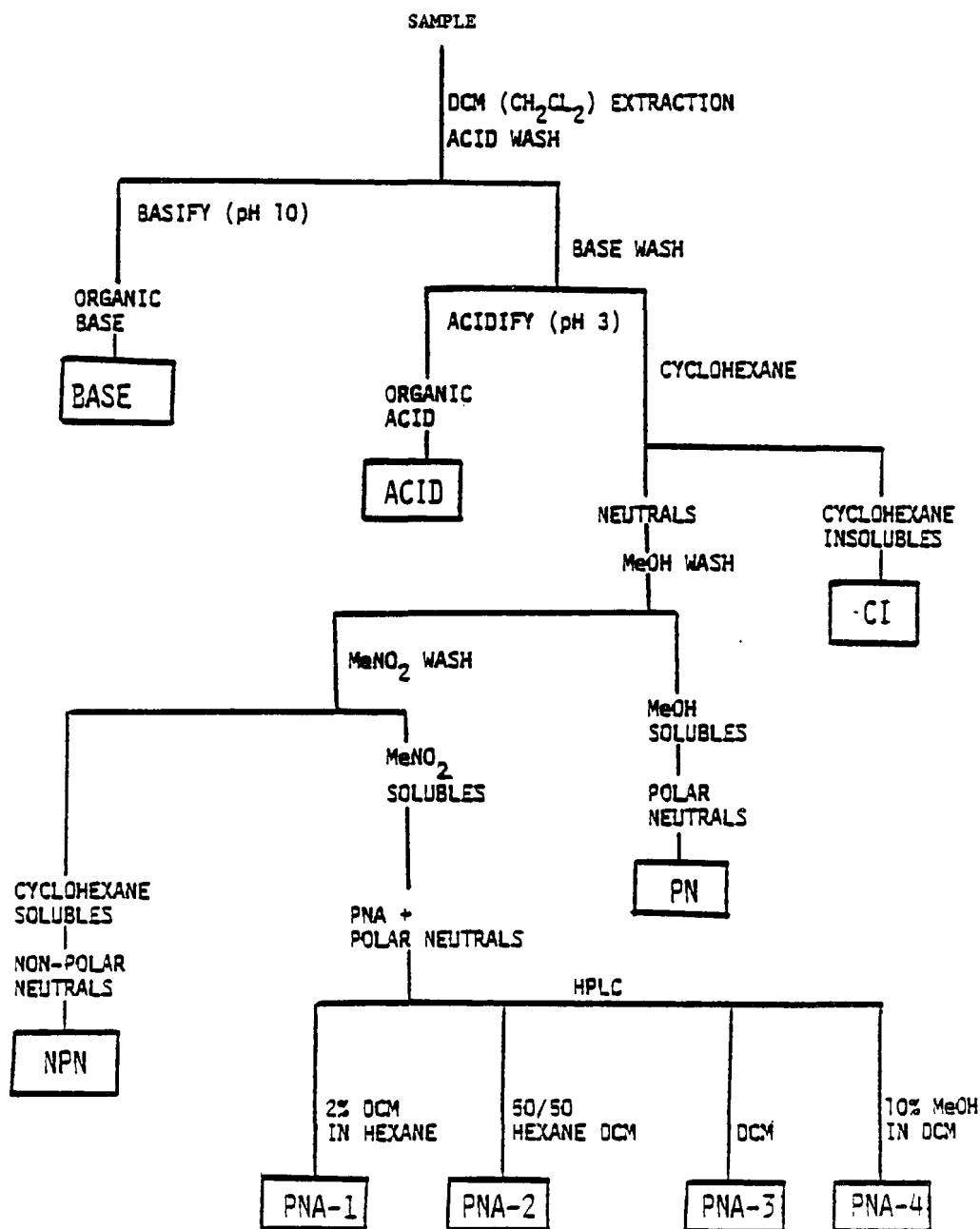
SwRI-SFRE DIESEL HEALTH EFFECTS
EXPOSURE FACILITY

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The future of the fuel efficient automotive diesel passenger car engine has been clouded by the possibility that its exhaust particulates have carcinogenic properties. To determine whether this is a possibility, auto manufacturers and the federal government are seeking to determine the possible long term health effects of diesel exhaust particulates. What the National Research Council called the most comprehensive effort in this area is being conducted by Southwest Research Institute and its sister organization, Southwest Foundation for Research and Education. The project is sponsored by General Motors Corporation.

The facility is the largest and most advanced of its kind. The building consists of three rooms. The engine room houses the diesel engine and mechanical equipment for conditioning the dilution air. The exposure levels are monitored and the operation of the experiment is handled from the control room. The four large exposure chambers are located in the chamber room and are in close proximity to the diesel engine exhaust, yet isolated by a soundproof wall.

Each chamber is 8 ft wide by 8 ft long by 8 ft high. They are large enough to handle about 1250 rats, mice, and hamsters for long term exposure to diesel exhaust for a total of about 5000 animals. We wished to simulate levels of exposure not unlike those that might be experienced on the street. From previous experience we know that the maximum dose one might experience behind a city bus is about 1 part of exhaust in 120 parts of air. So, one chamber is operating at that level. Another chamber is operating at twice that dose, or 1 part of exhaust to 60 parts of air. A third chamber is operating at about one-third that level, 1 part of exhaust to 360 parts of air. A fourth chamber receives no diesel exhaust at all, just purified air and therefore is the control group for comparison to the other three.

Two Oldsmobile 5.7 liter diesel engines are mounted on individual stationary dynamometers. One is operated and the other is a back-up when

necessary. The engine operates at an equivalent 40 mph cruise condition (1,350 rpm and 71 ft lbs torque) for 20 hours each day. Individual samples of exhaust are directed to the top entrance of each of the three pyrimidal shaped chambers for subsequent dilution and mixing. The engine operates on a type 2D emissions test fuel. The engine room also houses the air conditioning and air filtration equipment used to condition and purify the dilution air.

The control room contains the automatic controls to maintain engine speed and power output, the environmental controls for maintaining the chamber temperature at 74°F and relative humidity at 50 percent with a slight negative pressure of 0.5 in H_2O. Monitoring of the gaseous emissions is on a semi-continuous basis. Each chamber is automatically monitored for 10 minutes each hour for hydrocarbons by heated FID, CO and CO_2 by non-dispersive infrared analyzers, and for NO/NO_x by chemiluminescence analyzer. These and pertinent engine and chamber environment data are recorded each 10 minutes.

The most important measurement is that of total particulate in each chamber. This is performed by collecting a sample of the chamber atmosphere on a 47 mm diameter plastic coated fiberglass filter media. The weight gain is used with the sample volume to compute the particulate concentration within each chamber. These measurements are made at least once each day. The amount of exhaust sample admitted into the mixing area prior to the chamber is adjusted as required to maintain the concentration of particulates as close to specification as possible.

The entire facility was designed for long term, continuous, trouble-free operation. Redundancies are provided in terms of backup engine dynamometer and controls, backup power for emergency air conditioning, backup pumps and air conditioning units and backup controllers etc., that may malfunction and result in an emergency condition. The totally integrated design located equipment items to simulate exhaust exposures as close to that in the field as possible. The system typically operates 20 hours each day (4 hours for animal and cage hygiene) and on a 7 day per week basis.

POST-EXPOSURE DIESEL PARTICLE RESIDENCE IN THE LUNGS OF RATS FOLLOWING INHALATION OF DILUTE DIESEL EXHAUST FOR 6 MONTHS

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Due to its submicron size, 15-17% of inhaled diesel particulate deposits in the airways of the lung. In the alveoli of the lung, the alveolar macrophages scavenge the diesel particles, phagocytize them and diesel particulate-laden macrophages were found in lung lavage fluid even 90 days following an exposure of 16 days to diesel emissions at 6 mg/m^3 [1,2]. This indicated that the diesel particles may have a long residence time in the macrophages within the lung, rather than being rapidly eliminated via the ciliated airways or lymphatics. The studies describe the results of biochemical, morphological and physical measurements on the alveolar macrophages, as well as the histology of the lung after exposure of rats to 250 µg DP/m^3 for 6 months, and serial sacrifice up to 16 months post-exposure.

Male Fischer 344 rats (COBS CDF F-344/CrlBR) were exposed to diesel exhaust particulate concentration of 250 µg/m^3 for 20 hrs/day, 5-1/2 days/week for 6 months. Control animals were exposed to clean air. Lungs of exposed rats were lavaged *in situ* with Hank's Balanced Salt Solution (without calcium or magnesium). Differential cell counts and assays of the enzymatic activities of acid phosphatase and beta-glucuronidase were performed on the lavaged cells.

Light microscopic investigations of the lung revealed that immediately after the exposure, diesel-laden macrophages were diffusely distributed throughout the pulmonary alveoli and also focally aggregated in some alveoli. Some macrophages containing diesel particles were also observed in the bronchus-associated lymphoid tissue (BALT) and in the lymphatics. Subpleural pigmentation consisted primarily of aggregations of alveolar macrophages containing diesel particulate. Initially, ninety-five percent of the lavaged macrophages were completely pigmented with phagocytized diesel particles, such that only the nucleus of the cell was visible and the cytoplasm was opaque. The percentage of these macrophages declined with time, showing an exponential decay with a 6 week half-time. The percentage of lavaged macrophages which were free of diesel particulates rose linearly at a rate of 2.5 percent per week. The rest of the lavaged macrophages contained some diesel particulate-filled phagosomes within the cytoplasm. The macrophages were obtained in the same numbers as those from control animals, and had comparable cell size and activities of the lysosomal enzymes, beta-glucuronidase and acid phosphatase.

With increasing time post-exposure, diesel-laden macrophages were no longer observed diffusely distributed throughout the lung, but remained even after 16 months (life span) in focal accumulations in the alveoli (some of which had thickened alveolar walls), particularly noted in the subpleural regions, also in the BALT and in the lymphatics. Initially, the lung tissue contained 0.663 ± 0.075 mg (n=2) diesel particulate. After 16 months of clearance, diesel particulate in the lung had declined to 0.250 ± 0.090 mg (n=5) with only 0.014 mg (n=10) in the regional lymph nodes.

The rapid decline of the diesel-laden macrophages which was much faster than the overall diesel particulate removal from the lung, suggests that under conditions of prolonged exposures to high concentrations disappearance of particulate-laden macrophages from the lavageable pool of cells seems to be due to the formation of aggregates of alveolar macrophages rather than transport out of the lung. The overall clearance of diesel particulate after extensive exposures seems to be slow and proceeds by as yet unknown mechanisms. After 16 months post-exposure, alveolar macrophages containing small amounts of diesel particulate can still be identified in the lavage fluid. In addition, polymorphonuclear leukocytes are present among the aggregated macrophages suggesting that the incoming alveolar macrophages and polymorphonuclear leukocytes may be involved in or contribute to the breakdown of the macrophages aggregates.

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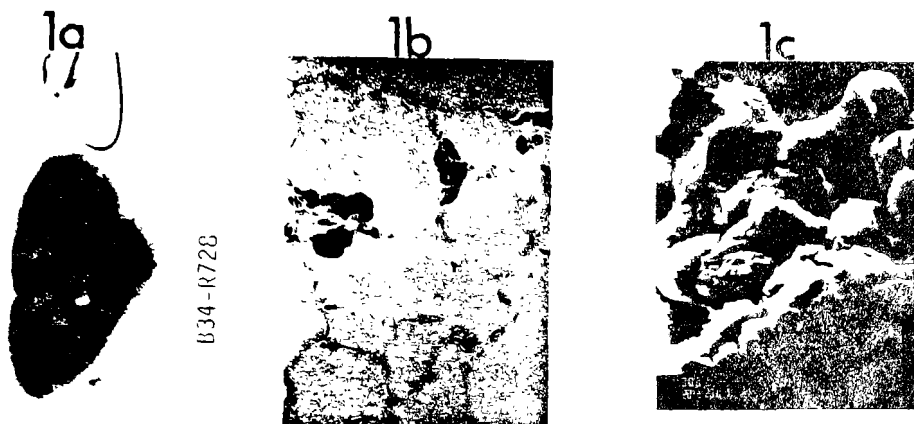
FIGURE LEGENDS

Fig. 1a   250 μg/m^3 for twenty-five weeks and 8 weeks post-exposure: speckled appearance of the exposed lung.

Fig. 1b   250 μg/m^3 for twenty-five weeks and sixty-nine weeks post-exposure. Diesel-laden macrophages are still present in association with the pleural surface region.

Fig. 1c   250 μg/m^3 for twenty-six weeks and forty-five weeks post-exposure. Scanning electron micrograph of macrophage aggregation in a pleural region from an area shown in Figure 1b.

TRAPPING GASEOUS HYDROCARBONS

by

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The gas phase mutagen research was initiated at the request of the Office of Mobile Source Air Pollution Control for the development of a procedure to trap gas phase hydrocarbons (HC) and then to use the method in a series of studies with the objective of comparing the Ames Bioassay activity of the gas phase with the particle-bound vehicle emissions.

Several of the hydrocarbon absorbents in use by other researchers were procured for evaluation as to applicability for HC collection in the diesel fuel range. A thorough examination of the known physical characteristis of the mediums resulted in the selection of coconut charcoal and XAD-2 resin (divinylbenzene polystyrene polymer) as the best possible candidates.

Preliminary evaluations consisted of making injections of diesel fuel into a small dilution tunnel and trapping the hydrocarbons with subsequent recovery determinations proving both to be excellent HC absorbers but the XAD-2 was the better release medium. Although, several different solvents and solvent combinations were used as extraction agents to improve recovery efficiency from the charcoal, a recovery greater than 60% could not be achieved and work with charcoal was discontinued. Further recovery work showed the XAD-2 to be quantitative by both chromatographic and gravimetric analysis for diesel fuel range hydrocarbons. The recovery and qualification experiments were performed using A.D. Little (ADL) developed odor traps, 3/8" O.D. x 2" length, filled with the absorbents.

With completion of the qualification tests several samples were taken from both a 1978 Oldsmobile and a 1978 Datsun 220C diesel powered passenger car with the resin filled ADL traps. An extended sample time in the order of 5-10 hours was necessary, due to the low trap flow rate and limited resin capacity, to obtain sufficient materials for Ames Bioassay. This long sample time not only tied up equipment and personnel excessively but also posed some

question as to the possibility of artifact formation on the resin. In order to help clarify the artifact question, an experiment was conducted by making injections of diesel fuel and NO_x on resin filled traps (ADL) and then submitting the extract for Ames testing using the TA 98 strain. The test results indicated that considerable artifact, sample level in some situations, could be generated under these long exposure conditions and further hydrocarbon collecting with these tubes was discontinued.

To eliminate the low flow and the long sample time problems, a larger 2" x 2" trap with about 100 times the resin capacity of the ADL tubes was fabricated and carried through the same qualification experiments as the smaller traps with comparable recovery efficiencies (greater than 95%).

These large traps were then used to sample a series of different cycles (FTP's, NYCC, HWFET's) from a VW Rabbit diesel passenger car. These samples were submitted for Ames testing with the results showing the gas phase materials to be active but this activity was only 9-16% (depending on test cycle) of the particle-bound activity. Although, artifact formation is probably still present the trap activity data indicates that it had been substantially reduced.

These tests completed the diesel studies. Since the gasoline system had little NO_2 present, with possible minimum artifact formation, an XAD-2 trapping system, 20" x 20" x 2" bed, having 100 times (due to low gasoline emissions) the resin capacity of the 2" x 2" traps was fabricated to collect the hydrocarbons. A series of three gasoline powered vehicles were tested: (1) a 1972 Chevrolet Impala using unleaded fuel; (2) a 1981 Dodge van with a light duty catalyst system also using unleaded fuel; and (3) a 1970 Ford van using commercially available leaded fuel.

A sample from each of the vehicles was then submitted for Ames Bioassay testing with the XAD-2 trapping results being quite different from that observed in the diesel vehicle. The activity of the gaseous materials trapped by the XAD-2 was at background level for all three vehicles. The Dodge van particle-bound HC, without S9 activation, had about twice the activity in reverents/microgram as the Impala, and four times the activity of the Ford, with the higher Dodge activity density probably due to the oxidative properties of the emissions control system since the catalyst was the major parameter difference between the Dodge and Impala. The Ford had the lowest activity of the three vehicles and this could possibly be attributed to the lower fuel aromatic content (44.4% unleaded and 27.8% leaded).

A comparison of the diesel and gasoline vehicles activity (reverents/ microgram) indicates that for the HWFET cycle,(the only cycle tested common to all vehicles) without activation the diesel and 1972 Chevrolet are about the same activity, the Dodge has twice the diesel activity, and the Ford about one half the diesel activity. Although the gasoline emissions have activities comparable on a revertent/microgram basis, when the emissions are observed on a reverent/mile basis, without activation, the worst of the gasoline vehicle emitters (Ford) has an activity that is only 11.8% and, with activation, only 4.5% of the diesel particle-bound activity.

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These trapping studies have clearly indicated the low level of activity associated with the diesel gas phase hydrocarbons and the extremely low or background levels present in gasoline gas phase emissions.

The ability of XAD-2 to effectively collect diesel fuel range HC has been well demonstrated and studies are currently in progress to characterize the XAD-2 collectability of gasoline fuel range hydrocarbons.

ANALYTICAL METHODS FOR NITROAROMATIC COMPOUNDS

by

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A number of methods have been used to detect and/or measure nitroaromatic compounds in environmental samples (1-5). The analysis usually involves a combination of fractionation schemes - solvent-solvent extraction, thin layer chromatography (TLC), open column chromatography, high performance liquid chromatography (HPLC) - and followed by analytical finish using TLC, HPLC with UV and fluorescence detection, gas chromatography (GC), and a variety of mass spectrometric (MS) techniques. Most of these methods are labor intensive and some are plagued by poor sensitivity and interference problems.

We have developed a reverse phase HPLC-fluorescence method using water-methanol solvent for the detection, identification and measurement of selected nitroaromatic compounds with sensitivity at low and sub-nanogram levels. The detection technique is based on on-column catalytic conversion of the non-fluorescent nitroaromatic compounds to the highly fluorescent amine analogs. Compound selectivity is achieved by appropriate choice of wavelengths for fluorescence measurements. Stop-flow techniques and spectral scanning of the trapped peaks were used to establish chemical identify by comparison with spectra of standard samples.

The heart of our analytical system is a platinum-rhodium catalyst column (maintained at 60-80 degrees Celsius) between two reverse phase ODS columns. Initial separation is achieved in the first ODS column, reduction to amine analog is immediately accomplished in the catalyst column and the final analytical separation of the aminoaromatic compounds from interferring components is achieved in the second ODS column. By allowing only selected aminoaromatic peaks through the second ODS column, we have managed to conveniently eliminate the tedious sample clean-up prior to analysis. Figures 1 and 2 illustrate the use of this technique in the analysis of nitro-pyrene. Precision of ± 3% at 1 nanogram level is routinely obtained for nitro-pyrene analysis. Minimum detectable quantity of nitropyrene under our present analytical configuration is about 20 picograms.

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The catalyst has been observed to reduce nitro compounds to the corresponding amine reproducibly under fixed conditions of flow rate, temperature and solvent composition. The following nitroaromatic compounds are converted with better than 99% conversion efficiency: nitro-naphthalenes, nitro-anthrance, nitro-fluorene, nitro-chrysene, nitro-BaP, dinitro-pyrenes and nitro-fluor-enones. We have obtained fluorescence spectra of the amine analogs of most of the nitroaromatic compounds available to us. The amines, especially the diamines, were observed to be unstable under UV light. Adjustment of the solvent pH to about 8 with NaOH helped to stabilized the diamines adeqately to make reproducible spectral scans of the trapped peaks.

The present analytical system has been used to measure nitro pyrene in complex matrices such as diesel exhaust particulate extracts, leaded and non-leaded automotive exhaust particulate extracts, gas trap extracts, fly ash extracts as well as biological extracts. Samples dissolved in DMSO intended for Ames tests are likewise amenable to analysis without additional sample clean-up. Other nitroaromatic compounds can be detected and measured by appropriate choice of chromatographic elution windows coupled with the optimum wavelengths for fluorescence measurements.

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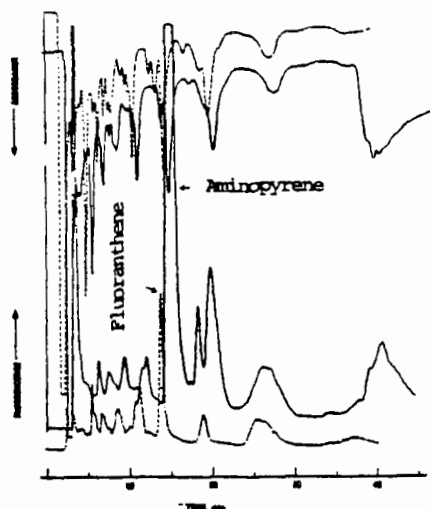
Figure 1

Neat diesel extract (25 ug) through ODS column only (——);
through ODS and catalyst columns (——). Note peak
enhancements due to formation of aminocompounds. Detection
wavelengths: Excitation (360 nm), Emission (430 nm), UV (254 nm).

Figure 2

Neat diesel extract (25 ug) through ODS-Catalyst-ODS columns.
Only the main aminopyrene peak was injected into the second
ODS column. (——) Uv and fluorescence profiles of the sample
through ODS-Catalyst columns. Note removal of interferent
peaks after passage through second ODS column.

TOTAL LUMINESCENCE SPECTROSCOPY
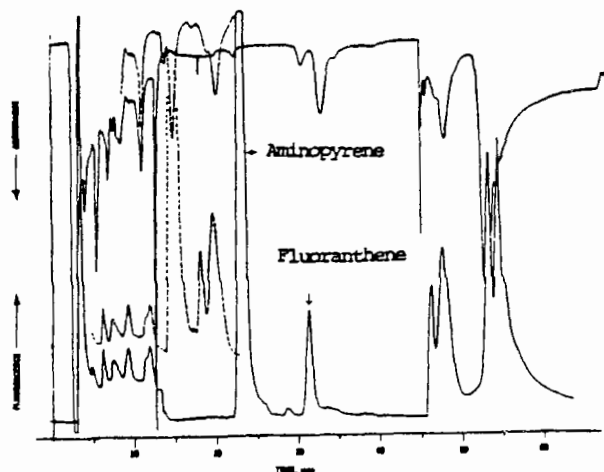## OF DIESEL EXHAUST PARTICULATE

by

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Total Luminescence Spectroscopy (TLS) analysis of organic extract material from diesel exhaust particulate matter has been previously described by Wotzak et al[1]. TLS is the determination of the luminescence intensity as a function of all accessible excitation and emission wavelengths. TLS data are typically obtained from between 50 and 200 emission spectra, each taken at a specific excitation wavelength. Contour mapping of the points of equal luminescence intensity on an excitation vs. emission wavelength grid has been chosen as a convenient means of representation for TLS data. TLS analysis thus encompasses the computerized data acquisition, manipulation, display, and interpretation of such luminescence data.

The New York State Department of Environmental Conservation, Automotive Emission Evaluation (AEE) unit has been studying the utilization of TLS in the characterization of diesel particulate organic extract using a Baird Corporation SFR-100 Ratio Recording Spectrofluorometer. Data acquisition and scan control for this instrument is locally provided by a Baird MP-100 microprocessor controller which are linked to a host Data General Nova 3 computer system with 128K words of core and a 10 megabyte disk. Software was provided by Baird Corporation and modified by AEE computer personnel to include data smoothing algorithms. Contour plots are produced on a Houston Instruments Complot[TM] X-Y recorder.

More recent advancement in raw data reduction has been achieved by wavelength correction and digital smoothing using Fast Fourier Transform methods. Extract samples, fractionated by column chromatography, have been analyzed, generating TLS contour maps for each fraction. These fraction contours may be added by computer software routines, using appropriate weighting by cut recovery factors, and compared with spectra for the original bulk sample.

This reconstitution work was performed as an internal consistency test for both the acidic, basic and neutral cuts of the raw extract, as well as the sub-cuts of the neutral fraction.

A variety of tasks have been performed in order to obtain a general indication of the utility of this relatively new analytical procedure. Sequential dilutions of extract sub-fractions were performed in order to determine the extent of internal absorption of fluorescent radiation. TLS spectra of the diesel fuel and lubricant were subtracted from appropriate spectra in order to facilitate further analysis, and determine the partition of unburned fuel and lubricant among extract sub-fractions. The neutral fraction was analyzed qualitatively and quantitatively for several known compounds.

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EVALUATION OF THE METABOLIC REQUIREMENTS OF DIESEL AND COMPARATIVE SOURCE SAMPLES IN THE <u>SALMONELLA</u> <u>TYPHIMURIUM</u> PLATE INCORPORATION ASSAY

by

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The mutagenic activity of a mobile source sample (Nissan diesel exhaust extract) and three comparative source samples (coke oven mains, cigarette smoke condensate, and roofing tar extract) were examined in the Ames plate incorporation assay using strain TA98. The mutagenic and carcinogenic activity of these complex mixtures had previously been determined in a bioassay test matrix (1). The comparative sources were less mutagenic in the Ames assay compared to the mobile source sample. However, they were more active in mammalian cell mutation and mouse tumor initiation bioassays. The objective of this study was to determine whether modifications in the S9 activation system would alter the mutagenic activity in the Ames bioassay. The modifications examined included altering both the species from which the liver S9 was prepared and the concentration of S9 on the plate.

Both Aroclor 1254-induced and -uninduced S9 were prepared from CD rats and Syrian golden hamsters. Livers were pooled from groups of at least 6 male animals for each preparation. The protein concentration of each S9 was determined by the method of Lowry et al. (2). Four doses of S9 were run: 0.31, 0.63, 1.25, and 2.5 mg/plate, chosen to encompass the usual concentration in the Ames test (ca 1.5 mg/plate). Aliquots were adjusted to contain the required dose in a 0.5-ml volume as used in the Ames test.

Three doses of each source sample (a low, medium, and high range) were tested at all four S9 concentrations for each S9 preparation. The dose varied between samples and were selected to be below the toxic level for each, yet high enough to have mutagenic activity. For Diesel Nissan and cigarette smoke, the doses were 30, 100, and 300 µg/plate. For coke oven mains and roofing tar, they were 5, 50, and 100 µg/plate.

Experiments were run in duplicate, on different days, using triplicate plates for each point. In any experiment all 3 doses of 2 samples were tested with all 4 concentrations of both uninduced and induced S9 from one species. For each experiment fresh dilutions were prepared from aliquots of the S9

preparations, which were held at -80°C. The time between duplicate experiments ranged from 3 to 9 days. The data is presented as the mean ± SE for duplicate experiments.

The optimum S9 dose for all samples, with the exception of the Diesel Nissan which did not require metabolic activation, was either 1.25 or 2.5 mg/plate for both induced and uninduced rat and hamster S9, as shown in Table 1 for the induced rat liver S9.

Recently, several studies have noted that induced hamster S9 is more effective than induced rat S9 in activating such compounds as aromatic amines (3) phenacetin (4) and diethylnitrosamine (5), while Aroclor-induced rat S9 is more effective than hamster S9 with polycyclic aromatic hydrocarbons (3). This study showed no difference in effectiveness between hamster S9 and rat S9 in activating the Diesel or the comparative source samples.

The mutagenic activity of the comparative source samples was higher when Aroclor-induced S9 was the metabolic activator as compared to uninduced S9, whether rats or hamsters were the source of the S9. The Diesel Nissan sample was again the exception; the mutagenic activity was higher with uninduced S9 than with induced.

In conclusion, for the three comparative source samples, all of which require metabolic activation for maximum mutagenic activity in the Ames test: the optimum S9 dose is 1.25 to 2.5 mg/plate; Aroclor-induced S9 is more effective as an activator than uninduced S9 regardless of species; and rat and Syrian golden hamster S9 are equally effective in activating these complex mixtures. These results would suggest the lower mutagenic activity of the comparative source samples in the Ames test as compared with other mutagenicity or carcinogenicity bioassays was not due to the exogenous metabolic activation system.

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Table 1. Effect of Metabolic Activation Dose in Mutagenicity of Diesel and Comparative Source Samples[a]

Sample	Revertants/plate[b]			
	0.31[c]	0.63[c]	1.25[c]	2.5[c]
Diesel Nissan	816 ± 26	625 ± 39	491 ± 50	334 ± 31
Coke oven mains	492 ± 57	727 ± 8	861 ± 21	874 ± 27
Cigarette smoke	68 ± 0	83 ± 4	74 ± 6	64 ± 3
Roofing tar	59 ± 1	86 ± 2	94 ± 3	113 ± 9

[a]_Salmonella typhimurium_ TA98. Samples at 100 µg/plate.
[b]Mean ± SE of two experiments with triplicate plates.
[c]Rat Aroclor 1254-induced S9, mg/plate.

MS/MS CHARACTERIZATION OF DIESEL PARTICULATES

by

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INTRODUCTION

Analysis of the organic constituents adsorbed on diesel exhaust particulates has become increasingly important with the increase in the number of diesel engine automobiles. Extraction followed by GC/MS has been the usual analytical method of choice for these unknown organic constituents. Mass spectrometry/mass spectrometry (MS/MS) offers a possible means of direct analysis with either minimal or no separation. This technique enables a fast characterization of the organic constituents of the whole diesel particulates. Selectivity and specificity of particular compounds or classes of compounds of interest using MS/MS can be improved by the appropriate choice of the chemical ionization reagent gas as well as the choice of positive or negative ion detection.

EXPERIMENTAL

The MS/MS experiments described in this study were run using a Finnigan triple stage quadrupole mass spectrometer.[1] The diesel particulate sample is introduced into the source with the direct insertion probe which is heated in steps to obtain temperature profiles of the organics adsorbed on the particulates. The sample is ionized using the chemical ionization technique. The ion of interest is mass selected by quadrupole 1, undergoes collisionally induced dissociations in quadrupole 2 with the resulting

fragment ions being mass analyzed with quadrupole 3. Typcial ion axial energies into quadrupole 2 are 20 eV relative to the source. The collision gas used for these studies was argon at a pressure ca. 2.2 mTorr.

The diesel engine employed in this study was an AVL model[2] 520.005 naturally aspirated single cylinder direct injection engine. The diesel exhaust particulates are sampled using a mini dilution tunnel system.[3] As this system was designed both the dilution ratio and the temperature of the particulate filter can be varied.

RESULTS

The initial emphasis of this study was to identify constituents of diesel exhaust particulates by direct analysis using MS/MS. This identification is done by comparing the MS/MS spectrum of a particular ion in the diesel exhaust particulate sample with the MS/MS spectrum of the corresponding ion of a standard reference compound. For example, the MS/MS spectrum of the m/z 143 ion from a diesel exhaust particulate sample has two major fragment ions, m/z 128 (100%) m/z 115 (15%) besides the main beam ion m/z 143 (20%). These ion ratios are nearly identical to that found in the MS/MS spectrum of 2-methylnaphthalene, suggesting its presence in the diesel exhaust particulate sample. It is not possible to say which methyl-naphthalene is present, if only one is, or the relative concentration of the two in a probable mixture of both. The case of the m/z 143 ion suggesting the presence of predominately only one constituent and its positional isomers is not unique. However, as would be expected the MS/MS spectra of many ions are suggestive of the presence of more than one type of structure. An example of this is the ion at m/z 139 in the diesel exhaust particulate sample which is strongly indicative of the presence of hydroxybenzoic acid isomers through comparison with standard reference compounds. However, there are other relatively intense fragment ions in the MS/MS spectrum which can-not be resulting from hydroxybenzoic acid. One other compound that may account for the remaining fragment ions is decalin.

Along these lines is the investigation of the selectivity and specifi-city of the MS/MS technique as it relates to the direct analysis of diesel exhaust particulates. For example the comparison of positive and negative ion isobutane chemical ionization of a diesel exhaust particulate sample can yield information about specific classes of compounds. An example of this is the identification of carboxylic acids in the diesel exhaust particulate samples. In positive ion chemical ionization a carboxylic acid will be protonated as will an aromatic hydrocarbon at the same nominal mass. How-ever, in negative ion chemical ionization the carboxylic acid will lose a proton to give a $(M-H)^-$ ion whereas the aromatic hydrocarbon will be ionized by electron transfer to give a M^- ion. This technique was used to confirm the identification of the components in the m/z 139 ion MS/MS spectrum discussed previously. Thus the use of negative ion chemical ionization allows the separation of different classes of compounds for MS/MS identification in the direct analysis of diesel exhaust particulates.

Another example of selective ionization to allow a more accurate MS/MS identification to be made is the use of differing reagent gases. While

isobutane chemical ionization is a general protonating agent, ammonia can be used to protonate relatively basic compounds, like amines or aza compounds. While this technique has been used successfully in the analysis of coal-derived liquids[4] it has not been as useful with diesel exhaust particulates.

Besides these studies, two variables associated with sampling the diesel exhaust particulates have also been investigated. These variables, dilution ratio and particulate filter tmperature were utilized to gain a better insight into the complex problem of particulate sampling.

CONCLUSION

MS/MS provides a means for the rapid direct analysis of diesel exhaust particulates. The use of selective ionization techniques further enhances the positive identification when different types of compounds are present at the same nominal mass.

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SECTION 9

PERSPECTIVES

PERSPECTIVES ON DIESEL EMISSIONS HEALTH RESEARCH

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I will not attempt to summarize this very excellent, information-packed symposium. Rather I will make some very personal comments as to what I have learned and what I've concluded.

The problem before us is one of major social importance. I think we can conclude quite straightforwardly that a major increase in the Diesel fleet is not going to produce a disastrous epidemic of lung cancer. I think at the other extreme that we are probably not in a position now, today, to reach a meaningful judgment as to the quantitative impact of such an expansion. It may be that it's going to be negligible — that's quite possible. However, it may be that the impact will be at a level that will require some difficult social decisions. I don't know.

We've learned an enormous lot in the last three years; this is attributable to the very intensive and very fine work on the part of EPA, on the part of industry and on the part of independent universities and institutes. The science that we've seen here in the last three days has been very impressive,; not all of it has been equally elegant, not all of it has been as sharply focused as it might have been, but the great bulk has been sound and to the point. We've moved ahead a great deal in the last three years, but we are not quite where we should be.

I start with the premise that I can't really see how there can be a serious doubt in anyone's mind at this stage that Diesel exhaust is potentially carcinogenic for humans. We know from chemical analyses that there are carcinogenic chemicals present in the Diesel exhaust. We know from a vast amount of study in simple systems — revertant studies, cell transformation — that they are mutagenic in a variety of ways; we know that mutagenicity is highly correlated in this class of compounds with carcinogenicity. We have biological data on whole animals which, although sometimes borderline, are sometimes clear and decisive and which support these findings. We know, therefore, that there is a potentiality for carcinogenicity.

We further know that the extractable materials are (at least largely) bioavailable. This has been debated intensely; but as far as I'm concerned, the data presented in this meeting yield clear evidence that the material in or

on the carbon particles is bioavailable.

This brings us to a much more complicated question, that is, recognizing that the carcinogenic materials are there and that they are leachable, are they leached in sufficient quantities in exposed persons to reach the intracellular biochemical unit (DNA) that is important for an outcome of malignancy? This question involves a whole series of issues about which we are in varying degrees still somewhat ignorant. It involves the pharmacokinetics of movement from where the chemicals are lodged to where they are active. Are they effectively sequestered, what happens in macrophages or in the lymph nodes? Do these chemicals reach the nucleus of the cells in the epithelial lining of the lung in still potentially active form in sufficient quantity to initiate malignant changes. These issues are largely measurable; we still have large uncertainties as to the critical steps determining dosages to the nucleus of the cell.

Let me back up for just a moment to comment on the beginning of the problem, that is the actual human exposure. Characterization of the emission of the particulates starts with their collection and analysis. To what extent do artifacts in sampling or analysis disturb the outcome? To what degree are nitropyrenes artifacts of sample collection or, on the other hand, of genuine human concern? Basic is what happens to the particles once they leave the exhaust pipe and reach the human in respired air. From what I've heard here, I'm not sure that we really have satisfied ourselves on this score. One of the central issues is what happens to the particulates and the vapor phase PNA's between the time they leave the manifold and the time they are ready for inhalation by man; I'm not sure we know. There is NO_x outside; there is also, I believe, some evidence, perhaps only suggestive, that photochemical reactions can participate in nitrating the PNA's. This may be a factor which affects the actual potency of the particles in ambient air as inhaled. Such issues need to be resolved.

Now reverting to the biological aspects, getting a positive response from known carcinogens in lifetime studies is sometimes a very difficult procedure. I spent a good part of my career coping with that problem. If we were to depend on inhalation studies of cigarette smoke in laboratory animals to decide that cigarettes are carcinogenic, we would give them essentially a clean bill of health. It took ten to fifteen years of intensive work to confirm in the laboratory that the known human carcinogens, chromium chemicals, were carcinogenic for the lung. We've known for years that inhaled arsenic compounds are human pulmonary carcinogens; only recently is there a promising

positive experiment. I caution, therefore, to beware of putting too much weight on negative experiments of this sort. Dosage is extremely important; the conduct of the experiments is extremely important. The strain chosen, the species chosen, are important. I want to commend our visitors from Germany for having recognized what we have not always recognized, namely that a simple arithmetic calculation shows that to get a positive lung cancer outcome in Diesel particulates, even if they have significant carcinogenicity, will take ingenious experimental design if we're interested in levels that are anything less than 10, 15 or 20 percent incidence. That is, if one wants to get data at socially important levels, one must use special approaches in designing such experiments.

I have no patience with the view that experimentation in this area must be relevant to field conditions in terms of concentrations, times of exposure and so on. It just won't work. If one wants to detect socially significant levels of carcinogenicity with what is an intrinsically insensitive system, one needs to devise experimental methods capable of detecting low but important cancer potencies.

The work that's been done on isolated systems is extraordinarily impressive, and very elegant. The work that's been done chemically is again very impressive.

One issue that has been dealt with very little, if at all, that may be of the deepest importance, is the issue of interaction and promotion. The view that one is concerned with a one-for-one outcome between a single carcinogen and a human malignancy is rarely correct. In almost every case interaction with one or several factors is involved. This is especially true where we're dealing with what is clearly in this instance something less than a high frequency occurrence of malignancy.

Passing from cancer for just a moment, there were data suggesting non-malignant histological changes and, in particular, fibrosis; we need to pursue such issues.

Finally, to summarize let me suggest a few things amongst those that have already been mentioned which I think need resolution. First, I think, is the issue having to do with the actual state of the particles in the air as breathed by people. Are there important changes between their departure from the exhaust pipe and breathing point of man? This ought to be a reasonably straightforward and attackable problem.

It would seem to me that it would be worthwhile attempting to see whether a small number of index PNA's could be identified. It is clear that

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benzo(a)pyrene is by no means the best index agent. Pyrene is not the best index agent. But is it possible that two or three or four (not more than four) could be identified that could be useful indicators for perhaps an equal number of classes of PNA's. Another alternative would perhaps be some kind of refinement of the HPLC fingerprint. Another possibility would be a small standardized set of revertant tests. The point is that work would go ahead much more rapidly if there could be developed simple, straightforward techniques for relatively quick identification and quantification of major classes of PNA's.

It would seem to me that a major advance (and challenge) is to move forcefully towards improving the utility of short term tests for quantitative estimates of potency for humans; the group that's worked here is eminently qualified to do this. There is no question but that the revertant and cell transformation tests have all been extremely powerful in this research. They are still, however, weak tools for quantitative estimates of potency for humans.

We need, as I mentioned, more ingenuity in the design of some of our long term experiments. We must refine of our understanding of the kinetics of movement of the important chemicals and their metabolites from the inhaled particles to the nucleus of the potentially responding cell, by which I mean the basal cells in the respiratory tract.

Now all of this, as far as I'm concerned, should have one objective, and that is estimation of the risk for man. What we now need in order to move on with extending our fuel, and meeting our transport requirements, is to make an estimate of whether the problem is trivial or spcially significant. Thus, risk assessment should be the ultimate goal and should be given the highest priority. There have been starts; I've been not totally impressed with the state of art. The weakness is not in mathematics. The mathematics are, by and large, relatively straightforward. A quantitative understanding of the relevant biological processes is much more needed now than is improvement in the mathematics. An important contribution to an improved biological understanding could come from a better knowledge base on low dose-response relationships in laboratory animals with and without tumor promoters.

I have only to add when controls have been developed and the decision is made to move ahead, we will need to maintain monitoring to assess the importance of changes in fuels, and in engines and engine designs. Procedures developed in the present studies should be extremely useful in monitoring such changes.

The last word I would like to leave is the urgency of moving ahead to secure the data to assess the human impact of the expansion of Diesel usage in such a way as to permit us to make rational and clearheaded decisions.