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Project Summary

Use of Short-Term Genotoxic Bioassays in the Evaluation of Unregulated Automobile Exhausts

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The levels of several products of fuel combustion in ambient air (nitrogen oxides, hydrocarbons and carbon monoxide) are currently regulated under the Clean Air Act. Amendments ([202(a)(4)] of 1977) also specify that new vehicles shall not be certified if they generate unregulated emissions which present a potential risk to human health. In addition, Section 211 of the Clean Air Act as amended in 1977 specifies that tests should be conducted to determine the mutagenic and carcinogenic effects (among other health effects) of automotive fuels and fuel additives and their emissions.

The objectives of this document are to review the data from selected short-term *in vitro* and *in vivo* bioassays to (a) determine if there is evidence suggesting potential human health risk either from uncombusted emissions or from emissions of combusted motor vehicle fuels or fuel additives, (b) identify the operational variables involved in generating products of concern for human health, (c) determine the probable nature of the health effects of concern, (d) estimate the ability of short-term tests to establish human risk estimates and (e) develop a short-term bioassay program to monitor the potential health hazard of fuel/fuel additives and unregulated combustion emissions.

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This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research pro-ject which is fully documented in a separate report of the same title (see project report ordering information at back).

Introduction

Automotive emission products from complete or partial combustion of fuels such as diesel fuel or gasoline are associated with genotoxic activity. Most of the genotoxic effects are found in the solid-phase (particulate) fraction as mutagenic and cell-transforming organic compounds condensed onto the carbonaceous core of the exhaust particle.

Air pollutants include both gaseous and solid phases. Gaseous-phase pollutants include carbon monoxide, ozone, nitrogen oxides, sulfur oxides, hydrocarbons, and other volatile organic compounds (e g., formaldehyde, benzene). The solid phase contains condensed organic polycyclic matter (POM) including polycyclic aromatic hydrocarbons (PAHs) and substituted PAHs, which are suspected of contributing to a potential lung cancer risk associated with long-term exposure to urban air pollution.

The possibility of human health risk from motor vehicle emissions has been a matter of concern for many years, and there has been substantial research into the effects of emissions from both gasoline spark ignition and diesel engines (1,2). Even with this data base, the extent to which motor vehicle emissions con-

tribute to human health problems is far from understood. The complex interactions between fuel (and its additives) and gases in the combustion chamber, environmental exposure conditions, and biology of the target species must be investigated in order to apply most conventional hazard assessment methods.

The objective of this report was to summarize the current status of research on unregulated automobile emissions using short-term tests for genotoxicity and to develop an approach for the application of these tests to the development of regulatory strategy. Based on the results of extensive studies with pure chemicals, short-term genetic tests are believed to be reliable quantitative indicators of carcinogenic and mutagenic potential. Thus, such tests should be useful in addressing the concern for carcinogenic effects.

Current Status of the Application of Genetic Assays to Studies of Motor Vehicle Emissions

Many of the characteristics of short-term tests measuring genotoxicity appear ideally suited to an analysis of automobile exhaust emissions; in fact, the current level of information available on the biological properties and possible health risks associated with particulate emissions has been accumulated largely using short-term test data. Certainly, the conduct of multi-dose experiments using relatively small quantities of particles collected from exhaust emissions was one of the true breakthroughs in the evaluation of motor vehicle emissions. Many attributes of short-term tests are important to the application of these tests and are summarized in the full report. The importance of the ability to conduct assays of very small samples cannot be overemphasized, since spark-ignition gasoline engines equipped with catalytic converters produce very low levels of particulate. Without short-term assays, valid comparisons of these samples with those of other emissions would not be possible

Application of Short-Term Bioassays to Risk Assessment of Automobile Emissions

Much of the data predicting qualitative hazard have been derived from short-term, especially *in vitro*, assays, because they have been found to be readily applicable to an evaluation of the solid-phase emissions. Risk analysis must draw upon all data available. Quantitative estimates developed for automobile exhaust emissions by the U.S. Environmental Protection Agency (EPA) and the National Academy of Sciences (NAS) have relied heavily upon comparative data obtained from *in vitro* assays for genotoxicity and short-term animal tests (1,3). Both approaches have been drawn upon the available bodies of short-term data and made comparative analyses of laboratory and epidemiological data on diesel and gasoline engines as well as chemically related environmental exposures—coke oven emissions, roofing tar emissions, and cigarette smoke condensate (CSC).

Both the EPA and the NAS risk estimates included analyses of short-term test results in terms of a linear, non-threshold, extrapolation model. The potency response of all sample emissions evaluated in the short-term bioassays was compared using linear extrapolation models. Relative potencies for gasoline and diesel emissions were then compared to those for coke oven, roofing tar, and cigarette smoke condensate. Assuming comparability between relative potency for the diesel emissions and the model emissions (coke oven or roofing tar), a relative potency value for humans was constructed using human lung cancer data from coke oven workers and roofers.

The estimate for risk of lung cancer from exposure to diesel exhaust emissions was derived from the ratio of short-term test activities between the two emissions and extrapolating that ratio to the incidence of human lung cancers for the two exposures after adjusting for dose. The calculated risks were quite similar

In summary, short-term tests for genotoxicity are amenable to use in human risk estimates in situations where conventional animal modeling or human epidemiology results cannot be obtained. An evaluation of two approaches conducted by EPA and NAS shows similar quantitative risk estimates for diesel exhaust emissions. These two values are also similar to a worst-case estimate for lung cancer in humans derived from negative epidemiological data. Risk estimation for heritable genetic effects cannot be derived from the available data.

Review of Bioassay Performance on Emission Samples

Review of the bioassay data is arranged by the type of toxic endpoint measured and is subdivided into mammalian *in vivo* tests and *in vitro* mammalian/submammalian tests. Table 1 outlines the stratification of endpoints included in this evaluation.

An evaluation of data from the bioassay data base was examined using the procedure outlined in Figure 1. The end product of this procedure was selection of a battery of tests amenable to routine evaluation of emissions.

Sample Collection and Preparation

Analysis of the published literature was presented and recommendations were made regarding collection and preparation of samples for genetic testing.

The full report suggested that a companion engineering document be prepared to establish specific test conditions for engine operation and emission collection.

Use of particle extracts in formulating comparisons of various engines, fuels, or fuel additives is considered appropriate, since most biological activity of the emissions appears to be associated with the particle-bound organics and suitable methods for gas phase or whole particle testing are not ready for routine application.

Selection of Bioassays to Form a Minimum Evaluation Matrix

Objective analysis of the current data base for automobile combustion emissions and test performance criteria resulted in the selection of three bioassay types which appear to merit further consideration as screens for automotive emission certification. The Ames *Salmonella*/microsome reverse mutation assay appears to be useful. Cytogenetic endpoints, especially sister chromatid exchange (SCE), showed very good sensitivity to the genotoxic components of automotive emissions. An *in vitro* mammalian cell gene mutation assay, especially the Mouse Lymphoma assay, is the third test considered applicable to emission screening, because it responded in a quantitative fashion to organic solvent extracts and in some cases to whole unextracted particles. Some of the information used to support the selection of these three tests is shown in Table 2, which identifies the range of susceptibilities of various bioassays to the types of samples evaluated.

Table 1. *Stratification of Bioassay Endpoints Reviewed*

Genotoxic Effects
A. Specific Locus Mutation in Prokaryotic and Eukaryotic Organisms
B. Chromosome Alterations (Including Aberrations, Sister Chromatid Exchange, and Aneuploidy)
C. Damage of the Primary DNA Level in Mammalian Cells In vivo *and* In vitro *(Including DNA Adduct Formation, DNA Repair Phenomena, Mitotic Crossing Over)*
Cell Transformation and/or Tumor Induction
A. In vitro *Cell Transformation in Mouse Cell Lines and Hamster Primary Cells*
B Short-Term Tumor Induction Assays in Mice
C. Long-Term Tumor Induction Assays in Rodent Species
D. Co-Carcinogenesis Studies in Rodents

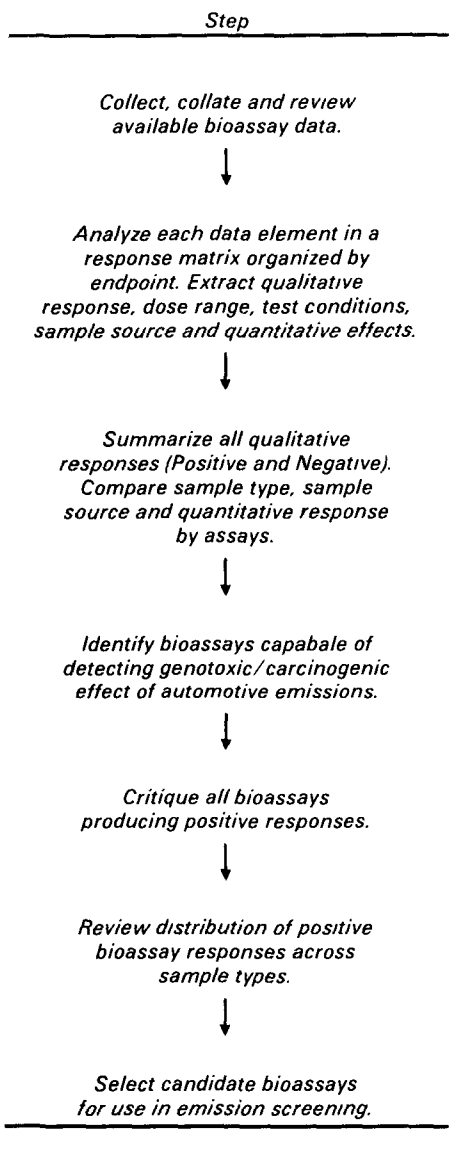

Figure 1. *Bioassay data evaluation matrix.*

Approach to Emission Testing for Comparisons of Biological Activity

The procedures described in the full report attempt to satisfy the need for data comparisons. To be useful, a description of biological activity must include an estimate of the specific activity of a sample in each bioassay employed. For the three tests recommended as the battery, the linear slope of the dose-response curve, expressed as revertants/μg, SCE/cells/μg/ml, and revertant mutant frequency/μg/ml, has been selected. The specific activity should then be adjusted to an estimate of particle potency by determining the level of organic extractability (percent extractable) from the material collected. The final calculation should consider the particle potency *vs.* the particle emission rate (PER). The following formula gives the relationship among these factors in developing a Sample Activity Rate (SAR):

$$\frac{\text{Genetic Activity}}{\mu\text{g organics}} \times \frac{\mu\text{g organics}}{100\ \mu\text{g particles}} \times \frac{\mu\text{g particles}}{\text{kilometer (km)}} = \frac{\text{Genetic Activity}}{\text{km}}$$

$$\frac{\text{Slope (Specific Activity)} \times \text{\% Extractables}}{\text{Particle Potency}} \times \text{Particle Emission Rate (PER)} = \text{Sample Activity Rate (SAR)}$$

The determination of samples that had significant biological activity employed an *analysis of variance* which compared the experimental means (using a .05 level of significance) for each sample in each bioassay. Several methods may be used to ask the question of how to identify the outliers among the samples. For example, it is possible to group samples using various statistical methods and find those tests which differ significantly from the remaining tests.

Several conclusions can be made based on analysis of a pilot set of samples.

1. Comparisons between nonactivated and S9-activated tests indicated that the set of samples studied in S9 had no effect on the responses.
2. The biological activities calculated for the emission samples did not appear to follow similar patterns in the three tests.
3. Among the three tests, the Ames assay was the most discriminating for this set of emission samples. It is possible, however, that greater use of replicate trials in the mammalian cell assays would increase their discriminating properties.
4. As a consequence of the analysis of this pilot data, it would appear that replication of sample collection, analysis, and biological testing is essential. Recommendations for replication of these components of the proposed scheme are shown in Table 3. By performing independent trials, the variability arising from replicating the collection and extraction procedures as well as bioassay techniques are important data components. Claxton and Kohan showed that there are small but consistent variances resulting from sample preparation differences (4).

Conclusions

1. Bioactive chemicals that exist in vapor and solid-phase exhaust emis-

Table 2. *Distribution of Responses in Susceptible Bioassays Across Sample Categories*

Bioassays	Organic Solvent Extracts of Particles	Biological Fluid Extracts of Particles	Unextracted Particles	Vapor Phase Alone	Whole Exhaust
Gene Mutation					
● *Microbial*	√	(√)		(√)	
● *Mammalian Cells* in vitro	√		(√)		
● *Plants*					
● *Insects*					
● *Mammals* in vivo					
Cytogenetic Endpoints					
● *Mammalian Cells* in vitro	√				
● *Plants*					√
● *Mammals* in vivo	√		√		√
Primary DNA Effects					
● In vitro *Mammalian*	√				
● In vivo/in vitro *Mammalian*	√				
Carcinogenesis Endpoints					
● *Mammalian Cells* in vitro	√				
● In vivo *Mammals (complete)*					
● In vivo *Initiation*	√				
● In vivo *Co-carcinogenesis*				√	√

(√) = data consist of limited experimentation that may not apply to all bioassays of this class.

Table 3. *Composition and Logistics of an Emission Assessment*

Step[a]	Trial A[b]			Trial B[b]		
1	Particle Collection from Filters(s)			Particle Collection from Filters(s)		
	↓			↓		
2	Extraction of Organics			Extraction of Organics		
	↓			↓		
	Bioassay			Bioassay		
	↓			↓		
	↓	↓	↓	↓	↓	↓
3	Ames Test Conducted with Triplicate Plates/Dose	Mouse Lymphoma Assay Conducted with Duplicate Cultures	SCE Assay Conducted with Duplicate Cultures	Ames Test Conducted with Triplicate Plates/Dose	Mouse Lymphoma Assay Conducted with Duplicate Cultures	SCE Assay Conducted with Duplicate Cultures
4	Data Analysis			Data Analysis		

[a]*Step 1 - Independent trials for determination of Particle Emission Rate (PER).*
Step 2 - Independent trials to measure percent extractables.
Step 3 - Replicate data within independent trials for each of the three bioassays.
Step 4 - Data analysis and comparison with other emission samples.

[b]*Definition of independent trials A and B has not been made. Trials may be different runs on the same day or different runs on two days. The total particulate required per trial is approximately 0.5 g.*

sions are generated by combustion and are believed to be derived from mono- or poly-substituted PAHs or nitroaromatics that can be activated to genotoxic agents by bacteria or animal cells (such as liver cells or macrophages).

2. Mutagens are bound to particles but can be extracted, to various degrees, with organic solvents and biological fluids. Thus, one can expect some level of bioavailability.
3. The levels and composition of chemicals found in emissions may vary significantly with engine types, fuel types, operating conditions, control devices, and environmental conditions of collection.
4. An efficient screening program to compare quantitative variations in one of the above parameters (by holding all other parameters constant) can be accomplished using a series of short-term *in vitro* tests; however, the assessment cannot be conducted on whole-emission collections; rather, it requires a standard sample collection and processing protocol for the preparation of organic compounds recovered from the solid phase.
5. A uniform data interpretation scheme has been defined in which the relative biological activity of each bioassay can be quantitatively compared to an existing data base

developed from similar engines, fuels, or fuel additives. Samples that show statistically significant deviations from the data base can be interpretated as a signal that an engine, fuel, or fuel additive might warrant further investigation for possible health impact.

The proposed approach should be adequate for making quantitative comparisons of the biological activity of emissions generated by different engines or fuels under standardized running and collection protocols and for identifying reponses which fall outside the normal range of engines, fuels, or fuel additives. The results will not be suitable for use in quantifying the absolute health risk of emissions because of the many uncertainties associated with bioavailability and dosimetry of emissions to heterogeneous human populations under normal exposure conditions and the lack of formal linkages between the responses in short-term tests and human disorders.

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The complete report, entitled "Use of Short-Term Genotoxic Bioassays in the Evaluation of Unregulated Automobile Exhausts," (Order No. PB 84-226 976; Cost: $14.50, subject to change) will be available only from:

National Technical Information Service
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