4. MUTAGENICITY

Since 1978, more than 100 publications have appeared in which genotoxicity assays were used with diesel emissions, the volatile and particulate fractions (including extracts), or individual chemicals found in diesel emissions. Although most of the studies deal with the question of whether gas-phase or particulate extracts from diesel emissions possess mutagenic activity in microbial and mammalian cell assays, a number of studies have employed bioassays (most commonly *Salmonella* TA98 without S9) to evaluate (1) extraction procedures, (2) fuel modifications, (3) bioavailability of chemicals from diesel particulate matter (DPM), and (4) exhaust filters or other modifications and variables associated with diesel emissions. As indicated in Chapter 2, the number of chemicals in diesel emissions is very large. Many of these have been determined to exhibit mutagenic activity in a variety of assay systems (see Table II in Claxton, 1983). Although a detailed discussion of those data is beyond the scope of this document, some of the mutagenically active compounds found in the gas phase are ethylene, benzene, 1,3-butadiene, acrolein, and several polycyclic aromatic hydrocarbons (PAHs) (see Table 2-21). Of the particle-associated chemicals, several PAHs and nitro-PAHs have been the focus of mutagenic investigations both in bacteria and in mammalian cell systems (see Table 2-22). Several review articles, some containing more detailed descriptions of the available studies, are available (IARC, 1989; Claxton, 1983; Pepelko and Peirano, 1983; Shirnamé-Moré, 1995). Discussions of genotoxicity are also found in the proceedings of several symposia on the health effects of diesel emissions (U.S. EPA, 1980; Lewtas, 1982; Ishinishi et al., 1986).

4.1. GENE MUTATIONS

Huisingh et al. (1978) demonstrated that dichloromethane extracts from DPM were mutagenic in strains TA1537, TA1538, TA98, and TA100 of *S. typhimurium*, both with and without rat liver S9 activation. This report contained data from several fractions as well as DPM from different vehicles and fuels. Similar results with diesel extracts from various engines and fuels have been reported by a number of investigators using the *Salmonella* frameshift-sensitive strains TA1537, TA1538, and TA98 (Siak et al., 1981; Claxton, 1981; Dukovich et al., 1981; Brooks et al., 1984). Similarly, mutagenic activity was observed in *Salmonella* forward mutation assays measuring 8-azaguanine resistance (Claxton and Kohan, 1981) and in *E. coli* mutation assays (Lewtas, 1983).

One approach to identifying significant mutagens in chemically complex environmental samples such as diesel exhaust or ambient particulate extracts is the combination of short-term bioassays with chemical fractionation (Scheutzle and Lewtas, 1986). The analysis is most frequently carried out by sequential extraction with increasingly polar or binary solvents.

Fractionation by silica-column chromatography separates compounds by polarity or into acidic, basic, and neutral fractions. The resulting fractions are too complex to characterize by chemical methods, but the bioassay analysis can be used to determine fractions for further analysis. In most applications of this concept, *Salmonella* strain TA98 without the addition of S9 has been used as the indicator for mutagenic activity. Generally, a variety of nitrated polynuclear aromatic compounds have been found that account for a substantial portion of the mutagenicity (Liberti et al., 1984; Schuetzle and Frazer, 1986; Schuetzle and Perez, 1983). However, not all bacterial mutagenicity has been identified in this way, and the identity of the remainder of the mutagenic compounds remains unknown. The nitrated aromatics thus far identified in diesel exhaust were the subject of review in the IARC monograph on diesel exhaust (IARC, 1989).

In addition to the simple qualitative identification of mutagenic chemicals, several investigators have used numerical data to express mutagenic activity as activity per distance driven or mass of fuel consumed. These types of calculations have been the basis for estimates that the nitroarenes (both mono- and dinitropyrenes) contribute a significant amount of the total mutagenic activity of the whole extract (Nishioka et al., 1982; Salmeen et al., 1982; Nakagawa et al., 1983). In a 1983 review, Claxton discussed a number of factors that affected the mutagenic response in *Salmonella* assays. Citing the data from the Huisingh et al. (1978) study, the author noted that the mutagenic response could vary by a factor of 100 using different fuels in a single diesel engine. More recently, Crebelli et al. (1995) used *Salmonella* to examine the effects of different fuel components. They reported that although mutagenicity was highly dependent on aromatic content, especially di- or triaromatics, there was no clear effect of sulfur content of the fuel. Later, Sjögren et al. (1996), using multivariate statistical methods with ten diesel fuels, concluded that the most influential chemical factors in *Salmonella* mutagenicity were sulfur contents, certain PAHs (1-nitropyrene), and naphthenes.

Matsushita et al. (1986) tested particle-free diesel exhaust gas and a number of benzene nitro-derivatives and PAHs (many of which have been identified as components of diesel exhaust gas). The particle-free exhaust gas was positive in both TA100 and TA98, but only without S9 activation. Of the 94 nitrobenzene derivatives tested, 61 were mutagenic, and the majority showed greatest activity in TA100 without S9. Twenty-eight of 50 PAHs tested were mutagenic, all required the addition of S9 for detection, and most appeared to show a stronger response in TA100. When 1,6-dinitropyrene was mixed with various PAHs or an extract of heavy-duty (HD) diesel exhaust, the mutagenic activity in TA98 was greatly reduced when S9 was absent but was increased significantly when S9 was present. These latter results suggest that caution should be used in estimating mutagenicity (or other toxic effects) of complex mixtures from the specific activity of individual components.

Mitchell et al. (1981) reported mutagenic activity of DPM extracts of diesel emissions in the mouse lymphoma L5178Y mutation assay. Positive results were seen both with and without S9 activation in extracts from several different vehicles, with mutagenic activity only slightly lower in the presence of S9. These findings have been confirmed in a number of other mammalian cell systems using several different genetic markers. Casto et al. (1981), Chescheir et al. (1981), Li and Royer (1982), and Brooks et al. (1984) all reported positive responses at the HPRT locus in Chinese hamster ovary (CHO) cells. Morimoto et al. (1986) used the APRT and Oua^r loci in CHO cells; Curren et al. (1981) used Oua^r in BALB/c 3T3 cells. In all of these studies, mutagenic activity was observed without S9 activation. Liber et al. (1981) used the thymidine kinase (TK) locus in the TK6 human lymphoblast cell line and observed induced mutagenesis only in the presence of rat liver S9 when testing a methylene chloride extract of diesel exhaust. Barfknecht et al. (1982) also used the TK6 assay to identify some of the chemicals responsible for this activation-dependent mutagenicity. They suggested that fluoranthene, 1-methylphenanthrene, and 9-methylphenanthrene could account for more than 40% of the observed activity.

Morimoto et al. (1986) injected DPM extracts (250 to 4,000 mg/kg) into pregnant Syrian hamsters and measured mutations at the APRT locus in embryo cells cultivated 11 days after i.p. injection. Neutral fractions from both light-duty (LD) and HD tar samples resulted in increased mutation frequency at 2,000 and 4,000 mg/kg. Belisario et al. (1984) applied the Ames test to urine from Sprague-Dawley rats exposed to single applications of DPM administered by gastric intubation, i.p. injection, or s.c. gelatin capsules. In all cases, dose-related increases were seen in TA98 (without and with S9) from urine concentrates taken 24 h after particle administration. Urine from Swiss mice exposed by inhalation to filtered exhaust (particle concentration 6 to 7 mg/m^3) for 7 weeks (Pereira et al., 1981a), or Fischer 344 rats exposed to DPM (2 mg/m^3) for 3 months to 2 years was negative in *Salmonella* strains.

Schuler and Niemeier (1981) exposed *Drosophila* males in a stainless steel chamber connected to the 3-m^3 chamber used for the chronic animal studies at EPA (see Hinners et al., 1980, for details). Flies were exposed for 8 h and mated to untreated females 2 days later. Although the frequency of sex-linked recessive lethals from treated males was not different from that of controls, the limited sample size precluded detecting less than a threefold increase over controls. The authors noted that, because there were no signs of toxicity, the flies might tolerate exposures to higher concentrations for longer time periods.

Driscoll et al. (1996) exposed Fischer 344 male rats to aerosols of carbon black (1.1, 7.1 and 52.8 mg/m^3) or air for 13 weeks (6 h/day, 5 days/week) and measured *hprt* mutations in alveolar type II cells in animals immediately after exposure and at 12 and 32 weeks after the end of exposure. Both the two higher concentrations resulted in significant increases in mutant frequency. Whereas the mutant frequency from the 7.1 mg/m^3 group returned to control levels by

12 weeks, the mutant frequency of the high-exposure group was still higher than controls even after 32 weeks. Carbon black particles have very little adsorbed PAHs, hence a direct chemically induced mechanism is highly unlikely. Induction of *hprt* mutations were also observed in rat alveolar epithelial cells after intratracheal instillation with carbon black, α-quartz and titanium dioxide (Driscoll et al., 1997). All three types of particles elicited an inflammatory response, as shown by significant increases of neutrophils in bronchalveolar lavage (BAL) fluid. Culturing the BAL from exposed rats with a rat lung epithelial cell line also resulted in elevation of *hprt* mutational response. This response was effectively eliminated when catalase was included in the incubation mixture, providing evidence for cell-derived oxidative damage.

Specific-locus mutations were not induced in (C3H × 101)F_1 male mice exposed to diesel exhaust 8 h/day, 7 days/week for either 5 or 10 weeks (Russell et al., 1980). The exhaust was a 1:18 dilution and the average particle concentration was 6 mg/m^3. After exposure, males were mated to T-stock females and matings continued for the reproductive life of the males. The results were unequivocally negative; no mutants were detected in 10,635 progeny derived from postspermatogonial cells or in 27,917 progeny derived from spermatogonial cells.

Hou et al. (1995) measured DNA adducts and *hprt* mutations in 47 bus maintenance workers and 22 control individuals. All were nonsmoking men from garages in the Stockholm area and the exposed group consisted of 16 garage workers, 25 mechanics, and 6 others. There were no exposure data but the three groups were considered to be of higher to lower exposure to diesel engine exhaust. Levels of DNA adducts determined by ^{32}P-postlabeling were significantly higher in workers than controls (3.2 versus 2.3 × 10^{-8}), but *hprt* mutant frequencies were not different (8.6 versus 8.4 × 10^{-6}). Both adduct level and mutagenicity were highest among the 16 most exposed; mutant frequency was significantly correlated with adduct level. All individuals were genotyped for glutathione transferase GSTM1 and aromatic amino transferase NAT2 polymorphism. Neither GSTM1 nulls nor NAT2 slow acetylators exhibited effects on either DNA adducts or *hprt* mutant frequencies.

4.2. CHROMOSOME EFFECTS

Mitchell et al. (1981) and Brooks et al. (1984) reported increases in sister chromatid exchanges (SCE) in CHO cells exposed to DPM extracts of emissions from both LD and HD diesel engines. Morimoto et al. (1986) observed increased SCE from both LD and HD DPM extracts in PAH-stimulated human lymphocyte cultures. Tucker et al. (1986) exposed human peripheral lymphocyte cultures from four donors to direct diesel exhaust for up to 3 h. Exhaust was cooled by pumping through a plastic tube about 20 feet long; airflow was 1.5 L/min. Samples were taken at 16, 48, and 160 min of exposure. Cell cycle delay was observed in all cultures; significantly increased SCE levels were reported for two of the four cultures. Structural

chromosome aberrations were induced in CHO cells by DPM extracts from a Nissan diesel engine (Lewtas, 1983) but not by similar extracts from an Oldsmobile diesel engine (Brooks et al., 1984).

DPM dispersed in an aqueous mixture containing dipalmitoyl lecithin (DPL), a component of pulmonary surfactant or extracted with dichloromethane (DCM), induced similar responses in SCE assays in Chinese hamster V79 cells (Keane et al., 1991), micronucleus tests in V79 and CHO cells (Gu et al., 1992) and unscheduled DNA synthesis (UDS) in V79 cells (Gu et al., 1994). After separating the samples into supernatant and sediment fractions, mutagenic activity was confined to the sediment fraction of the DPL sample and the supernatant of the DCM sample. These findings suggest that the mutagenic activity of DPM inhaled into the lungs could be made bioavailable through solubilization and dispersion nature of pulmonary surfactants. In a later study in the same laboratory, Liu et al. (1996) found increased micronuclei in V79 cells treated with crystalline quartz and a noncrystalline silica, but response was reduced after pretreatment of the particles with the simulated pulmonary surfactant.

Pereira et al. (1981a) exposed female Swiss mice to diesel exhaust 8 h/day, 5 days/week for 1, 3, and 7 weeks. The incidence of micronuclei and structural aberrations was similar in bone marrow cells of both control and exposed mice. Increased incidences of micronuclei, but not SCE, were observed in bone marrow cells of male Chinese hamsters after 6 months of exposure to diesel exhaust (Pereira et al., 1981b).

Guerrero et al. (1981) observed a linear concentration-related increase in SCE in lung cells cultured after intratracheal instillation of DPM at doses up to 20 mg/hamster. However, they did not observe any increase in SCE after 3 months of inhalation exposure to diesel exhaust particles (6 mg/m^3).

Pereira et al. (1982) measured SCE in embryonic liver cells of Syrian hamsters. Pregnant females were exposed to diesel exhaust (containing about 12 mg/m^3 particles) from days 5 to 13 of gestation or injected intraperitoneally with diesel particles or particle extracts on gestational day 13 (18 h before sacrifice). Neither the incidence of SCE nor mitotic index was affected by exposure to diesel exhaust. The injection of DPM extracts but not DPM resulted in a dose-related increase in SCE; however, the toxicity of the DPM was about twofold greater than the DPM extract.

In the only studies with mammalian germ cells, Russell et al. (1980) reported no increase in either dominant lethals or heritable translocations in males of T-stock mice exposed by inhalation to diesel emissions. In the dominant lethal test, T-stock males were exposed for 7.5 weeks and immediately mated to females of different genetic backgrounds (T-stock; [C3H × 101]; [C3H × C57BL/6]; [SEC × C57BL/6]). There were no differences from controls in any of the parameters measured in this assay. For heritable translocation analysis, T-stock males were exposed for 4.5 weeks and mated to (SEC × C57BL/6) females, and the F_1 males were tested for

the presence of heritable translocations. Although no translocations were detected among 358 progeny tested, the historical control incidence is less than 1/1,000.

4.3. OTHER GENOTOXIC EFFECTS

Pereira et al. (1981b) exposed male strain A mice to diesel exhaust emissions for 31 or 39 weeks using the same exposure regimen noted in the previous section. Analyses of caudal sperm for sperm-head abnormalities were conducted independently in three separate laboratories. Although the incidence of sperm abnormalities was not significantly above controls in any of the three laboratories, there were extremely large differences in scoring among the three (control values were 9.2%, 14.9%, and 27.8% in the three laboratories). Conversely, male Chinese hamsters exposed for 6 months (Pereira et al., 1981c) exhibited almost a threefold increase in sperm-head abnormalities. It is noted that the control incidence in the Chinese hamsters was less than 0.5%. Hence, it is not clear whether the differing responses reflect true species differences or experimental artifacts.

A number of studies measuring DNA adducts in animals exposed to DPM, carbon black, or other particles have been reported and are reviewed by Shirnamé-Moré (1995). Although modest increases in DNA adducts have been observed in lung tissue of rats after inhalation of DPM (Wong et al., 1986; Bond et al., 1990), the increases are small in comparison with those induced by chemical carcinogens present in diesel exhaust (Smith et al., 1993). While Gallagher et al. (1994) found no increases in total DNA adducts in lung tissue of rats exposed to diesel exhaust, carbon black or titanium dioxide, they did observe an increase in an adduct with migration properties similar to nitrochrysene and nitro-benzo(a)pyrene adducts from diesel but not carbon black or titanium dioxide exposures. The majority of the studies used the ^{32}P-postlabeling assay to detect adducts. Although this method is sensitive, chemical identity of adducts can only be inferred if an adduct spot migrates to the same location as a known prepared adduct.

DNA adducts have also been measured in humans occupationally exposed to diesel exhaust. Distinct adduct patterns were found among garage workers occupationally exposed to diesel exhaust when compared with nonexposed controls (Nielsen and Autrup, 1994). Furthermore, the findings were concordant with the adduct patterns observed in groups exposed to low concentrations of PAHs from combustion processes. Hemminki et al. (1994) also reported significantly elevated levels of DNA adducts in lymphocytes from garage workers with known diesel exhaust exposure compared with unexposed mechanics. Hou et al. (1995) found elevated adduct levels in bus maintenance workers exposed to diesel exhaust. Although no difference in mutant frequency was observed between the groups, the adduct levels were significantly different (3.2 vs. 2.3×10^{-8}). Nielsen et al. (1996) reported significantly increased levels of three

biomarkers (lymphocyte DNA adducts, hydroxyethylvaline adducts in hemoglobin, and 1-hydroxypyrene in urine) in DE-exposed bus garage workers.

The role of oxidative damage in causing mutations has received increasing focus recently. More than 50 different chemicals have been studied in rodents, usually measuring the formation of 8-hydroxydeoxyguanosine (8-OH-dG), a highly mutagenic adduct (Loft et al., 1998). Increases in the mutagenic DNA adduct 8-hydroxydeoxyguanosine were found in mouse lung DNA after intratracheal instillation of diesel particles (Nagashima et al., 1995). The response was dose dependent. Mice fed on a high-fat diet showed an increased response whereas the responses were partially reduced when the antioxidant β-carotene was included in the diet (Ichinose et al., 1997). Oxidative damage has also been measured in rat lung tissue after intratracheal instillation of quartz (Nehls et al., 1997) and in rat alveolar macrophages after in vitro treatment with silica dust (Zhang et al., 2000). Arimoto et al. (1999) demonstrated that redissolved methanol extracts of DPM also induced the formation of 8-OH-dG adducts in L120 mouse cells. The response was dependent on both DPM concentration and P450 reductase. A detailed discussion of the potential role of oxidative damage in diesel exhaust carcinogenesis is presented in Chapter 7.4.

4.4. SUMMARY

Extensive studies with *Salmonella* have unequivocally demonstrated mutagenic activity in both particulate and gaseous fractions of diesel exhaust. In most of the studies using *Salmonella,* DPM extracts and individual nitropyrenes exhibited the strongest responses in strain TA98 when no exogenous activation was provided. Gaseous fractions reportedly showed greater response in TA100, whereas benzo(a)pyrene and other unsubstituted PAHs are mutagenic only in the presence of S9 fractions. The induction of gene mutations has been reported in several in vitro mammalian cell lines after exposure to extracts of DPM. Note that only the TK6 human cell line did not give a positive response to DPM extracts in the absence of S9 activation. Mutagenic activity was recovered in urine from animals treated with DPM by gastric intubation and i.p. and s.c. implants, but not by inhalation of DPM or diluted diesel exhaust. Dilutions of whole diesel exhaust did not induce sex-linked recessive lethals in *Drosophila* or specific-locus mutations in male mouse germ cells.

Structural chromosome aberrations and SCE in mammalian cells have been induced by particles and extracts. Whole exhaust induced micronuclei but not SCE or structural aberrations in bone marrow of male Chinese hamsters exposed to whole diesel emissions for 6 months. In a shorter exposure (7 weeks), neither micronuclei nor structural aberrations were increased in bone marrow of female Swiss mice. Likewise, whole diesel exhaust did not induce dominant lethals or heritable translocations in male mice exposed for 7.5 and 4.5 weeks, respectively.

The application of mutagenicity data to the question of the potential carcinogenicity of diesel emissions is based on the premise that genetic alterations are found in all cancers and that several of the chemicals found in diesel emissions possess mutagenic activity in a variety of genetic assays. These genetic alterations can be produce by gene mutations, deletions, translocations, aneuploidy, or amplification of genes, hence no single genotoxicity assay should be expected to either qualitatively or quantitatively predict rodent carcinogenicity. With diesel emissions or other mixtures, additional complications arise because of the complexity of the material being tested. Exercises that combined the *Salmonella* mutagenic potency with the total concentration of mutagenic chemicals deposited in the lungs could not account for the observed tumor incidence in exposed rats (Rosenkranz, 1993; Goldstein et al., 1998). However, such calculations ignored the contribution of gaseous-phase chemicals which have been estimated to contribute from less than 50% (Rannug et al., 1983) to over 90% (Matsushita et al., 1986) of the total mutagenicity. This wide range is partly reflective of the differences in material tested: semivolatile extracts in the former and whole gaseous emission in the latter. Of greater importance is that these calculations are based on a reverse mutation assay in bacteria with metabolic processes strikingly different from mammals. This is at least partly reflected in the observations that different nitro-PAHs give different responses in bacteria and in CHO cells (Li and Dutcher, 1983) or in human hepatoma-derived cells (Eddy et al., 1986).

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