Research | Article

Bioassay-Directed Fractionation and *Salmonella* Mutagenicity of Automobile and Forklift Diesel Exhaust Particles

David M. DeMarini,[1] Lance R. Brooks,[1] Sarah H. Warren,[1] Takahiro Kobayashi,[2] M. Ian Gilmour,[1] and Pramila Singh[1]

[1]National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA; [2]Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba, Japan

Many pulmonary toxicity studies of diesel exhaust particles (DEPs) have used an automobile-generated sample (A-DEPs) whose mutagenicity has not been reported. In contrast, many mutagenicity studies of DEPs have used a forklift-generated sample (SRM 2975) that has been evaluated in only a few pulmonary toxicity studies. Therefore, we evaluated the mutagenicity of both DEPs in *Salmonella* coupled to a bioassay-directed fractionation. The percentage of extractable organic material (EOM) was 26.3% for A-DEPs and 2% for SRM 2975. Most of the A-EOM (~55%) eluted in the hexane fraction, reflecting the presence of alkanes and alkenes, typical of uncombusted fuel. In contrast, most of the SRM 2975 EOM (~58%) eluted in the polar methanol fraction, indicative of oxygenated and/or nitrated organics derived from combustion. Most of the direct-acting, base-substitution activity of the A-EOM eluted in the hexane/dichloromethane (DCM) fraction, but this activity eluted in the polar methanol fraction for the SRM 2975 EOM. The direct-acting frameshift mutagenicity eluted across fractions of A-EOM, whereas > 80% eluted only in the DCM fraction of SRM 2975 EOM. The A-DEPs were more mutagenic than SRM 2975 per mass of particle, having 227× more polycyclic aromatic hydrocarbon–type and 8–45× more nitroarene-type mutagenic activity. These differences were associated with the different conditions under which the two DEP samples were generated and collected. A comprehensive understanding of the mechanisms responsible for the health effects of DEPs requires the evaluation of DEP standards for a variety of end points, and our results highlight the need for multidisciplinary studies on a variety of representative samples of DEPs. *Key words:* bioassay-directed fractionation, diesel particulates, *Salmonella* mutagenicity, SRM 2975. *Environ Health Perspect* 112:814–819 (2004). doi:10.1289/ehp.6578 available via *http://dx.doi.org/* [Online 31 October 2003]

Long-term exposure to particulate air pollution, including diesel exhaust, is associated with an increasing incidence of respiratory allergy, cardiopulmonary mortality, and risk of lung cancer (Pope et al. 2002; Sydbom et al. 2001). Although the health effects of diesel exhaust particles (DEPs) have been studied for many years (Lewtas 1982), a comprehensive identification of the chemical components responsible for the biologic effects and a full understanding of the underlying mechanisms remain incomplete (Mauderly 2001; Rosenkranz 1996).

The two most-studied health effects of DEPs are pulmonary toxicity and mutagenicity, and different chemical and physical features of DEPs have been associated with the induction of these two end points. For mutagenicity, early studies suggested that nitroarenes were a primary class of mutagens in organic extracts of DEPs (Austin et al. 1985; Claxton 1981; Claxton and Huisingh 1980); analytical studies confirmed this (Paputa-Peck et al. 1983; Salmeen et al. 1982) and also identified a role for polycyclic aromatic hydrocarbons (PAHs) (Rosenkranz 1996). For pulmonary effects, some PAHs have been shown to enhance pro-inflammatory and allergic responses induced by DEPs in the airways (Diaz-Sanchez 1997; Kawasaki et al. 2001; Tsien et al. 1997). However, the size and

surface reactivity of particles also may play a role in the induction of pulmonary effects (Dick et al. 2003; Donaldson et al. 1996).

Many studies of the mutagenicity of DEPs have been conducted using a standard reference material (SRM), such as SRM 2975, which was derived from a forklift truck and developed by the National Institute of Standards and Technology (NIST) (Claxton et al. 1992; Hughes et al. 1997); however, only a few studies have characterized the pulmonary effects of this sample (Lovik et al. 1997; Madden et al. 2000). In contrast, many studies on the pulmonary toxicity of DEPs have used an automobile-derived DEP sample (A-DEP) (Kobayashi and Ito 1995; Sagai et al. 1993), but the mutagenicity of this DEP sample has not been reported. Although Seagrave et al. (2002) have examined the same DEPs for pulmonary effects as well as mutagenicity, no one has done so for the extensively studied A-DEP sample.

The chemical composition of DEPs is influenced by the age and type of engine, fuel composition, load characteristics, lube oil components, presence and efficiency of control devices, and sampling procedures (Claxton 1983; Mauderly 2001; Rosenkranz 1996; Schuetzle 1983). Given that these factors vary for the SRM and A-DEP samples, we reasoned that the biologic activities of these DEPs were

likely to be different. To assess the potential impact of these differences on mutagenicity, we evaluated the two DEP samples using a bioassay-directed fractionation (Schuetzle and Lewtas 1986) coupled with the *Salmonella* mutagenicity assay.

Various DEP samples have been subjected to such analyses since the first report (Huisingh et al. 1979), including an earlier SRM of DEPs, SRM 1650 (Savard et al. 1992). In this study, we sequentially extracted an organic extract of each DEP with solvents of increasing polarity on a silica-gel column and then evaluated the fractions for mutagenicity in various strains of *Salmonella*. The results were expressed as the distribution of mass and mutagenicity among the fractions. Combined with physical and chemical features of these DEPs, as well as their pulmonary toxicities (Singh et al. 2004), we propose that DEP samples should be characterized chemically, physically, and biologically at multiple end points to understand the mechanisms associated with the health effects of DEPs.

Materials and Methods

Generation and collection of DEPs. A-DEPs were provided by one of the authors (T.K.), and the generation and collection conditions of these DEPs have been described previously (Kobayashi and Ito 1995; Sagai et al. 1993). Briefly, DEPs were collected "cold" at a sampling temperature of 50°C onto glass-fiber filters (GD-100R, 203 mm × 254 mm) in a constant-volume sampling system fitted at the end of a dilution tunnel. The particles were generated using a light-duty (2,740 cc), 4-cylinder, 4JB1-type Isuzu diesel engine. The engine had a torque load of 6 kg/m generated by an

ECDY dynamometer (Meiden-Sya, Tokyo, Japan) and was run at 2,000 rpm.

SRM 2975 was generated by a forklift truck and was purchased from NIST (Gaithersburg, MD, USA). The DEPs were generated by a heavy-duty diesel engine and collected using a filtering system designed for diesel forklifts under "hot" conditions without a dilution tunnel at the Donaldson Company, Inc. (Minneapolis, MN, USA; personal communication). The certified analyses of these particles are available (NIST 2000).

Organic extractions and fractionation. DEPs were sonicated for 20 min in dichloromethane (DCM) at 2× the estimated volume of the particles, and the tube was centrifuged at approximately 2,000 rpm for 10 min. The solvent was transferred to another glass tube, and the extraction was repeated two more times. The pooled solvent extract was concentrated, and the percentage of extractable organic material (EOM) was determined by gravimetric measurement. The remaining extract was concentrated to 1 mL and readjusted to 5 mL with hexane.

Silica gel (10 g of grade 62, 60–200 mesh) was added to a 40 × 300 mm open column with a medium-porosity ground-glass frit. The silica was washed with DCM followed by hexane. The extract was added to the column, and the EOM was eluted serially with hexane, 50:50 hexane:DCM, DCM, and methanol. Each fraction was then concentrated under nitrogen, and the mass of EOM for each fraction was determined as described above for the whole extract. Stock solutions at 20 μg of EOM/mL in dimethyl sulfoxide (DMSO) of the whole extract and each fraction were prepared for bioassay by solvent exchange.

Mutagenicity assays. The EOM and fractions from each DEP sample were evaluated for mutagenicity in the standard plate-incorporation *Salmonella* (Ames) mutagenicity assay (Maron and Ames 1983). The strains used were the base-substitution strain TA100 (*hisG46, rfa, ΔuvrB,* pKM101) and the frameshift strain TA98 (*hisD3052, rfa, ΔuvrB,* pKM101) (Maron and Ames 1983); the nitroreductase- and dinitroreductase-deficient strains TA98NR and TA98/1,8-DNP6, respectively, which are derivatives of TA98 (McCoy et al. 1983; Rosenkranz 1981); and the acetyltransferase- and nitroreductase-overexpressing strains YG1024 (Watanabe et al. 1990) and YG1021 (Watanabe et al. 1989), respectively, which are also derivatives of TA98.

Aroclor-induced Sprague-Dawley rat liver S9 was obtained from Moltox (Boone, NC, USA) and used at 1 mg S9 protein/plate. Plates were incubated for 3 days, the colonies were counted, and linear regressions were calculated over the linear portion of the dose–response curves to determine the mutagenic potencies expressed as revertants (rev) per microgram.

A positive result was defined as a reproducible, dose-related response that at least approached a 2-fold increase in rev relative to the control. All experiments were performed twice using either two plates per dose (whole extracts) or one plate per dose (fractions). Thus, results are the average of two independent experiments. DMSO was the negative control, and the positive controls were 2-aminoanthracene (0.5 μg/plate) in TA98 +S9 and TA100 +S9; 2-nitrofluorene (3 μg/plate) in TA98 –S9, TA98NR –S9, TA98/1,8-DNP6 –S9, YG1024 –S9, and YG1021 –S9; and sodium azide (3 μg/plate) in TA100 –S9.

Results

Mutagenic potencies of EOM. The basis for the interpretation of the mutagenicity data is summarized in Table 1, and the mutagenicity dose–response data for the EOM samples are shown in Table 2. The mutagenic potencies derived from the dose–response data for both the EOM and particles are shown in Table 3.

The A-EOM exhibited 18× more PAH-type mutagenic activity (TA100 +S9) than did the SRM 2975 EOM (Table 3). In the absence of S9, the base-substitution mutagenic potency (TA100) of the SRM 2975 EOM was 3× greater than that of the A-EOM. The two EOM samples had rather similar frameshift mutagenic potencies (TA98); however, the SRM 2975 EOM had 2.3× more frameshift activity in the absence of S9 than in the presence of S9 (Table 3). Also, the SRM 2975 EOM had 5× more frameshift potency than base-substitution potency in the presence of S9. In the absence of S9, the rankings of the mutagenic potencies among the strains, with the exception of TA100, were similar for the two EOM samples (Table 3).

Table 4 shows the inferred contribution of nitroarenes to the mutagenic activity of the DEPs. Based on comparative results between TA98 and either TA98/1,8-DNP6 or YG1024, the EOM of SRM 2975 had approximately 2× more nitroarene-type mutagenic activity than

Table 1. Interpretation of *Salmonella* data.

Mutagenicity	Inference
TA100 +S9 > TA98 +S9	PAH-type mutagenicity because PAHs are more mutagenic in TA100 than in TA98 (DeMarini et al. 1994)
TA98 –S9 > TA98NR –S9	Nitroarene-type mutagenicity because nitroarenes require nitroreductase for mutagenicity, and some of this is missing in TA98NR (Rosenkranz 1981)
TA98 –S9 > TA98/1,8-DNP6 –S9	Dinitroarene-type mutagenicity because TA98/1,8-DNP6 is missing a reductase that activates some dinitroarenes (McCoy et al. 1983)
YG1021 –S9 > TA98 –S9	Nitroarene-type mutagenicity because YG1021 contains additional nitroreductase to activate nitroarenes to mutagens (Einistö et al. 1991)
YG1024 –S9 > TA98 –S9	Nitroarene- and/or aromatic amine-type mutagenicity because YG1024 contains acetyltransferase that activates these chemical classes to mutagens (Einistö et al. 1991)

Table 2. Mutagenicity of organic extracts of DEPs in *Salmonella*.

Strain	EOM/plate (μg)	Rev/plate[a]			
		A-DEP		SRM 2975	
		+S9	–S9	+S9	–S9
TA100	0.0	29	18	29	18
	0.5	114	103	87	110
	1.0	191	214	121	208
	2.0	314	299	225	456
TA98	0.0	81	75	81	75
	0.5	303	113	92	101
	1.0	497	161	99	99
	2.0	780	245	118	132
TA98NR	0.0		28		28
	0.5		30		47
	1.0		43		60
	2.0		41		71
TA98/1,8-DNP6	0.0		16		16
	0.5		26		28
	1.0		35		29
	2.0		47		46
YG1024	0.0		24		24
	0.5		287		663
	1.0		563		1,204
	2.0		1,049		1,992
YG1021	0.0		27		27
	0.5		188		129
	1.0		353		226
	2.0		652		435

[a]Data are the average of two independent experiments, each having two plates per dose; thus, the data are the average of four plates per dose.

did the A-EOM. However, based on data from strain YG1021, the opposite result was found, with the A-EOM having approximately 174× more nitroarene-type mutagenic activity than did the SRM 2975 EOM.

Mutagenic potencies of particles. To convert the mutagenic potencies of the EOM (rev per microgram of EOM) to mutagenic potencies of the particles (rev per microgram of particle), the potencies of the EOM were multiplied by the percent EOM of the particle. The percent EOM was 26.3% for A-DEPs and

2% for SRM 2975. Based on these calculations, the A-DEPs had 227× more PAH-type mutagenic activity (TA100 +S9) than did the SRM 2975 particles. In addition, the A-DEPs had approximately 8× more nitroarene-type activity than did the SRM 2975 particles based on data from TA98NR, TA98/1,8-DNP6, or YG1024 (Table 4). Based on data from strain YG1021, the A-DEPs had even more (45×) nitroarene-type mutagenic activity than did the SRM 2975 particles. The A-DEPs had greater mutagenic potency in both TA98 and TA100

than did the SRM 2975 particles (Table 3), perhaps due to higher amounts of nitroarene- and aromatic amine-type mutagenic activity in the A-DEP sample relative to the SRM 2975 sample. Except for the juxtaposition of TA98 and TA100, the mutagenic potency rankings of the two DEPs were similar among the strains (Table 3).

Mutagenic potencies of fractions of EOM. The dose–response data for the fractions are shown in Table 5, and the mutagenic potencies of the fractions of the two EOM are shown in Table 6. For example, in TA100 +S9, the most potent A-EOM eluted in the hexane/DCM and DCM fractions, whereas the most potent SRM 2975 EOM eluted in the DCM fraction. Thus, the classes of compounds accounting for S9-dependent, base-substitution mutagenicity in the A-EOM were less polar than were those in the SRM 2975 EOM. When comparing the reduction in mutagenic potencies in TA98NR relative to TA98, which is an indication of the presence of nitroarenes, the greatest reduction for the SRM 2975 EOM occurred in the hexane/DCM fraction, whereas the greatest reduction for the A-EOM occurred in the DCM and methanol fractions, which are much more polar than hexane/DCM. A variety of other differences of this sort can be noted by comparing the results in Table 6.

Distribution of recovered mass and mutagenicity of EOM. Most (84%) of the mass of the A-EOM and all (103%) of the mass of the SRM 2975 EOM were recovered from the silica-gel column after the fractionation (Tables 7 and 8). However, the distribution of the mass across the fractions was the opposite for the two EOM (Tables 7 and 8, Figure 1). Thus, approximately 55% of the A-EOM eluted in the hexane fraction and approximately 33% in the highly polar methanol fraction, whereas approximately 29% of the SRM 2975 EOM eluted in the hexane fraction and approximately 58% in the methanol fraction.

Likewise, the distribution of mutagenicity across the fractions was different for the two EOMs. For example, 3× more PAH-type mutagenic activity (TA100 +S9) eluted in the hexane/DCM fraction from the A-EOM than from the SRM 2975 EOM (Tables 7 and 8, Figure 2), confirming our other data that the A-DEP sample had more PAH-type mutagenic activity than did the SRM 2975. The distribution of direct-acting, base-substitution mutagenic activity (TA100 –S9) was completely the opposite for the two EOMs, with most of this activity eluting in the hexane/DCM fraction for the A-EOM but in the polar methanol fraction for the SRM 2975 EOM (Tables 7 and 8). The direct-acting frameshift mutagenic activity requiring minimal nitroreductase (TA98NR –S9) was due to compounds having a range of polarity for the A-EOM, because this activity

Table 3. Mutagenic potencies of EOM and particles of DEPs in *Salmonella*.

	Rev/μg EOM[a]				Rev/μg particle[a]			
	A-DEP		SRM 2975		A-DEP		SRM 2975	
Strain	+S9	–S9	+S9	–S9	+S9	–S9	+S9	–S9
TA100	345.2	85.9	19.0	262.0	90.8	22.6	0.4	5.2
TA98	155.3	138.5	96.0	218.1	40.8	36.4	1.9	4.4
TA98NR		15.5		27.3		4.1		0.6
TA98/1,8-DNP6		15.3		14.0		4.0		0.3
YG1024		512.7		970.0		134.8		19.4
YG1021		312.5		203.4		82.2		4.1

[a]Data are the average slopes of linear regressions calculated over the linear portion of the dose–response curves from two independent experiments, each of which had two plates per dose (Table 2).

Table 4. Comparative amounts of nitroarene-type mutagenic activity between the two DEPs.

Strain[a]	EOM	Particles
TA98NR	SRM 1.6× > A-DEP	A-DEP 8.5× > SRM
TA98/1,8-DNP6	SRM 1.7× > A-DEP	A-DEP 7.9× > SRM
YG1024	SRM 2× > A-DEP	A-DEP 6.5× > SRM
YG1021	A-DEP ~174× > SRM	A-DEP 45× > SRM

[a]Comparisons were made by determining the difference in mutagenic potency for each EOM or particle in Table 3 between TA98 –S9 and the strains listed above –S9. For example, for EOM in TA98NR, 138.5 – 15.5 (rev/μg in TA98 – TA98NR) = 123 rev/μg; for SRM 2975 this was 218.1 – 27.3 = 190.8 rev/μg. Then, 190.8 ÷ 123 = 1.6; thus, based on data in TA98NR, SRM 2975 EOM had 1.6× more nitroarene-type mutagenic activity than did A-EOM.

Table 5. Mutagenicity and mutagenic potencies of fractions of organic extract of DEPs in *Salmonella*.

		Rev/plate[a]							
	EOM/	A-DEP				SRM 2975			
Strain	plate (μg)	H	H/DCM	DCM	M	H	H/DCM	DCM	M
TA100 +S9	0.0	110	110	110	110	89	89	89	89
	0.25		731	532					
	0.5	112	1,008	849	167	90	135	176	133
	1.0	108	1,573	1,015	231	95	163	153	146
	2.0	111	1,486	1,010	398	90	235	523	199
TA100 –S9	0.0	110	110	110	110	90	90	90	90
	0.25		136						
	0.5	110	149	255	149	84	137	408	155
	1.0	115	215	322	181	99	163	662	184
	2.0	120	347	508	256	92	239	1,017	258
TA98 +S9	0.0	46	46	46	46	32	32	32	32
	0.5	56	221	231	80	38	60	172	80
	1.0	47	347	381	102	41	75	351	126
	2.0	47	570	658	161	45	123	960	204
TA98 –S9	0.0	31	31	31	31	22	22	22	22
	0.25							986	
	0.5	46	89	197	80	28	86	1,962	108
	1.0	44	118	394	113	24	162	2,702	211
	2.0	39	200	736	189	27	314	2,448	432
TA98NR –S9	0.0	24	24	24	24	17	17	17	17
	0.25							573	
	0.5	30	45	55	40	21	30	848	37
	1.0	27	57	80	36	20	37	1,494	55
	2.0	30	74	117	46	22	48	1,743	81

Abbreviations: H, hexane; H/DCM, hexane/DCM; M, methanol.
[a]Data are the average of two independent experiments, each of which had one plate per dose; thus, data shown are the average of two plates per dose.

was dispersed across three fractions, whereas for the SRM 2975 EOM, > 80% of this activity eluted in only the DCM fraction. Despite the many differences, one feature was identical for both EOM: neither had detectable mutagenic activity in the hexane fraction. This was true even for the A-EOM, despite approximately 55% of its mass eluting in the hexane fraction.

Discussion

Mutagenicity of EOM and DEPs. DCM is the most effective solvent for the extraction of mutagenic organics from diesel exhaust (Montreuil et al. 1992; Petersen and Chuang 1982); therefore, it was used here to prepare the initial organic extract of both DEP samples. Although the mutagenicity of SRM 2975 EOM in several strains of *Salmonella* had been reported previously (Hughes et al. 1997), this sample had not been subjected previously to a bioassay-directed fractionation of the type described here, and no mutagenicity data had been reported previously for the A-EOM. With regard to the whole, unfractionated SRM 2975 EOM, the mutagenic potency ranking in the absence of S9 among the strains obtained here was similar to that obtained previously by Hughes et al. (1997) except that our sample

ranked as more potent in TA100 than that of Hughes et al. (1997). One possible reason is that Hughes et al. (1997) evaluated the soxhlet extract of SRM 2975 purchased from NIST, whereas we prepared our own extract by sonication from SRM 2975.

In the absence of S9, the mutagenic potency of the SRM 2975 EOM was generally greater than that of the A-EOM; the opposite was the case in the presence of S9 (Table 3). However, when the mutagenic potencies of the EOM were combined with the percent EOM of the particles, the mutagenic potency of the A-DEPs per mass of particle was greater in all strains than that of the SRM 2975 DEP regardless of S9 (Table 3). This occurred partly because the A-DEPs had > 10× the percent EOM than did SRM 2975. The A-DEPs had 227× more PAH-type activity and approximately 8–45× more nitroarene-type activity than did the SRM 2975 particles. The A-DEPs also had 3–21× more frameshift mutagenic activity than did the SRM 2975 particles, which was most likely due to the excess amount of nitroarene and possibly aromatic amine activity in the A-DEPs. Considering the various strains used to infer the proportion of nitroarene-type mutagenic activity in the

samples, all the strains but YG1021 led to similar conclusions (Table 4). Perhaps the additional nitroreductase present in YG1021 activated many compounds present at low concentrations that were not activated by the normal levels of nitroreductase present in TA98. Thus, continued caution must be exercised regarding inferences about the role of nitroarenes in the mutagenicity of DEPs using these and other such strains (Rosenkranz 1981).

Mutagenicity of fractions of EOM. Various methods have been used to fractionate organic extracts of diesel particles, including acid/base/neutral fractionation procedures (Crebelli et al. 1991) and solid-phase extractions using Sephadex (Bechtold et al. 1985) or silica gel (Hayakawa et al. 1997; Strandell et al. 1994). Hexane is a neutral solvent in which chemical classes such as alkanes and alkenes elute (Hayakawa et al. 1997), whereas methanol is a highly polar solvent in which

Figure 1. Distribution of mass of EOM of DEPs across four fractions of increasing polarity. A DCM extract of each DEP resulted in the EOM, which was then fractionated on a silica-gel column by sequential extraction with solvents of increasing polarity: hexane (H), H/DCM, DCM, and methanol (M).

Table 6. Mutagenic potencies (rev/µg) of fractions of EOM of DEPs in *Salmonella*.

Strain	A-DEP[a]				SRM 2975[a]			
	H	H/DCM	DCM	M	H	H/DCM	DCM	M
TA100 +S9	0.0	1430.4	1204.0	145.0	0.0	71.5	217.4	52.0
TA100 −S9	0.0	122.7	191.9	72.7	0.0	73.0	452.9	80.9
TA98 +S9	0.0	256.7	302.2	56.7	0.0	44.8	471.5	85.8
TA98 −S9	0.0	94.2	354.9	77.7	0.0	149.5	2662.1	206.9
TA98NR −S9	0.0	31.5	53.2	9.3	0.0	14.7	1407.4	31.5

Abbreviations: H, hexane; H/DCM, hexane/DCM; M, methanol.
[a]Data are average slopes of linear regressions calculated from the linear portion of the dose–response curves from two independent experiments, each of which had one plate per dose (Table 5).

Table 7. Distribution of mass and mutagenicity among fractions of organic extract of A-DEP.

Sample	Mass			Distribution of recovered mutagenicity (%)[a]				
	EOM (µg)	Recovery (%)	Distribution of recovered mass (%)	TA100 +S9	TA100 −S9	TA98 +S9	TA98 −S9	TA98NR −S9
Whole	53,680							
Hexane	24,850	46.3	54.8	0.0	0.0	0.0	0.0	0.0
Hexane/DCM	2,690	5.0	5.9	40.6	80.7	28.7	10.4	22.5
DCM	2,890	5.4	6.4	36.7	13.7	36.3	42.1	40.8
Methanol	14,880	27.7	32.9	22.7	5.6	35.0	47.5	36.7
Σ Fractions	45,310	84.4	100.0	100.0	100.0	100.0	100.0	100.0

[a]Calculated by multiplying the number of rev/µg for each fraction from Table 6 by the number of micrograms of EOM recovered for each fraction as noted in the second column of this table. These values, rev/fraction, were then expressed as a percentage relative to the sum (Σ) of the recovered mass of the fractions noted in the second column of this table.

Table 8. Distribution of mass and mutagenicity among fractions of organic extract of SRM 2975.

Sample	Mass			Distribution of recovered mutagenicity (%)[a]				
	EOM (µg)	Recovery (%)	Distribution of recovered mass (%)	TA100 +S9	TA100 −S9	TA98 +S9	TA98 −S9	TA98NR −S9
Whole	8,430							
Hexane	2,550	30.3	29.1	0.0	0.0	0.0	0.0	0.0
Hexane/DCM	560	6.6	6.4	13.7	5.8	3.5	3.2	0.9
DCM	550	6.5	6.3	41.0	35.6	36.0	56.3	82.1
Methanol	5,090	60.4	58.2	45.3	58.6	60.5	40.5	17.0
Σ Fractions	8,750	103.8	100.0	100.0	100.0	100.0	100.0	100.0

[a]Calculated by multiplying the number of rev/µg for each fraction from Table 6 by the number of micrograms of EOM recovered for each fraction as noted in the second column of this table. These values, rev/fraction, were then expressed as a percentage relative to the sum (Σ) of the recovered mass of the fractions noted in the second column of this table.
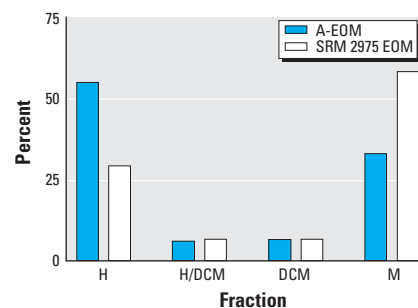
compounds such as oxygenated aromatics elute (Strandell et al. 1994). PAHs, aromatic amines, and nitroarenes can elute in the hexane/DCM and DCM fractions (Hayakawa et al. 1997; Schuetzle 1983). Hayakawa et al. (1997) showed that as much as 53% of the mutagenicity of the DCM fraction of DEPs was due to nitroarenes.

As summarized by Singh et al. (2004), the two DEP samples had different physical properties, chemical compositions, and pulmonary toxicities. The mass and mutagenicity distributions of the two samples described here reflected clear differences in the chemical composition of these DEPs. The mass distributions of the fractionated extracts of the two DEP samples were the opposite of each other, with most of the A-EOM eluting in the hexane fraction but most of the SRM 2975 EOM eluting in the highly polar methanol fraction. The lack of mutagenic activity in the hexane fraction (Tables 7 and 8) was consistent with the presence of unsubstituted alkanes and alkenes, which are not mutagenic. These results are also supported by the photomicrographs and other chemical and combustion data demonstrating that much of the A-EOM is uncombusted fuel, possibly neutral alkanes and alkenes (Singh et al. 2004), which would have eluted in the hexane fraction.

As reported previously (Hayakawa et al. 1997), the sum of the mutagenic potencies of the EOM across the fractions was generally much higher than that of the whole, unfractionated material, and this was true for all the strains used here. For example, in TA98 –S9, the mutagenic potency of the A-EOM was 138.5 rev/µg (Table 3), but the sum of the potencies of the fractions in this strain was

526.8 rev/µg (Table 7). Likewise, the mutagenic potency of the SRM 2975 EOM was 218.1 rev/µg, but that of the sum of its fractions was 3018.5 rev/µg, an approximately 14× increase. This showed that the fractionation unmasked the mutagenic potency of the compounds by separating some of the nonmutagenic cytotoxic compounds from the mutagenic compounds in the mixture. The ability of the fractionation to separate a large amount of nonmutagenic mass from mutagenic mass (Tables 7 and 8, Figure 1) is one of the goals of a successful bioassay-directed fractionation (Schuetzle and Lewtas 1986).

As shown previously, the distribution of mutagenic activity of the EOM across chemical fractions can vary depending on the type of engine (Clark et al. 1981; Sjogren et al. 1996), type of fuel (Clark et al. 1982; Crebelli et al. 1995; Sjogren et al. 1996; Westerholm et al. 2001), the running conditions (Bechtold et al. 1984; Courtois et al. 1993), and the collection conditions (Claxton and Barnes 1981; Lies et al. 1986). As demonstrated by extensive studies, much of the mutagenic activity of diesel exhaust is due to PAHs and nitroaromatics, especially nitropyrenes (Austin et al. 1985; Claxton 1981, 1983; Lies et al. 1986; Nakagawa et al. 1983; Rosenkranz 1996; Tokiwa and Ohnishi 1986). An important role for direct-acting acid/neutral compounds that are not nitropyrenes has also been noted (Crebelli et al. 1991). In this regard, the direct-acting, base-substitution mutagenicity (TA100 –S9) of the two EOM eluted in opposite fractions, indicating that different chemicals accounted for this activity for the two samples.

The mutagenic potencies of most of the fractions of the A-EOM were enhanced by S9

(Table 6), whereas the opposite result was found for the SRM 2975 EOM. This indicated that, across chemical classes, A-EOM contained more S9-dependent mutagenicity than did the SRM 2975 EOM. Consistent with this is the evidence presented here and by Singh et al. (2004) for greater amounts of PAH-type mutagenicity and PAHs in the A-DEPs compared with SRM 2975. There is ample evidence that the genotoxic activity associated with the EOM of DEPs inhaled into the lung may be bioavailable by virtue of the solubilization and dispersion properties of pulmonary surfactant components (Belisario et al. 1984; Keane et al. 1991; King et al. 1981).

Conclusions

A summary of some of the relative differences between the two DEPs (Table 9) shows that they have disparate mutagenic activities and chemical compositions due to the different conditions under which they were generated and collected. The fact that one sample (A-DEPs) has been used extensively in pulmonary toxicity studies but never studied previously for mutagenicity, and virtually the opposite situation pertains for the other sample (SRM 2975), illustrates the need for scientists to engage in collaborative, multidisciplinary research efforts in this area. Similar to the mutagenic activities of these particles, the pulmonary toxicities of these two DEPs were also strikingly different (Singh et al. 2004). These biologic data, combined with the physical and chemical features of these two DEPs (Singh et al. 2004), provide a basis for comparing these two DEPs that was not available previously.

A screening battery for a variety of DEPs involving pulmonary toxicity and mutagenicity has been proposed by Seagrave et al. (2002), and this could be extended to include a bioassay-directed fractionation as shown here. Until comparative data among a variety of DEP samples for various end points are available, a comprehensive understanding of the mechanisms associated with the health effects of any DEPs will be hindered (HEI 2002).

Figure 2. Distribution of mutagenicity of EOM in strain TA100 of *Salmonella* +S9 (*A*) and –S9 (*B*). Abbreviations: H, hexane; M, methanol. The percentage of recovered mutagenic activity across the four fractions was calculated by multiplying the rev/µg of each fraction by the micrograms of EOM recovered from that fraction. These values, rev/fraction, were then expressed as a percentage relative to the sum of the recovered mass of the fractions. Data are the average of two mutagenesis experiments.

Table 9. Summary of results.

Characteristics	A-DEP	SRM 2975
Percent EOM	26.3	2.0
Relative PAH-type potency of EOM	18	1
Relative PAH-type potency of particles	227	1
Relative nitroarene-type potency of particles	8–45	1
Relative distribution of PAH-type activity of EOM in hexane/DCM fraction	3	1
Relative distribution of direct-acting, base-substitution activity of EOM in		
hexane/DCM fraction	14	1
methanol fraction	1	10
Relative distribution of S9-dependent frameshift activity of EOM in methanol fraction	1	~2

REFERENCES

Austin AC, Claxton LD, Lewtas J. 1985. Mutagenicity of the fractionated organic emissions from diesel, cigarette smoke condensate, coke oven, and roofing tar in the Ames assay. Environ Mutagen 7:471–487.

Bechtold WE, Dutcher JS, Brooks AL, Henderson TR. 1985. Fractionation of diesel particle extracts by sephadex LH-20 and thin-layer chromatography. J Appl Toxicol 5:295–300.

Bechtold WE, Dutcher JS, Mokler BV, Lopez JA, Wolf I, Li AP, et al. 1984. Chemical and biological properties of diesel exhaust particles collected during selected segments of a simulated driving cycle. Fundam Appl Toxicol 4:370–377.

Belisario MA, Buoncore V, De Marinis E, De Lorenzo F. 1984. Biological availability of mutagenic compounds adsorbed onto diesel exhaust particulate. Mutat Res 135:1–9.

Clark CR, Henderson TR, Royer RE, Brooks AL, McClellan RO, Marshall WF, et al. 1982. Mutagenicity of diesel exhaust particle extracts: influence of fuel composition in two diesel engines. Fundam Appl Toxicol 2:38–43.
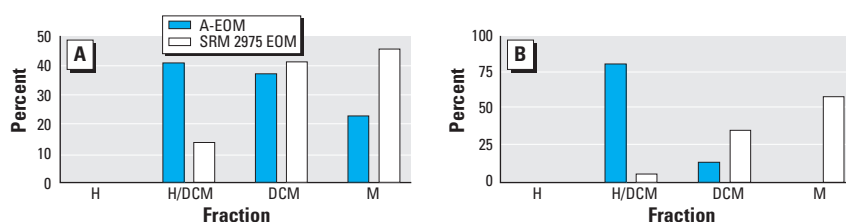
Clark CR, Royer RE, Brooks AL, McClellan RO, Marshal WF, Naman TM, et al. 1981. Mutagenicity of diesel exhaust particle extracts: influence of car type. Fundam Appl Toxicol 1:260–265.

Claxton L, Huisingh JL. 1980. Comparative mutagenic activity of organics from combustion sources. In: Pulmonary Toxicology of Respirable Particles (Sander CL, Cross FT, Dagle GE, Mahaffey JA, eds). DOE Symposium Series, Vol 53. Washington, DC:Technical Information Center, Department of Energy, 453–465.

Claxton L, Lewtas J, Becking G, Shelby M, eds. 1992. Collaborative Study on Complex Mixtures. Mutat Res 276(1-2):1–144.

Claxton LD. 1981. Mutagenic and carcinogenic potency of diesel and related environmental emissions: *Salmonella* bioassay. Environ Intl 5:389–391.

Claxton LD. 1983. Characterization of automotive emissions by bacterial mutagenesis bioassay: a review. Environ Mutagen 5:609–631.

Claxton L, Barnes HM. 1981. The mutagenicity of diesel-exhaust particle extracts collected under smog-chamber conditions using the *Salmonella typhimurium* test system. Mutat Res 88:255–272.

Courtois Y, Molinier B, Pasquereau M, Degobert P, Festy B. 1993. Influence of the running conditions of diesel engine on the mutagenic effects of its emissions. Sci Total Environ 134:61–70.

Crebelli R, Conti L, Crochi B, Carere A, Bertoli C, Del Giacomo N. 1995. The effect of fuel composition on the mutagenicity of diesel engine exhaust. Mutat Res 346:167–172.

Crebelli R, Fuselli S, Conti G, Conti L, Carere A. 1991. Mutagenicity spectra in bacterial strains of airborne and engine exhaust particulate extracts. Mutat Res 261:237–248.

DeMarini DM, Shelton ML, Bell DA. 1994. Mutation spectra in *Salmonella* of complex mixtures: comparison of urban air to benzo[a]pyrene. Environ Mol Mutagen 24:262–275.

Diaz-Sanchez D. 1997. The role of diesel exhaust particles and their associated polyaromatic hydrocarbons in the induction of allergic airway disease. Allergy 52(38 suppl):52–56.

Dick CAJ, Brown DM, Donaldson K, Stone V. 2003. The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types. Inhal Toxicol 15:39–52.

Donaldson K, Beswick PH, Gilmour PS. 1996. Free radical activity associated with the surface of particles: a unifying factor in determining biological activity? Toxicol Lett 88:293–298.

Einistö P, Watanabe M, Ishidate M Jr, Nohmi T. 1991. Mutagenicity of 30 chemicals in *Salmonella typhimurium* strains possessing different nitroreductase or *O*-acetyltransferase activities. Mutat Res 259:95–102.

Hayakawa K, Nakamura A, Terai N, Kizu R, Ando K. 1997. Nitroarene concentrations and direct-acting mutagenicity of diesel exhaust particulates fractionated by silica-gel column chromatography. Chem Pharm Bull (Tokyo) 45:1820–1822.

HEI. 2002. Understanding the health effects of components of the particulate matter mix: progress and next steps. HEI Perspect, April, 1–20. Available: http://www.healtheffects.org/Pubs/Perspectives-2.pdf [accessed 9 October 2003].

Hughes TJ, Lewtas J, Claxton LD. 1997. Development of a standard reference material for diesel mutagenicity in the *Salmonella* plate incorporation assay. Mutat Res 391:243–258.

Huisingh J, Bradow R, Jungers R, Claxton L, Zweidinger R, Tejada S, et al. 1979. Application of bioassay to the characterization of diesel particle emissions. In: Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures (Waters MD, Nesnow S, Huisingh JL, Sandhu SS, Claxton L, eds). New York:Plenum, 383–418.

Kawasaki S, Takizawa H, Takami K, Desaki M, Okazaki H, Kasama T, et al. 2001. Benzene-extracted components are important for the major activity of diesel exhaust particles: effect on interleukin-8 gene expression in human bronchial epithelial cells. Am J Respir Cell Mol Biol 24:419–426.

Keane MJ, Xing SG, Harrison JC, Ong T, Wallace WE. 1991. Genotoxicity of diesel-exhaust particles dispersed in simulated pulmonary surfactant. Mutat Res 260:233–238.

King LC, Kohan MJ, Austin AC, Claxton LD, Huisingh JL. 1981. Evaluation of the release of mutagens from diesel particles in the presence of physiological fluids. Environ Mutagen 3:109–121.

Kobayashi T, Ito T. 1995. Diesel exhaust particulates induce nasal mucosal hyperresponsiveness to inhaled histamine aerosol. Fund Appl Toxicol 27:195–202.

Lewtas J, ed. 1982. Toxicological Effects of Emissions from Diesel Engines. New York:Elsevier.

Lies KH, Hartung A, Postulka A, Gring H, Schulze J. 1986. Composition of diesel exhaust with particular reference to particle bound organics including formation of artifacts. Dev Toxicol Environ Sci 13:65–82.

Lovik M, Hogseth A-K, Gaarder PI, Hagemann R, Eide I. 1997. Diesel exhaust particles and carbon black have adjuvant activity on the local lymph node response and systemic IgE production to ovalbumin. Toxicology 121:165–178.

Madden MC, Richards JH, Dailey LA, Hatch GE, Ghio AJ. 2000. Effect of ozone on diesel exhaust particle toxicity in rat lung. Toxicol Appl Pharmacol 168:140–148.

Maron DM, Ames BN. 1983. Revised methods for the *Salmonella* mutagenicity test. Mutat Res 113:173–215.

Mauderly JL. 2001. Diesel emissions: is more health research still needed? Toxicol Sci 62:6–9.

McCoy EC, Anders M, Rosenkranz HS. 1983. The basis of the insensitivity of *Salmonella typhimurium* strain TA98/1,8-DNP6 to the mutagenic action of nitroarenes. Mutat Res 121:17–23.

Montreuil CN, Ball JC, Gorse RA Jr, Young WC. 1992. Solvent extraction efficiencies of mutagenic components from diesel particles. Mutat Res 282:89–92.

Nakagawa R, Kitamori S, Horikawa K, Nakashima K, Tokiwa H. 1983. Identification of dinitropyrenes in diesel-exhaust particles. Their probable presence as the major mutagens. Mutat Res 1224:201–211.

NIST. 2000. Certificate of Analysis, Standard Reference Material 2975. Gaithersburg, MD:National Institute of Standards and Technology. Available: http://patapsco.nist.gov/srmcatalog/certificates/2975.pdf [accessed 1 July 2003].

Paputa-Peck MC, Marano RS, Schuetzle D, Riley TL, Hampton CV, Prater TJ, et al. 1983. Determination of nitrated polynuclear aromatic hydrocarbons in particulate extracts by capillary column gas chromatography with nitrogen selective detection. Anal Chem 55:1946–1954.

Petersen BA, Chuang CC. 1982. Methodology of fractionation and partition of diesel exhaust particulate samples. In: Toxicological Effects of Emissions from Diesel Engines (Lewtas J, ed). New York:Elsevier, 51–67.

Pope CA, Burnett RT, Thun MJ, Calle EE, Krewski D, Ito K, et al. 2002. Lung cancer, cardiopulmonary mortality and long-term exposure to fine particulate air pollution. JAMA 287:1132–1141.

Rosenkranz HS. 1981. A cautionary note on the use of nitroreductase-deficient strains of *Salmonella typhimurium* for the detection of nitroarenes as mutagens in complex mixtures including diesel exhausts. Mutat Res 91:103–105.

Rosenkranz HS. 1996. Mutagenic nitroarenes, diesel emissions, particulate-induced mutations and cancer: an essay on cancer-causation by a moving target. Mutat Res 367:65–72.

Sagai M, Saito H, Ichinose T, Kodama M, Mori Y. 1993. Biological effects of diesel exhaust particles. I. *in vitro* production of superoxide and *in vivo* toxicity in mouse. Free Rad Biol Med 14:37–47.

Salmeen I, Durisin AM, Prater TJ, Riley T, Schuetzle D. 1982. Contribution of 1-nitropyrene to direct-acting Ames assay mutagenicities of diesel particulate extracts. Mutat Res 104:17–23.

Savard S, Otson R, Douglas GR. 1992. Mutagenicity and chemical analysis of sequential organic extracts of airborne particulates. Mutat Res 276:101–115.

Schuetzle D. 1983. Sampling of vehicle emissions for chemical analysis and biological testing. Environ Health Perspect 47:65–80.

Schuetzle D, Lewtas J. 1986. Bioassay-directed chemical analysis in environmental research. Anal Chem 58:1060A–1075A.

Seagrave J, McDonald JD, Gigliotti AP, Nikula KJ, Seilkop SK, Gurevich M, et al. 2002. Mutagenicity and in vivo toxicity of combined particulate and semivolatile organic fractions of gasoline and diesel engine emissions. Toxicol Sci 70:212–226.

Singh P, DeMarini DM, Dick CAJ, Tabor DG, Ryan JV, Linak WP, et al. 2004. Sample characterization of automobile and fork-lift diesel exhaust particles and comparative pulmonary toxicity in mice. Environ Health Perspect 112:820–825.

Sjogren M, Li H, Banner C, Rafter J, Westerholm R, Rannug U. 1996. Influence of physical and chemical characteristics of diesel fuels and exhaust emissions on biological effects of particle extracts: a multivariate statistical analysis of ten diesel fuels. Chem Res Toxicol 9:197–207.

Strandell M, Zakrisson S, Alsberg T, Westerholm R, Winquist L, Rannug U. 1994. Chemical analysis and biological testing of a polar fraction of ambient air, diesel engine, and gasoline engine particulate extracts. Environ Health Perspect 102(suppl 4):85–92.

Sydbom A, Blomberg A, Parnia S, Stenfors N, Sandstrom T, Dahlen S-E. 2001. Health effects of diesel exhaust emissions. Eur Respir J 17:733–746.

Tokiwa H, Ohnishi Y. 1986. Mutagenicity and carcinogenicity of nitroarenes and their sources in the environment. Crit Rev Toxicol 17:23–60.

Tsien A, Diaz-Sanchez D, Ma J, Saxon A. 1997. The organic component of diesel exhaust particles and phenanthrene, a major polyaromatic hydrocarbon constituent, enhances IgE production by IgE-secreting EBV-transformed human B cells *in vitro*. Toxicol Appl Pharmacol 142:256–263.

Watanabe M, Ishidate M Jr, Nohmi T. 1989. A sensitive method for the detection of mutagenic nitroarenes: construction of nitroreductase-overproducing derivatives of *Salmonella typhimurium* strains TA98 and TA100. Mutat Res 216:211–220.

Watanabe M, Ishidate M Jr, Nohmi T. 1990. Sensitive method for the detection of mutagenic nitroarenes and aromatic amines: new derivatives of *Salmonella typhimurium* tester strains possessing elevated *O*-acetyltransferase levels. Mutat Res 234:337–348.

Westerholm R, Christensen A, Törnqvist M, Ehrenberg L, Rannug U, Sjögren M, et al. 2001. Comparison of exhaust emissions from Swedish environmental classified diesel fuel (MK1) and European Program on Emissions, Fuels and Engine Technologies (EPEFE) reference fuel: a chemical and biological characterization with viewpoints on cancer risk. Environ Sci Technol 35:1748–1754.