

DIESEL AND GASOLINE ENGINE EXHAUSTS AND SOME NITROARENES

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4. MECHANISTIC AND OTHER RELEVANT DATA

4.1 Overview of the mechanisms of carcinogenesis of polycyclic aromatic hydrocarbons

The chemical exposures of greatest concern are to polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs with which diesel engine exhaust particles may be impregnated and to PAHs that are present in gasoline engine exhaust. Mechanistic and other relevant data associated with exposure to individual nitro-PAHs are reviewed elsewhere in this Volume (see Sections 4 in the individual *Monographs*). Exposure to PAHs is associated with an increased risk of cancer and was recently reviewed in the *IARC Monographs* ([IARC, 2010a](#)) and comprehensive reviews by the Agency of Toxic Substances and Disease Registry ([ATSDR, 1995](#)) and the International Programme on Chemical Safety ([IPCS, 1998](#)). A major finding of [IARC \(2010a\)](#) was the reclassification of the representative PAH, benzo[*a*]pyrene, as a Group 1 or known human carcinogen. Benzo[*a*]pyrene is the most extensively studied PAH, for which an exhaustive set of literature exists. Its reclassification as a known human carcinogen considered epidemiological data, data on metabolism, bioactivation, mutagenicity and tumorigenicity, and the existence of a mode of action or plausible mechanism that accounts for the end-points observed. Many of these data are relevant to the risk assessment of other PAHs found in diesel

and gasoline engine exhausts, which contain many of the 16 Environmental Protection Agency (EPA) priority pollutants, e.g. naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[*1,2,3-cd*]pyrene, benzo[*ghi*]perylene and dibenz[*a,h*]anthracene. An examination of the structure–activity relationships of these compounds showed that four or more of their fused benzene rings need to create an indentation known as a bay-region for the compound to be a potent carcinogen. Substitution of the bay-region by a methyl group or the introduction of another benzene ring to create a *fiord*-region often increases the carcinogenicity of the PAH. A summary of the mechanistic data that support the role of benzo[*a*]pyrene as a human carcinogen is presented below, and other PAHs present in diesel and gasoline engine exhausts are discussed in this context.

4.1.1 Mechanistic data to support the role of benzo[*a*]pyrene as a human carcinogen

(a) Toxicokinetics

PAHs, including benzo[*a*]pyrene, are present in airborne fine particles, and absorption occurs via the respiratory tract, as shown by studies on [¹⁴C]benzo[*a*]pyrene-labelled particles ([Withey](#)

[et al., 1993](#); [ATSDR, 1995](#); [IPCS, 1998](#)). Because of their lipophilic character, PAHs can be cleared by the epithelial cell mucillary elevator which can lead to ingestion via the gastrointestinal tract ([Withey et al., 1994](#)). Benzo[*a*]pyrene is a complete multiorgan and multispecies carcinogen and forms tumours in the lungs of newborn mice after intraperitoneal administration ([Kapitulnik et al., 1978](#)), in A/J mouse lung after intraperitoneal administration ([Nesnow et al., 1995, 1998](#)), in mouse skin after topical application ([Levin et al., 1977](#)) and in the murine forestomach and gastrointestinal tract after oral administration ([Wattenberg et al., 1980](#); [Culp & Beland, 1994](#)). Thus, many of the sites of tumour formation in rodents correspond to sites of human exposure to diesel and gasoline engine exhausts.

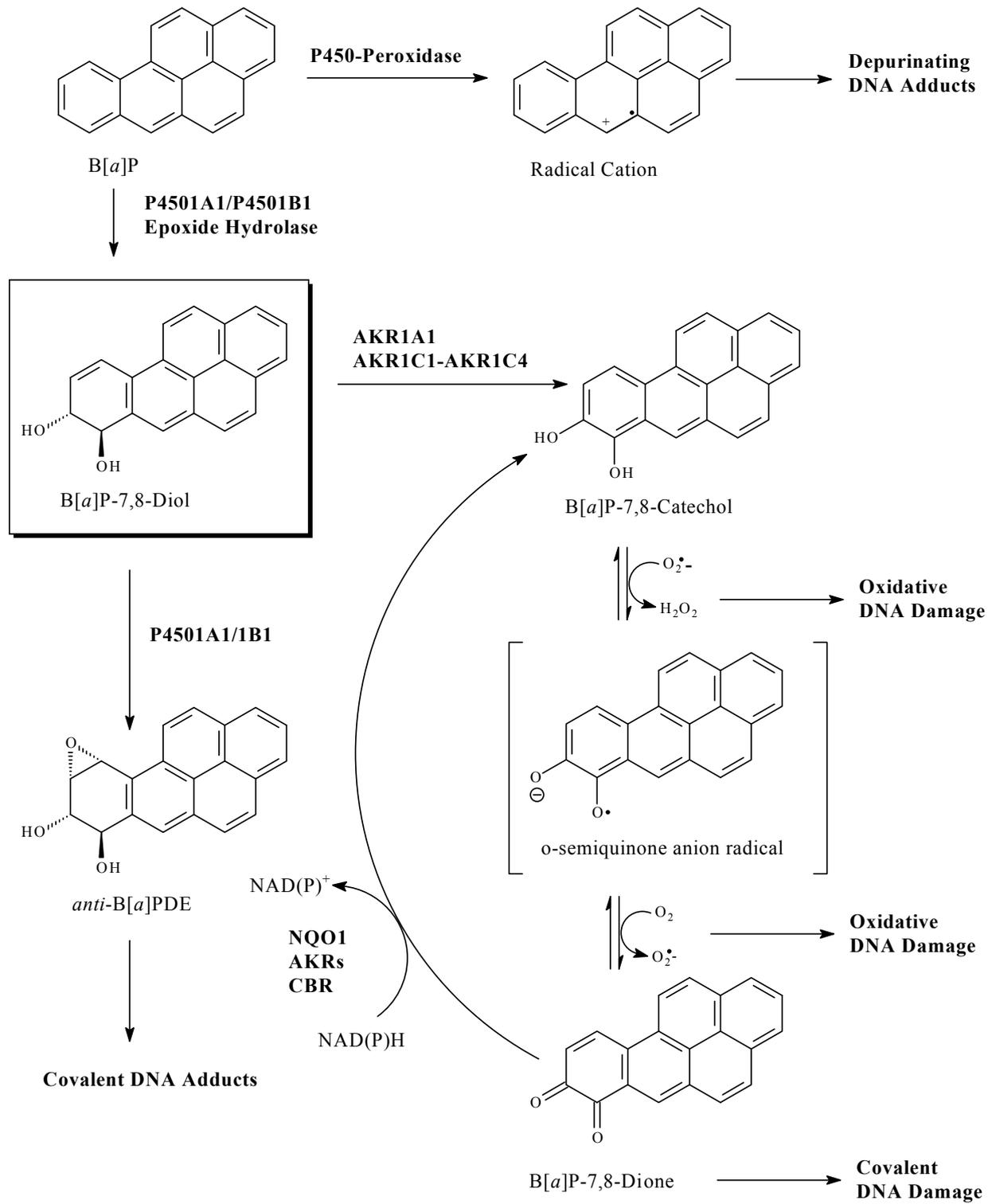
PAHs are metabolically inert and must be metabolized before both their elimination and activation can occur; the balance of these events determines the carcinogenicity of PAHs in a given species and at a given organ site. For benzo[*a*]pyrene and other PAHs, a general consensus is that phase I enzymes – cytochrome P450 (CYP) and aldo-keto reductases (AKRs) – form phenols, dihydrodiols and catechols, and that each of these metabolites are then available for conjugation by phase II enzymes, leading to elimination. The phase II enzymes of interest are the sulfotransferases (SULTs) and the uridine glucuronosyl transferases (UGTs), while catechols are uniquely conjugated by catechol-*O*-methyl transferase. In each instance, the conjugate is eliminated by excretion. In addition, phase I metabolism to epoxides, diol epoxides and quinones, which are biologically reactive intermediates, may occur, and, if they are not conjugated with glutathione by glutathione *S*-transferases (GSTs), they can react with DNA, RNA and protein. These detoxication and toxication pathways of benzo[*a*]pyrene have been studied extensively *in vitro* and *in vivo* in mouse, rat and human subcellular fractions and tissues, and in cell lines. The metabolic pathways observed appear to be consistent

([Gelboin, 1980](#); [Conney, 1982](#)). Features that can determine the balance of detoxication and activation are the induction of phase I and phase II enzymes, and the existence of polymorphic variants in enzyme isoforms that can determine individual susceptibility to exposure to PAHs. The mechanisms of phase I and phase II enzyme induction are discussed elsewhere. For a discussion of the polymorphic variants of the major enzyme families involved in the metabolism of PAHs, the reader is referred to [IARC \(2010a\)](#). The pathways that lead to the metabolic activation of benzo[*a*]pyrene and the production of genotoxic species are discussed below.

(b) *Metabolic pathways of activation*

Benzo[*a*]pyrene is a pro-carcinogen that is metabolically activated to electrophilic species, which can react with bases in DNA to form DNA adducts. When these adducts persist, they give rise to mutations and, when these mutations activate proto-oncogenes or deactivate tumour-suppressor genes, the cell is altered in a manner consistent with the hallmarks of cancer, as described by [Hanahan & Weinberg \(2011\)](#). The mutations of most interest are G→T transversions, which are the dominant mutations observed in the genes most commonly mutated in human lung cancer, e.g. *K-ras* and *p53*, and any molecular mechanism that explains the carcinogenicity of PAHs must provide a process that leads to these mutations. Three pathways have been proposed for the metabolic activation of benzo[*a*]pyrene that can lead to DNA adducts that can ultimately induce G→T transversions ([Fig. 4.1](#)). These include the diol epoxide pathway (mediated by CYP isozymes and epoxide hydrolase), the radical cation pathway (mediated by CYP peroxidase activity or other peroxidases) and the *ortho*-quinone pathway (mediated by AKRs following the action of CYP and epoxide hydrolase). An additional pathway involving L-region mesomethylation, e.g. the conversion of benzo[*a*]pyrene to 6-methyl-benzo[*a*]pyrene

Fig. 4.1 Three pathways of metabolic activation of benzo[a]pyrene



AKR, aldo-keto reductase; B[a]P, benzo[a]pyrene; CBR, carbonyl reductase; NAD(P)H/NAD(P)⁺, nicotinamide adenine dinucleotide phosphate; NQO1, NADPH quinone oxidoreductase; P450, cytochrome P450

followed by hydroxylation and sulfation, was evaluated by IARC but no compelling evidence was found to support this mechanism (IARC, 2010a), and this pathway is not discussed further.

(i) *Diol epoxides*

Overwhelming evidence shows that benzo[*a*]pyrene and other carcinogenic PAHs are metabolically activated to diol epoxide ultimate carcinogens (Gelboin, 1980; Conney, 1982), which can account for the observation that bay-region PAHs are potent carcinogens. In this pathway, the principal CYP enzymes involved are CYP1A1 (ubiquitous) and CYP1B1 (extrahepatic), although others may play a minor role, e.g. CYP1A2 (Shimada *et al.*, 1996, 1999, 2001). These monooxygenases form an arene oxide on the terminal benzo-ring of benzo[*a*]pyrene to form the major 7*R*,8*S*,-benzo[*a*]pyrene-7,8-oxide and minor 7*S*,8*R*-benzo[*a*]pyrene-7,8-oxide, which, in the presence of epoxide hydrolase, are converted to (-)*R,R*,-7,8-dihydro-7,8-dihydroxy-benzo[*a*]pyrene [(-)benzo[*a*]pyrene-7,8-dihydrodiol] and (+)*S,S*,-7,8-dihydro-7,8-dihydroxy-benzo[*a*]pyrene [(+)benzo[*a*]pyrene-7,8-dihydrodiol], respectively. Each non-*K*-region benzo[*a*]pyrene-7,8-dihydrodiol isomer undergoes a second round of monooxygenation to form the corresponding diol epoxide (Shimada *et al.*, 1996, 1999), (+)-*anti*-7β,8α-dihydroxy-9α,10α-oxo-7,8,9,10-tetrahydro-benzo[*a*]pyrene [(+)-*anti*-B[*a*]PDE] and (+)-*syn*-7α,8β-dihydroxy-9α,10α-oxo-7,8,9,10-tetrahydro-benzo[*a*]pyrene [(+)-*syn*-B[*a*]PDE], respectively (Thakker *et al.*, 1976). These diol epoxides can undergo hydrolysis with either *cis* or *trans* ring-opening which gives rise to four stereoisomeric tetraols. Alternatively, they can undergo nucleophilic attack at the C10 position by either a guanine or adenine DNA base to form either 8-stereoisomeric *N*²-deoxyguanosine adducts or 8-stereoisomeric *N*⁶-deoxyadenosine adducts, resulting in a total of 16 possible adducts (Jennette *et al.*, 1977; Jeffrey *et al.*, 1979). The major pathway

proceeds through the (-)benzo[*a*]pyrene-7,8-dihydrodiol and the major DNA adduct formed is the (+)-*anti-trans*-B[*a*]PDE-*N*²-deoxyguanosine adduct [(+)-*anti*-B[*a*]PDE-dG] (Jennette *et al.*, 1977). This stereochemistry of metabolic activation is an important consideration when comparing other pathways that may be involved.

Compelling evidence demonstrates that (+)-*anti*-B[*a*]PDE is an ultimate carcinogen, and is produced metabolically in target tissues that develop tumours (Melikian *et al.*, 1987). (+)-*Anti*-B[*a*]PDE adducts have been detected at sites of tumour formation: in mouse skin using [³H]benzo[*a*]pyrene as a precursor followed by isolation of the DNA adducts by digestion and their co-chromatography with standards of (+)-*anti*-B[*a*]PDE-dG (Koreeda *et al.*, 1978) and by ³²P-postlabelling (Bodell *et al.*, 1989; Suh *et al.*, 1995); in A/J mouse lung using ³²P-postlabelling approaches (Mass *et al.*, 1993); in the lung and forestomach of mice (Adriaenssens *et al.*, 1983); and in human bronchial and colon explants using [³H]benzo[*a*]pyrene as a precursor followed by reversed phase high-performance liquid chromatography (HPLC) of the adducts obtained from DNA digests (Jeffrey *et al.*, 1977; Autrup *et al.*, 1978). (+)-*Anti*-B[*a*]PDE-dG adducts have also been detected in a variety of human cell lines by ³²P-postlabelling (Li *et al.*, 1996) and quantified by stable-isotope dilution liquid chromatography/mass spectrometry (SID-LC/MS) in human lung tissues (Beland *et al.*, 2005) and in human bronchoalveolar (H358) cells (Ruan *et al.*, 2006, 2007). These adducts can be repaired by nucleotide-excision repair or by transcription-coupled repair. If they persist, (+)-*anti*-B[*a*]PDE-dG adducts can undergo translesional synthesis by specific by-pass DNA polymerases to produce G→T transversions (Zhao *et al.*, 2006; Choi *et al.*, 2011). Many of these DNA polymerases show low fidelity and low processivity; thus, once the lesion is by-passed, the replication complex becomes stalled until the by-pass polymerase is substituted with replicative DNA polymerase II

(Choi *et al.*, 2011). This route to G→T transversions is considered to be less than straightforward because it requires multiple steps.

(+)-Anti-B[a]PDE is the most mutagenic of the known benzo[a]pyrene metabolites in the Ames test (Malaveille *et al.*, 1977) and is mutagenic in mammalian mutagenicity assays in Chinese hamster ovary cells or fibroblasts that express the *hypoxanthine-guanine phosphoribosyltransferase* (HPRT) gene (MacLeod *et al.*, 1988; Chen *et al.*, 1990; Wei *et al.*, 1993). (+)-Anti-B[a]PDE is also the most tumorigenic of the known benzo[a]pyrene metabolites in the lung of newborn mice (Buening *et al.*, 1978; Kapitulnik *et al.*, 1978) but it is less tumorigenic than benzo[a]pyrene in mouse skin (Slaga *et al.*, 1977). (+)-Anti-B[a]PDE can mutate *H-ras* at codons 12 and 61 which activates this proto-oncogene and leads to the transformation of NIH3T3 cells (Marshall *et al.*, 1984); it preferentially forms DNA adducts in 'hot-spots' in the *p53* tumour-suppressor gene which correspond to codons mutated in tumours from lung cancer patients (Denissenko *et al.*, 1996; Hainaut & Pfeifer, 2001).

Ample evidence indicates that this pathway of metabolic activation and hence adduct formation occurs in humans. Phenanthrene is similar to benzo[a]pyrene because it contains a bay-region and can be metabolically transformed to a diol epoxide that can be hydrolysed to a tetraol. Biomonitoring studies on the urine of smokers has led to the detection of phenanthrene tetraols as a surrogate biomarker of exposure to PAHs. More recently, benzo[a]pyrene tetraols have been detected as exposure biomarkers in the urine of smokers using SID-LC/MS (Hecht *et al.*, 2010; Hochalter *et al.*, 2011). (+)-Anti-B[a]PDE-dG-adducts have also been detected in human maternal and umbilical white blood cells following exposure to air pollution using enzyme-linked immunosorbent assay-based methods (Whyatt *et al.*, 1998; Santella, 1999). Using SID-LC/MS, it was found that (+)-anti-B[a]PDE-dG adducts in human lung tissue from 26

donors with different smoking histories contributed to only a small portion of adducts that were measured by ³²P-postlabelling (Beland *et al.*, 2005). These studies showed that other stable, bulky DNA adducts arise in lung tissue due to cigarette smoke, including (+)-anti-B[a]PDE-dG adducts.

Other PAHs have been shown to be activated to diol epoxides and account for either the mutagenic and/or tumorigenic properties of the parent hydrocarbon from which they are derived. A key feature of this generalized mechanism is the formation of a resonance-stabilized carbocation following epoxide ring-opening, which is stabilized further by the inductive group of a bay-region methyl group or *fford*-region benzo group, as originally proposed and predicted by Jerina *et al.* (1976, 1986). The following PAHs are activated to diol epoxides which react with DNA to form diol epoxide-DNA adducts: benz[a]anthracene is converted to benz[a]anthracene-3,4-diol-1,2-oxide (Cooper *et al.*, 1980); benzo[g]chrysene is activated to benzo[g]chrysene-11,12-diol-13,14-oxide (Giles *et al.*, 1996; Agarwal *et al.*, 1997); benzo[b]fluoranthene is converted to benzo[b]fluoranthene-9,10-diol-11,12-oxide (Ross *et al.*, 1992; Weyand *et al.*, 1993); benzo[j]fluoranthene is activated to benzo[j]fluoranthene-4,5-diol-6,6a-oxide and the 9,10-diol-11,12-oxide (LaVoie *et al.*, 1980); benzo[c]phenanthrene is converted to the benzo[c]phenanthrene-3,4-diol-1,2-oxide (Wood *et al.*, 1980); dibenz[a,h]anthracene is activated to dibenz[a,h]anthracene-3,4-diol-1,2-oxide and can be converted to a *bis*-diol-epoxide because it has two bay-regions (Platt *et al.*, 1990); dibenz[a,j]anthracene is activated to the dibenz[a,j]anthracene-3,4-diol-1,2-oxide; dibenzo[a,h]pyrene is converted to dibenzo[a,h]pyrene-1,2-diol-3,4-oxide; dibenzo[a,l]pyrene is activated to dibenzo[a,l]pyrene-11,12-diol-13,14-oxide (Luch *et al.*, 1997, 1999); and 5-methylchrysene is activated to 5-methylchrysene-7,8-diol-9,10-oxide and 5-methylchrysene-1,2-diol-3,4-oxide (Melikian *et al.*, 1983; Koehl *et al.*, 1996). Of these

PAHs, chrysene, benz[*a*]anthracene and dibenzo[*a,l*]pyrene, as well as benzo[*a*]pyrene, belong to the 16 EPA priority PAHs found in air pollution which arise from incomplete combustion and are probably present in diesel or gasoline engine exhaust. Thus, the weight of the evidence suggests that many PAHs are activated to reactive diol epoxides that form DNA adducts which give rise to mutations, and that this mechanism is responsible for the initiation of cancer.

(ii) *Radical cations*

In the radical cation pathway, CYP peroxidases or other peroxidases use benzo[*a*]pyrene as a co-reductant. The pathway does not require a bay-region in the parent PAH but is governed by the ionization potential (the ease at which an already electron-deficient PAH will donate an electron) (Cavalieri & Rogan, 1985, 1995). Peroxidases require protoporphyrin IX and, when a peroxide (ROOH) is cleaved to yield an alcohol and water, the equivalent of iron(V⁺) is produced (compound I) which is reduced back to iron(III⁺) in the presence of a reductant. Benzo[*a*]pyrene can be a source of these electrons and generate a radical cation at C6. This reactive radical can form unstable C8-guanine [8-(benzo[*a*]pyren-6-yl)guanine], N7-guanine [7-benzo[*a*]pyren-6-yl)guanine] and N7-adenine [7-benzo[*a*]pyren-6-yl)adenine] depurinating adducts. These adducts have been detected *in vitro* following the incubation of benzo[*a*]pyrene with hydrogen peroxide horseradish peroxidase (Rogan *et al.*, 1988) or the incubation of 3-methylcholanthrene-induced rat liver microsomes with benzo[*a*]pyrene, DNA and cumene hydroperoxide (Cavalieri *et al.*, 1990), *in vivo* in mouse skin following the topical application of benzo[*a*]pyrene (Chen *et al.*, 1996) and in the urine of smokers (Casale *et al.*, 2001). These depurinating adducts give rise to abasic sites, and DNA polymerases most frequently insert an A opposite an abasic site (Sagher & Strauss, 1983; Shibutani *et al.*, 1997) providing a straightforward route

to the G→T transversions found in *K-ras* and *p53* in human lung cancers. The radical cation pathway could explain the higher tumorigenicity of benzo[*a*]pyrene than that of (+)-*anti*-B[*a*]PDE in mouse skin (Slaga *et al.*, 1977) and would provide an explanation for the tumorigenicity of 1,2,3,4-tetrahydro-7,12-dimethylbenz[*a*]anthracene (DiGiovanni *et al.*, 1982), which is incapable of forming a diol epoxide. If the radical cation is not intercepted by bases in DNA, it can undergo hydroxylation and/or subsequent air oxidation to produce the remote quinones, benzo[*a*]pyrene-1,6-dione, benzo[*a*]pyrene-3,6-dione and benzo[*a*]pyrene-6,12-dione (Lesko *et al.*, 1975; Cavalieri *et al.*, 1988). These diones can undergo one-electron and two-electron redox cycling to produce reactive oxygen species (ROS) (Chesis *et al.*, 1984). The one-electron pathway leads to mutagenesis in the Ames test with a fortified exogenous metabolic activation system and is unaffected by dicoumarol but is blocked by catalase and super oxide dismutase, indicating that ROS are the culprit mutagens (Chesis *et al.*, 1984). In subsequent experiments, it was shown by ³²P-postlabelling that benzo[*a*]pyrene-3,6-dione produced stable covalent benzo[*a*]pyrene-3,6-dione-dG adducts provided that CYP1A1 and CYP-nicotinamide adenine dinucleotide phosphate (NADPH) oxidoreductase were present but that this was suppressed by the presence of NADPH quinone oxidoreductase 1 (NQO1), suggesting that the one-electron-reduced semiquinone radical was responsible for the adducts (Joseph & Jaiswal, 1994).

Similar pathways for the activation of PAHs that involved radical cations have been observed for 7,12-dimethylbenz[*a*]anthracene and dibenzo[*a,l*]pyrene in mouse skin, in which depurinating DNA adducts were shown to be responsible for the bulk of the covalent DNA adducts that were formed from the parent PAH (Devanesan *et al.*, 1990, 1993; Cavalieri *et al.*, 2005). Of the PAHs that are activated via this pathway, only

benzo[*a*]pyrene is found in diesel and gasoline engine exhausts.

(iii) *ortho*-Quinones

The third pathway is known as the *ortho*-quinone pathway (Penning *et al.*, 1999), through which (-)benzo[*a*]pyrene-7,8-*trans*-dihydrodiol and other non-K-region PAH *trans*-dihydrodiols undergo an enzymatic NAD(P)⁺-dependent oxidation catalysed by human AKRs (AKR1A1, AKR1C1–AKR1C4) to yield a ketol (Burczynski *et al.*, 1998; Palackal *et al.*, 2001, 2002). The ketol undergoes a spontaneous rearrangement to produce benzo[*a*]pyrene-7,8-catechol. Because the transformation occurs on the terminal benzo-ring, this pathway (similarly to the diol epoxide pathway) explains why the parent PAH requires the presence of a bay-region to be tumorigenic. Benzo[*a*]pyrene-7,8-catechol is air-sensitive and undergoes primary one-electron oxidation to produce the *ortho*-semiquinone radical followed by secondary one-electron oxidation to produce the *ortho*-quinone, benzo[*a*]pyrene-7,8-dione and concomitant ROS (Smithgall *et al.*, 1988; Penning *et al.*, 1996). Benzo[*a*]pyrene-7,8-dione is a reactive Michael acceptor that can form stable and depurinating DNA adducts *in vitro* (Shou *et al.*, 1993; McCoull *et al.*, 1999; Balu *et al.*, 2004, 2006). Benzo[*a*]pyrene-7,8-dione is also redox-active and, in the presence of NQO1 and AKRs, is reduced back to the corresponding catechol establishing a futile redox cycle in which ROS are amplified until cellular reducing equivalents are depleted (Shultz *et al.*, 2011).

The catalytic efficiency of an AKR to convert the *trans*-dihydrodiol to the *ortho*-quinone is several orders of magnitude lower than that of the same AKR to reduce the quinone back to the catechol, indicating that, once the *ortho*-quinone is formed, AKRs can play an important role in exacerbating the formation of ROS (Shultz *et al.*, 2011). This formation can be attenuated if the catechol is intercepted by conjugation that is catalysed by catechol-*O*-methyl transferase,

SULTs or UGTs or if the *ortho*-quinone is intercepted by conjugation that is catalysed by GST. Evidence has been obtained for the formation of 8-*O*-monomethyl-benzo[*a*]pyrene-7,8-catechol in three different human lung cell lines (H35A, A549 and HBEC-tk) (Zhang *et al.*, 2011). In addition, benzo[*a*]pyrene-7,8-dione reacts rapidly and non-enzymatically with glutathione to form 10-glutathionyl-benzo[*a*]pyrene-7,8-dione and 10-glutathionyl-benzo[*a*]pyrene-7,8-catechol (Murty & Penning, 1992).

The conversion of benzo[*a*]pyrene-7,8-*trans*-dihydrodiol to benzo[*a*]pyrene-7,8-dione, concomitant formation of intracellular ROS and the generation of 8-oxo-2'-deoxyguanosine (8-OH-dG) adducts via this pathway was recently demonstrated in human lung adenocarcinoma (A459) cells (Park *et al.*, 2008a); evidence of the entire pathway for benzo[*a*]pyrene was also found in human H358 bronchoalveolar cells (Lu *et al.*, 2011).

Benzo[*a*]pyrene-7,8-dione was found to be a *p53* mutagen at nanomolar concentrations *in vitro* only under conditions in which it can redox cycle (Yu *et al.*, 2002). In this assay, a linear correlation was observed between the frequency of *p53* mutagenicity and the formation of 8-OH-dG adducts (Park *et al.*, 2008b). The *p53* mutations observed were G→T transversions, which are consistent with the formation of 8-oxoguanine and its base mispairing with adenine. When selected for dominance, the *p53* mutations were found in 'hot-spots' in the tumours of lung cancer patients (Shen *et al.*, 2006). *In vivo*, only 8-OH-dG gives rise to G→T transversions if it is not repaired by base-excision repair. In human lung cancer, one allele of the human 8-oxoguanine glycosylase gene (*OGG1*) is deleted, suggesting that oxidative lesions are more prone to lead to mutation (Wikman *et al.*, 2000). Covalent, stable and depurinating *ortho*-quinone adducts have not yet been detected in cells or in the A/J mouse model of benzo[*a*]pyrene-induced lung cancer (Nesnow *et al.*, 2010). *In vitro*, the dominant

mutagenic lesion from this pathway is 8-OH-dG (Yu *et al.*, 2002; Park *et al.*, 2005). Furthermore, in many studies of benzo[*a*]pyrene–DNA adduct formation, the ³²P-postlabelling assay used would not have detected 8-OH-dG. Unlike the diol epoxide pathway, no evidence has shown that PAH-*ortho*-quinones are mutagenic in mammalian cells, have transformation potential or can cause tumours in rodent models.

Other PAH *trans*-dihydrodiols that are activated by this pathway are: chrysene-1,2-*trans*-dihydrodiol that is activated to chrysene-1,2-dione; 5-methyl-chrysene-1,2-*trans*-dihydrodiol that is converted to 5-methyl-chrysene-1,2-dione; benz[*a*]anthracene-3,4-*trans*-dihydrodiol that is activated to benz[*a*]anthracene-3,4-dione; 7-methylbenz[*a*]anthracene-3,4-dihydrodiol that is converted to 7-methylbenz[*a*]anthracene-3,4-dione; 12-methylbenz[*a*]anthracene-3,4-dihydrodiol that is activated to 12-methylbenz[*a*]anthracene-3,4-dione; 7,12-dimethylbenz[*a*]anthracene-3,4-dihydrodiol that is converted to 7,12-dimethylbenz[*a*]anthracene-3,4-dione; benzo[*g*]chrysene-11,12-diol that is activated to benzo[*g*]chrysene-11,12-dione; and benzo[*c*]phenanthrene-3,4-*trans*-dihydrodiol that is converted to benzo[*c*]phenanthrene-3,4-dione (Palackal *et al.*, 2001, 2002). As stereochemistry is a critical determinant of the downstream metabolism of PAHs, AKR1A1 was found to have the highest catalytic efficiency for (–) benzo[*a*]pyrene-7,8-*trans*-dihydrodiol and to be stereoselective for this major isomeric metabolite (Palackal *et al.*, 2001). In contrast, AKR1C1–AKR1C4 showed no stereoselectivity and can oxidize the racemic *trans*-dihydrodiols listed. These isoforms also display a higher catalytic efficiency than AKR1A1 for *trans*-dihydrodiols that contain either a methylated bay-region or *ford*-region (Palackal *et al.*, 2002). Of the PAHs affected by this pathway, chrysene, benz[*a*]anthracene and benzo[*a*]pyrene are found in air pollution.

(c) Induction of metabolism and/or activation

The relative contribution of these three pathways to the activation of PAHs is influenced by enzyme induction. Planar PAHs can induce their own metabolism. Compounds such as benzo[*a*]pyrene can bind to the aryl hydrocarbon receptor (AhR) (Nebert *et al.*, 1979, 1993, 2004), which is found in the cytosol and, after binding to the ligand, dissociates from heat-shock protein 90 (a chaperone protein) and translocates to the nucleus where the liganded receptor heterodimerizes with the aryl hydrocarbon nuclear translocator (Hankinson, 1995). The AhR:aryl hydrocarbon nuclear translocator heterodimer binds to the xenobiotic response element on the promoter of *CYP1A1* and *CYP1B1* genes to cause gene transcription and enzyme induction (Denison *et al.*, 1988a, b, 1989), resulting in increased monooxygenation of the parent PAH.

PAHs have been referred to as bi-functional inducers (Prochaska & Talalay, 1988), because their metabolism frequently results in the production of electrophiles that can then activate the Nrf2 (transcription factor)-Keap1 (negative regulator) system (Itoh *et al.*, 1997, 1999), which is also activated by other electrophiles, redox-active compounds, heavy metals and ROS (Dinkova-Kostova *et al.*, 2005). This activation leads to the dissociation of Nrf2 from the electrophilic/ROS sensor Keap1 followed by its translocation to the nucleus where it heterodimerizes with small c-Maf proteins (Dinkova-Kostova *et al.*, 2005). The Nrf2:small c-Maf complex then binds to the antioxidant response element in the promoter regions of responsive genes (Rushmore *et al.*, 1991; Nguyen *et al.*, 2003). The relevant genes in humans that are regulated by an antioxidant response element are: γ -glutamyl cysteine synthase, *NQO1*, *AKR1C1*–*AKR1C3* and *AKR1B10* (Burczynski *et al.*, 1999; Jin & Penning, 2007; Penning & Drury, 2007). In contrast, human GSTs lack an antioxidant response element in their gene promoters (Hayes

et al., 2005). Among the human genes, *AKR1C1*–*AKR1C3* are involved in the metabolic activation of PAH *trans*-dihydrodiols to electrophilic and redox-active *ortho*-quinones (Palackal *et al.*, 2002), when a positive feedback loop is created in which the quinone and ROS can further induce metabolism via this pathway. In addition, PAH *ortho*-quinones can also operate as bi-functional inducers by activating the Nrf2-Keap1 system and acting as ligands for the AhR (Burczynski & Penning 2000). Thus, benzo[*a*]pyrene-7,8-dione promotes nuclear translocation of the AhR and induction of the *CYP1A1* and *CYP1B1* genes. *AKR1C1*, *AKR1C2*, *AKR1B10* and *CYP1B1* have been shown to be upregulated in the bronchial epithelial cells of tobacco smokers (Woenckhaus *et al.*, 2006), downregulated in those of smokers who quit (Zhang *et al.*, 2008) and upregulated in oral dysplastic cells induced by cigarette-smoke condensate (Nagaraj *et al.*, 2006; Gümüş *et al.*, 2008), which led to the concept that they form part of a battery of genes that are induced by cigarette smoking (Penning & Lerman 2008). The same genes are probably regulated in response to particulate matter of 2.5 mm diameter (PM_{2.5}) and similar mixtures found in diesel and gasoline engine exhausts.

(d) *Relative contributions of the three pathways of activation*

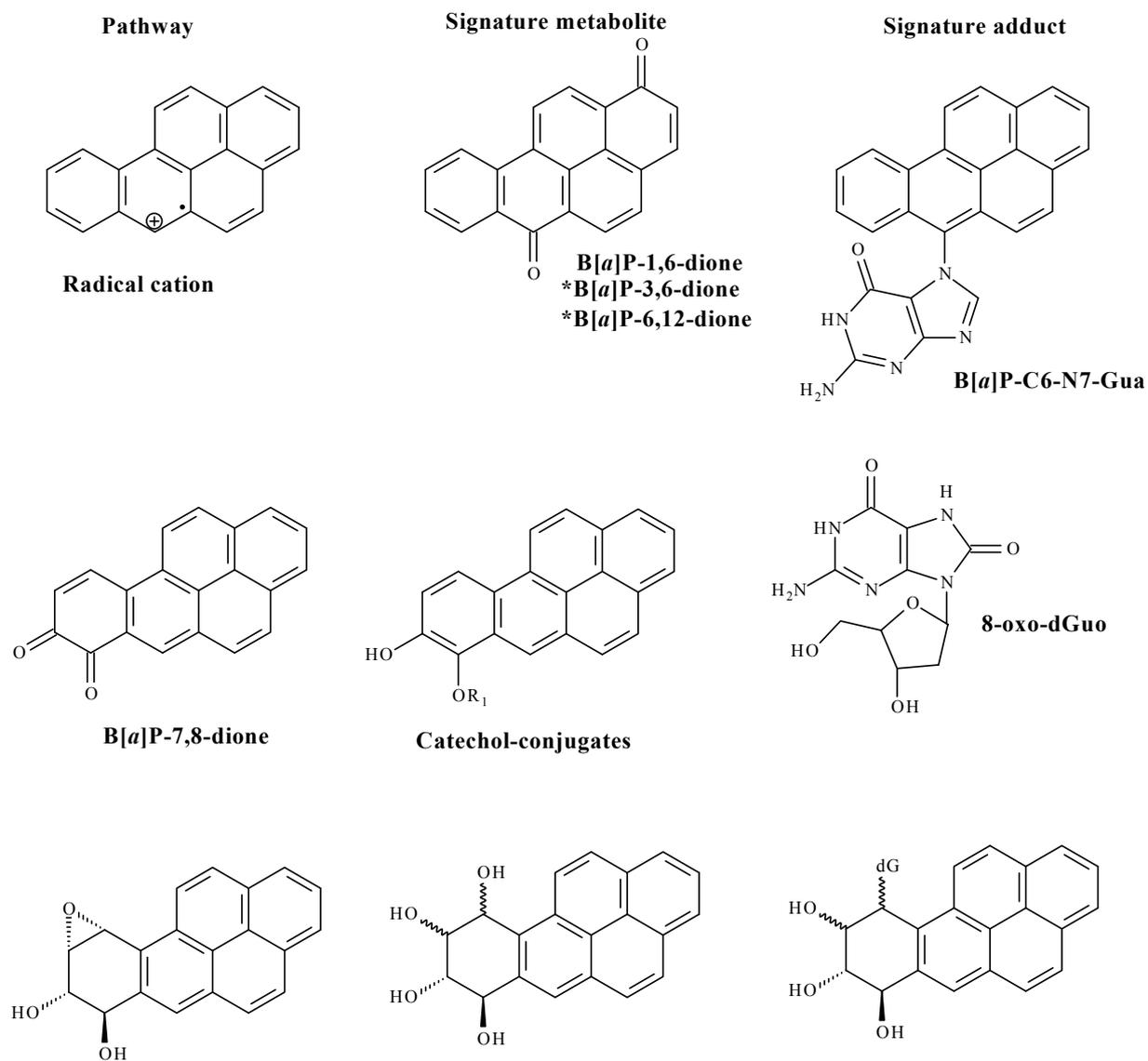
While compelling evidence shows that the diol epoxide pathway of PAH activation is mechanistically important and explains how PAHs may initiate lung cancer in mice and humans, relatively few studies have been conducted to compare the contribution of all three pathways to their activation in the same cell line, tissue, organ or animal model. One approach is to compare the relative formation of signature metabolites from each benzo[*a*]pyrene pathway of activation, e.g. benzo[*a*]pyrene tetraols (diol-epoxide pathway), benzo[*a*]pyrene-1,6-dione and benzo[*a*]pyrene-3,6-dione (radical cation pathway) and benzo[*a*]pyrene-7,8-dione (*ortho*-quinone pathway).

Another approach is to compare the formation of signature benzo[*a*]pyrene–DNA adducts following exposure to benzo[*a*]pyrene; these would include (+)-*anti*-B[*a*]PDE-dG adducts (diol epoxide pathway), the formation of abasic sites or the corresponding depurinating adducts (C6-C8-guanine, C6-N7-guanine and C6-N7-adenine adducts of benzo[*a*]pyrene) (radical cation pathway) and the formation of 8-OH-dG (*ortho*-quinone pathway) following treatment with benzo[*a*]pyrene (Fig. 4.2). Both approaches have been used with variable success.

Recently, SID-LC/MS/MS assays were developed to profile benzo[*a*]pyrene metabolism in human H358 bronchoalveolar cells in the absence and presence of the AhR ligand, 2,3,7,8-tetrachlorodibenzodioxin. Signature metabolites of each pathway were measured, e.g. benzo[*a*]pyrene tetraols, benzo[*a*]pyrene-1,6-dione, benzo[*a*]pyrene-3,6-dione and benzo[*a*]pyrene-7,8-dione. It was found that, in the absence and presence of 2,3,7,8-tetrachlorodibenzodioxin, all three pathways made an equal contribution to the metabolism of benzo[*a*]pyrene, and that 2,3,7,8-tetrachlorodibenzodioxin abolished its lag phase (Lu *et al.*, 2011).

Other studies compared the relative formation of dibenzo[*a,l*]pyrene-diol epoxide–DNA adducts with that of abasic sites (evidence of depurination and radical cation formation) following the treatment of HL60 cells with dibenzo[*a,l*]pyrene (Melendez-Colon *et al.*, 1997, 1999). Dibenzo[*a,l*]pyrene was chosen because it is the most carcinogenic PAH known and HL60 cells were chosen because of their high level of endogenous peroxidase activity. Only diol epoxide adducts were detected by ³²P-postlabelling, whereas abasic sites could not be detected with the aldehyde-reactive probe. In other studies, unstable depurinating DNA adducts of benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene and dibenzo[*a,l*]pyrene were detected in mouse skin and found to exceed the formation of stable DNA adducts that would be formed from the diol epoxide

Fig. 4.2 Signature metabolites and signature DNA adducts arising from the three pathways of benzo[*a*]pyrene activation



* Structures not shown

B[a]P, benzo[*a*]pyrene; B[a]PDE, benzo[*a*]pyrene-7,8-diol-9,10-epoxide; 8-oxo-dGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine

pathway ([RamaKrishna et al., 1992](#); [Chen et al., 1996](#); [Cavalieri et al., 2005](#)). In a comparison of the formation of *anti*-B[a]PDE-dG and 8-OH-dG adducts in human H358 bronchoalveolar cells following treatment with the proximate carcinogen (-)benzo[a]pyrene-7,8-*trans*-dihydrodiol using SID-LC/MS, the levels of both adducts were similar ([Ruan et al., 2007](#); [Mangal et al., 2009](#)). The level of 8-OH-dG increased following treatment of the cells with a catechol-*O*-methyl transferase inhibitor, suggesting that a futile redox cycle between benzo[a]pyrene-7,8-catechol and benzo[a]pyrene-7,8-dione was responsible for the increased formation of lesions. Using a low dose to initiate DNA adducts in the A/J mouse lung model of benzo[a]pyrene carcinogenesis, the relative level of stable adducts formed was measured with ³²P-postlabelling. While (+) *anti*-B[a]PDE-dG adducts were detected, the corresponding stable covalent adducts that can be formed with benzo[a]pyrene-7,8-dione were not found ([Nesnow et al., 2010](#)). These data were consistent with the concept that 8-OH-dG may be the dominant lesion formed from benzo[a]pyrene-7,8-dione *in vivo*.

[IARC \(2010a\)](#) examined four mechanisms for the activation of benzo[a]pyrene. Overwhelming evidence supported the formation of bay-region diol epoxides as the mechanism that generates biological reactive intermediates which form the DNA adducts that lead to mutation. Other pathways, such as the radical cation pathway or the *ortho*-quinone pathway, may also play a role but the data for each of these pathways are still incomplete, and no compelling data support L-region mesomethylation as a viable pathway.

4.2 Deposition, clearance, retention and metabolism

The general principles of inhalation, deposition, clearance and retention of poorly soluble particles have been described previously ([IARC,](#)

[2010b](#)). Modelling of inhaled particle deposition in the human lung has also been reviewed ([Hofmann, 2009, 2011](#)).

4.2.1 Humans

(a) Diesel engine exhaust

The number, concentration and size distribution of aerosol particles in the submicrometre range generated from diesel combustion that were inhaled and exhaled by 14 nonsmoking human volunteers were determined using a scanning mobility particle sizer. The subjects were in a relaxed state and inhaled nasally using a spontaneous breathing pattern. Total deposition was determined by the difference between the total measured number and concentrations of the inhaled and exhaled size distributions. The average total deposition for diesel smoke was $30 \pm 9\%$ (\pm standard deviation [SD]) and the count median diameter was $0.124 \pm 0.025 \mu\text{m}$ ([Morawska et al., 2005](#)). A Monte Carlo deposition morphometric stochastic model was applied to this data set. The theoretical prediction for total deposition was $24 \pm 2.7\%$, which was lower than the values obtained experimentally. The apparent discrepancy between experimental data on total deposition and modelling results was reconciled by considering the non-spherical shape of the test aerosols by diameter-dependent dynamic shape factors that account for differences between mobility-equivalent and volume-equivalent or thermodynamic diameters ([Hofmann et al., 2009](#)).

Other models have been developed to predict the deposition of diesel engine exhaust in humans and rats ([Yu & Xu, 1986](#)).

A group of selective studies of metabolism have been conducted on humans exposed to diesel engine exhaust, generally in the workplace. Many of these studies (described below) focused on measurements of urinary levels of hydroxylated and amino PAHs, mainly of pyrene, and demonstrated the ability of humans exposed

to diesel engine exhaust to adsorb, distribute, metabolize and excrete metabolites of PAHs. Additional studies have described haemoglobin adducts of PAHs and low-molecular-weight alkenes.

The levels of urinary 1-hydroxypyrene and haemoglobin-bound adducts (hydroxyethylvaline and hydroxypropylvaline) were measured in four groups of nonsmoking men: urban bus drivers (27), suburban bus drivers (23), taxi drivers (21) and suburban controls (22). No differences were found between the groups ([Hemminki *et al.*, 1994a](#)).

Several biomarkers were evaluated for their ability to assess differences in exposure to diesel engine exhaust and used lubricating oil in bus garage workers and mechanics, and controls by measuring the levels of both urinary 1-hydroxypyrene and hydroxyethylvaline adducts in haemoglobin. Samples were taken from 10 nonsmoking bus garage workers and mechanics and 12 nonsmoking healthy men in the administrative department. The exposed workers had significantly higher levels of the biomarkers than the controls. The level of hydroxyethylvaline haemoglobin adducts was 33.3 pmol/g haemoglobin in exposed workers versus 22.1 pmol/g haemoglobin in controls, and that of 1-hydroxypyrene in the urine was 0.11 pmol/mol creatinine in the workers compared with 0.05 pmol/mol creatinine in controls. The levels of hydroxyethylvaline adducts in the blood correlated with those of urinary 1-hydroxypyrene. The study indicated that skin absorption might be an important factor to consider when studying exposure to PAHs from air pollution sources ([Nielsen *et al.*, 1996a](#)).

Fifty Estonian underground oil-shale miners (35 smokers) who drove diesel-powered excavators and 50 surface workers (31 smokers) engaged in various production assignments above-ground that were not associated with the use of diesel-powered engines were examined for levels of urinary 1-hydroxypyrene, S-phenylmercapturic acid and

t,t-muconic acid. Samples were collected before the first of a series of work shifts and after several shifts over the course of a work week. The group of underground miners had slightly but significantly increased levels of all three biomarkers at the end of the first and last shifts while no significant changes in the levels over time were observed for the surface controls ([Scheepers *et al.*, 2002](#)).

The levels of urinary 1-hydroxypyrene in groups of smoking and nonsmoking Chilean workers exposed to diesel engine exhaust emissions in the transport industry were compared with those of an unexposed rural population. The groups comprised 59 diesel-exposed workers (38 involved in diesel revision (emission compliance) and 21 in an urban area working as established street vendors) and 44 unexposed subjects living in a rural area. The levels of urinary 1-hydroxypyrene in the urban and rural populations showed significant differences between smokers and nonsmokers ($P < 0.04$), but no significant differences between smokers and nonsmokers were found among the diesel plant workers. Nonsmoking subjects from the diesel plants and the urban area showed similar levels which were significantly higher ($P < 0.05$) than those of subjects living in the rural area ([Adonis *et al.*, 2003](#)).

Levels of 1-hydroxypyrene were determined in 60 nonsmoking bus drivers in city and rural areas on a work day and on a day off and in 88 nonsmoking mail deliverers working outdoors (in the streets) and indoors (in the office). Twenty-four hour urine samples were collected on a working day and a day off. Bus drivers excreted significantly more 1-hydroxypyrene in the urine than mail deliverers ($P < 0.001$). These results indicated that bus drivers have greater exposure to PAHs than mail deliverers. Mail deliverers who worked outdoors had significantly higher urinary concentrations of 1-hydroxypyrene than those who worked indoors ($P < 0.001$) ([Hansen *et al.*, 2004](#)). [The Working Group noted that the

exposures of the study groups to specific exhaust emissions were not identified.]

In Helsinki, Finland, urinary hydroxylated metabolites of naphthalene, phenanthrene and pyrene were examined as markers of exposure to diesel engine exhaust in a group of exposed workers comprising 30 nonsmoking men and two women (20 bus garage workers and 12 waste-collection truck drivers), who were exposed daily to diesel engine exhaust. A group of 46 nonsmoking white-collar workers served as controls. Urine samples were collected from workers at three bus garages who repaired, refuelled and cleaned diesel-powered buses indoors and were exposed to diesel engine exhaust through work activities near the buses inside the garages, and from truck drivers who worked for four companies that collected household waste in a suburb. The sum of seven PAH metabolites (mean, 3.94 ± 3.40 (SD) and 5.60 ± 6.37 (SD) $\mu\text{mol/mol}$ creatinine in winter and summer, respectively) was higher ($P = 0.01$) in the exposed group than in the control group (mean, 3.18 ± 3.99 (SD) and 3.03 ± 2.01 (SD) $\mu\text{mol/mol}$ creatinine in winter and summer, respectively). The mean concentrations of 2-naphthol ranged from 3.34 to 4.85 $\mu\text{mol/mol}$ creatinine for the exposed workers and 2.51 to 2.58 $\mu\text{mol/mol}$ creatinine for the controls ($P < 0.01$ in winter, $P < 0.03$ in summer). The mean levels of hydroxyphenanthrenes were between 0.40 and 0.70 $\mu\text{mol/mol}$ creatinine in the samples from exposed workers and between 0.40 and 0.60 $\mu\text{mol/mol}$ creatinine in those from controls. The concentration of 1-hydroxypyrene was higher among exposed workers in both pre-shift and post-shift samples (mean, 0.10–0.15 $\mu\text{mol/mol}$ creatinine) than in the control group (mean, 0.05–0.06 $\mu\text{mol/mol}$ creatinine) in winter ($P = 0.002$) and in summer ($P < 0.001$). The authors concluded that the presence of urinary hydroxylated metabolites of naphthalene, phenanthrene and pyrene indicated low exposure to diesel-derived PAHs, and was higher in exposed workers than in the control group. Urinary monohydroxylated PAH

metabolites measured in this study did not correlate with PAHs in the air samples as reported previously ([Kuusimäki et al., 2004](#)).

Levels of 1-hydroxypyrene were measured in the urine of 47 Italian road construction workers exposed to diesel engine exhaust (17 nonsmokers and 30 smokers), who also refrained from consuming smoked and grilled food, tea and coffee the evening before and during the day of sampling. Samples were taken at several time-points: after 2 days of vacation (baseline), and before and at the end of the monitored work shift during the second part of the work week. At all sampling time-points, the levels of urinary 1-hydroxypyrene of the smoking construction workers were higher than those of the nonsmoking group ($P < 0.01$). No differences were found in the urinary levels of 1-hydroxypyrene between samples taken at baseline, before the shift and at the end of the shift in either smoking or nonsmoking workers ([Campo et al., 2006](#)). The subjects served as their own controls.

Levels of 1-hydroxypyrene were determined in pre- and post-shift urine samples collected on 4 consecutive days from 17 Chinese workers (73% smokers) exposed to diesel engine exhaust at a locomotive engine inspection plant. Increased levels were observed over at least 3 consecutive sampling days. The biological kinetics of pyrene metabolism was studied using one-compartment pharmacokinetics and nonlinear mixed-effects models. The mean half-life of urinary 1-hydroxypyrene was estimated to be 29 hours ([Huang et al., 2007](#)).

Urinary levels of 1-aminopyrene were compared between three diesel mechanics working in a repair shop for train engines and two office clerks. Both cumulative and average excretion of urinary 1-aminopyrene over 48 and 72 hours were significantly enhanced ($P < 0.05$) in samples from diesel mechanics compared with those in samples from the office clerks ([Scheepers et al., 1994](#)).

Blood samples from 29 bus garage workers (occupationally exposed to diesel engine exhaust), 20 urban hospital workers and 14 rural council workers (controls) were analysed for sulfinic acid-type amino-haemoglobin adducts derived from 1-nitropyrene, 2-nitrofluorene, 3-nitrofluoranthene, 9-nitrophenanthrene and 6-nitrochrysene. The most abundant cleavage products were 1-aminopyrene and 2-amino-fluorene at levels ranging from 0.01 to 0.68 pmol/g haemoglobin. No significant difference in the levels of 1-nitropyrene- and 2-nitrofluorene-derived haemoglobin adducts was observed between the groups. A comparison of the sum of the five nitro-PAH-derived haemoglobin adducts also indicated no differences between the three groups ([Zwirner-Baier & Neumann, 1999](#)). The same methodology was applied to a group of 30 former East German miners exposed to diesel engine exhaust emitted from heavy-duty equipment, a group of 40 Danish bus drivers and the 29 bus garage workers cited above. The sum of the five nitro-PAH-derived haemoglobin adducts was higher (no statistical comparison) in the miners and bus drivers compared with the garage workers ([Neumann, 2001](#)).

The 24-hour urine samples from 18 underground salt miners (nine smokers, nine nonsmokers) exposed to diesel engine exhaust were collected during and after their shift. Nonsmoking workers exposed to diesel engine exhaust excreted an average level of ~4 µg phenanthrene metabolites (phenol and dihydrodiols), whereas the urinary levels in smokers were up to threefold higher. 1-Aminopyrene and 3-aminobenzanthrone were detected in the urine of all miners ([Seidel et al., 2002](#)).

The urinary levels of 1-hydroxypyrene of 50 Estonian underground shale-oil miners who drove diesel-powered excavators were compared with those of 50 surface workers who had no known occupational exposure to diesel engine exhaust. Personal exposure measurements of particle-associated 1-nitropyrene were collected.

Urine samples were collected at the start and after the first and last shift of the same week. Levels of particle-associated 1-nitropyrene were approximately eightfold higher for miners (as determined by personal air monitoring) than for surface workers, while levels of urinary 1-hydroxypyrene showed only a small increase ([Scheepers et al., 2004](#)).

Concentrations of 1-aminopyrene were measured in spot urine samples from 38 individuals collected before and during a 24-hour period following the initiation of 1-hour controlled exposures to diesel engine exhaust (target concentration, 300 µg/m³ as PM₁₀) or clean air. Urinary samples of the *N*-acetyl-1-aminopyrene conjugate were deconjugated to 1-aminopyrene and analysed. Time-weighted average concentrations of urinary 1-aminopyrene were significantly greater following exposure to diesel engine exhaust compared with clean air (median, 138.7 versus 21.7 ng/g creatinine; $P < 0.0001$). Comparing exposures to diesel engine exhaust and clean air, significant increases in the concentration of 1-aminopyrene from pre-exposure to either first post-exposure void (24-hour time-point) or peak spot urine concentration following exposure ($P = 0.027$ and $P = 0.0026$, respectively) were recorded. Large interindividual variability, in both the concentration of urinary 1-aminopyrene and the time course of appearance in the urine following the standardized exposure to diesel engine exhaust, was observed ([Laumbach et al., 2009](#)). In a follow-up study, pharmacokinetic analyses of the data on 1-aminopyrene excretion found that two subgroups could be determined within the exposed group in terms of the timing of the excretion. Approximately 63% of the subjects had a median maximal excretion time of 5.37 hour whereas 30% of the subjects had maximal excretion times of > 24 hours ([Huyck et al., 2010](#)). Genetic polymorphisms, metabolic enzymes and/or differences in respiration rates may play a role in the differences in excretion rates.

(b) Gasoline engine exhaust

The results of three studies on the deposition of gasoline engine exhaust have been reported previously ([IARC, 1989](#)). In two controlled studies, volunteers were exposed to exhaust from an engine run on gasoline containing ^{203}Pb -tetraethyl lead. Total deposition was relatively constant (30%) over a wide range of breathing patterns for sizes of typical aerosols ([Chamberlain, 1985](#)). As the size of primary particles decreased (below $0.1\ \mu\text{m}$), deposition increased sharply and the length of the respiratory cycle (start of the time between respiratory breaths) significantly affected deposition ([Heyder et al., 1983](#); [Schiller et al., 1986](#)). In a separate analysis of the same data, deposition was shown to increase with the respiratory cycle (time between the start of successive breaths) in an approximately linear fashion, ranging from 10% at 3 seconds to 55% at 20 seconds; the slope of the gradient was somewhat dependent on tidal volume ([Wells et al., 1977](#)). In the third study, measurements of total deposition were taken in the field by comparing inhaled and exhaled concentrations of lead; the method was found to give results comparable with those of studies on ^{203}Pb -tetraethyl lead. Total deposition was measured for inhalation at an average breathing pattern of 0.81 and a respiratory cycle of 5.2 seconds in persons seated by a motorway (69%), by a roundabout (64%), in an urban street (48%) and in a car park (48%). Median particulate sizes in the breath of persons near fast-moving traffic ($0.04\ \mu\text{m}$) were found to be much smaller than those in persons in the urban environment or in a car park ($0.3\ \mu\text{m}$), although the air near roundabouts also contained a large proportion by mass of adventitious particles ($2\ \mu\text{m}$) ([Chamberlain et al., 1978](#)).

Lung clearance was best described as a four-component exponential. The first two phases (half-times, 0.7 and 2.5 hours) were similar for exhaust particles, lead nitrate (which is soluble)

and lead oxide (which is insoluble), and therefore probably represented mucociliary clearance. On average, 40% of lung deposition of the $0.35\text{-}\mu\text{m}$ aerosols was in the pulmonary region and 60% in the tracheobronchial region. The removal of lead compounds from the pulmonary region was described by a two-compartment exponential with half-times of 9 and 44 hours; one exception was the removal of lead from highly carbonaceous particles which exhibited half-times of 24 and 220 hours ([Chamberlain et al., 1975, 1978](#); [Chamberlain, 1985](#)).

4.2.2 Experimental systems

(a) Diesel engine exhaust

Engine exhaust contains material in gaseous, vapour and particulate phases, and the absorption, distribution and excretion of individual constituents are influenced by the phase in which they occur and by the properties of each compound. After inhalation, highly soluble compounds in the gaseous phase, such as sulfur dioxide, are absorbed in the upper airways and do not penetrate significantly beyond the level of the bronchioles. Compounds that interact biochemically with the body are also retained in significant quantities; thus, processes such as the binding of carbon monoxide to haemoglobin normally occur in the gas-exchange (pulmonary) region of the lung. Retention characteristics of materials not associated with the particulate phase are highly compound-specific. Factors that affect the uptake of a wide variety of vapours and gases have been summarized and modelled ([Davies, 1985](#); [Tsuji no et al., 2005](#)).

A proportion of a given compound in the vapour phase condenses onto the particulate material produced in the engine exhaust. The association of a compound with the particulate phase modifies its deposition pattern and affects its lung retention; following continuous exposure, the lung burden of a compound coated on particles may be many times higher than that

after continuous exposure to the compound alone (IARC, 1984; Tsujino *et al.*, 2005).

Deposition in the respiratory tract is a function of particle size (Smith *et al.*, 2001; Hofmann *et al.*, 2009). The median particle size in a variety of long-term exposure systems has been determined to be between 0.19 and 0.54 μm (IARC, 1984; Yu & Xu, 1986; Lapuerta *et al.*, 2003; IARC, 2010a), representative of that in an urban environment. However, some of the carbonaceous mass in environmental samples results from airborne suspensions of material collected in automobile exhaust pipes and is $> 5 \mu\text{m}$ in size (Lapuerta *et al.*, 2003); such particles are unlikely to be produced in a static exposure system.

At the time of the last review of diesel engine exhaust (IARC, 1989), it was known that long-term, high rates of exposure to diesel engine exhaust lead to lung tumour development in rats, but interpretation of this response for the risk of human lung cancer was in its early stages (Vostal, 1986). No evidence had been found to demonstrate that heavy doses of inhaled particles lead to the rat-specific mechanisms of lung clearance overload that trigger the release of inflammatory molecules in the lungs. The species specificity of the response of rat lungs to 'overload', and its commonality to low-solubility particles, such as those in diesel engine exhaust, are now better understood. The previous review noted that tumours were found in rats but not in other species [except for one study in mice that reported an abnormally low background incidence of tumours], and that the tumours were associated with the solid particulate and not gaseous components of the mixtures, based on the reduction in the incidence of tumours when particle traps were used. Further studies that compared the relative rates of tumour formation between similar concentrations of carbon black and diesel engine exhaust particles suggested that the tumour formation caused by diesel engine exhaust was not dependent on its organic compounds, but rather on its poorly soluble solid

carbon core (Mauderly *et al.*, 1994; Heinrich *et al.*, 1995; Nikula *et al.*, 1995; Driscoll *et al.*, 1997). Recently, Stinn *et al.* (2005) reported that chronic inhalation of diesel engine exhaust at high particle concentrations resulted in tumour formation in the absence of direct genotoxicity, as measured by the levels of DNA adducts. Because of the importance of this mechanism, this section elaborates on the evidence and its potential implications for hazard identification.

This section addresses the mechanisms of carcinogenesis of particles and is based on an extensive database for poorly soluble, respirable particles of low toxicity that was reviewed previously (IARC, 2010b). The extent to which these mechanisms are fully relevant for particles generated from combustion is not known.

(i) Lung overload

The concept of 'overload' is central to the relevance of using studies in rodents to evaluate human health hazards from inhaled particles. Overload is a biological mechanism that involves the dose-dependent impairment of alveolar macrophage-mediated clearance of respirable particles. In the alveolar region of the respiratory tract, the primary mechanism for particle clearance is phagocytosis by alveolar macrophages with subsequent removal of particle-containing macrophages by mucociliary clearance.

High particle burdens in the lungs can result in overload because alveolar macrophage-mediated clearance is overwhelmed, which results in a decreased rate of clearance and an increased retention of particles. Overloading of lung clearance has been observed in rats, mice and hamsters exposed to different insoluble respirable particles (e.g. carbon black, titanium dioxide, talc, toner and diesel engine exhaust particulates) (Strom *et al.*, 1989; Muhle *et al.*, 1990a, b; Bellmann *et al.*, 1991; NTP, 1993; Warheit *et al.*, 1997; Bermudez *et al.*, 2002, 2004; Elder *et al.*, 2005) and asbestos fibres (Davis *et al.*, 1978; Bolton *et al.*, 1983).

Mechanisms that underlie lung overload

Experimentally, overloading of lung clearance has been inferred from the observation of a greater lung burden of particles or fibres than that expected on the basis of results with lower concentrations or shorter durations of exposure ([Davis et al., 1978](#)). A steady-state lung burden should be achieved when the rate of deposition equals the rate of clearance, and overloading represents a deficit in that clearance. Impaired clearance attributed to overloading has been expressed as a reduction in the clearance rate coefficient ([Muhle et al., 1990a](#); [Bellmann et al., 1991](#)) or an increase in the quantity of particles retained in the lungs following exposure ([Strom et al., 1989](#); [Bermudez et al., 2002, 2004](#); [Elder et al., 2005](#)). Increased translocation of particles to the lung-associated lymph nodes has also been observed at doses at which overload occurs ([Strom et al., 1989](#); [Bellmann et al., 1991](#)).

[Morrow \(1988\)](#) hypothesized that overload was a consequence of macrophages that become progressively immobilized and aggregated. When the dose of particles reaches a critical particle volume, clearance by macrophages is suppressed and particles accumulate in the lungs. Based on the lung burden of particle mass associated with increased retention in rat lungs (approximately 1 mg/g of lung tissue for unit density particles) and data on the volume and number of alveolar macrophages in rat lungs, it was hypothesized that impairment of clearance would be initiated when the particle volume exceeded an average of 6% of the macrophage volume, and would be completely impaired when particle volume exceeded an average of 60% of the macrophage volume. The upper particle volume estimate (60%) was supported by [Oberdörster et al. \(1994\)](#), who showed that clearance was no longer detectable 200 days after instillation of polystyrene particles 10 µm in diameter into rat lungs. The overload mechanism pertains specifically to poorly soluble respirable (< 10 µm) particles of low

toxicity. Factors other than the volumetric overload can lead to impaired alveolar clearance. For example, particles that are toxic to macrophages (e.g. crystalline silica) can cause impaired clearance at doses lower than those of low-toxicity particles ([Bellmann et al., 1991](#)). Ultrafine particles have been found to differ from fine particles with regard to overloading. [Morrow \(1992\)](#) noted that ultrafine particles impair clearance at lower mass or volume concentrations than those expected for larger respirable particles. [Oberdörster \(1996\)](#) confirmed this observation and showed that increased particle retention and inflammation were related to particle surface area.

One mechanism for the impaired clearance of ultrafine particles may be their ineffective phagocytosis ([Churg et al., 1998](#); [Renwick et al., 2001, 2004](#); [Geiser et al., 2005](#)), which leaves the particles free in the alveolar region and more readily able to translocate to the lung interstitium ([Ferin et al., 1992](#); [Ferin, 1994](#)). The surface properties of particles may also influence phagocytosis. For example, [Castranova et al. \(2000\)](#) found that chronic inhalation exposure to 2 mg/m³ of coal dust activated alveolar macrophages, while the same exposure to diesel engine exhaust depressed phagocytic activity. [Wolff et al. \(1986\)](#) noted that additional factors other than non-specific particle effects must be important, because the exposure level that resulted in overloading and lung tumours was higher for some particles than for others (e.g. 250 mg/m³ of fine-sized titanium dioxide versus ~7 mg/m³ of diesel engine exhaust).

Mechanisms that underlie lung response to overload

An increase in neutrophilic inflammation has been defined as the critical biological response to lung overload ([ILSI Risk Science Institute Workshop Participants, 2000](#)). An increase in polymorphonuclear leukocytes (granulocytes) in bronchoalveolar lavage (BAL) fluid in rats

has been associated with increased retention of particles in the lungs ([Tran *et al.*, 1999](#)). Mice also appear to be susceptible to overloading doses and adverse pulmonary responses, but regain normal clearance more readily after cessation of exposure. Hamsters clear particles much faster than rats or mice, experience overloading at higher doses and recover more easily. Lung responses follow the clearance kinetics for inhaled particles: rats show a more severe, sustained response to inhaled particles than mice, while hamsters demonstrate only a temporary inflammatory response ([Bermudez *et al.*, 2002, 2004](#); [Elder *et al.*, 2005](#)). In rats, lung responses to overloading include increased lung weight, chronic inflammation, fibrosis and lung cancer ([Muhle *et al.*, 1991](#)).

The series of events that are involved in the biological process that begins with particle deposition in critical target cells or tissues within the rat lung and results in tumours include: sustained inflammation, production of ROS, depletion of antioxidants and/or impairment of other defence mechanisms, cell proliferation and gene mutations. These individual steps comprise an overall mode of action that can be used to compare the responses of rats with those of other species including humans ([IARC, 2010b](#)).

At a lung burden of particle mass at which overload is observed in rats (estimated to begin at ~0.5 mg/g of lung tissue and to be fully developed at ~10 mg/g), a sustained and widespread cellular inflammatory response occurs. The cell population is dominated by activated and probably (under these conditions) persistent neutrophil granulocytes, and secretes a collection of mediators (pro- and anti-inflammatory cytokines, proteases, cytotoxins, fibrogenic mediators and other growth factors) that act through the pulmonary milieu on surrounding cells or tissues and surrounding structures ([Castranova *et al.*, 2000](#); [IARC, 2010b](#)).

The degree of sustained inflammation experienced by rodents (most notably rats) at

high lung burdens is not observed in humans. However, humans may experience sustained inflammation during certain diseases. One such condition (which may be particle-stimulated, e.g. by silica, or cryptogenic) is late-stage interstitial pulmonary fibrosis. Patients who have interstitial pulmonary fibrosis and chronic inflammation have been reported to experience a higher incidence of lung tumours ([Daniels & Jett, 2005](#)). [Rom \(1991\)](#) found a statistically significant increase in the percentage of neutrophil granulocytes in the BAL fluid of workers with respiratory impairment who had been exposed to asbestos, coal or silica (4.5% in cases versus 1.5% in controls). Elevated levels (sevenfold increase over controls) of neutrophil granulocytes have been observed in the BAL fluid of miners who had simple coal workers' pneumoconiosis ([Vallyathan *et al.*, 2000](#)) and in patients with acute silicosis (a 10-fold increase over controls) ([Goodman *et al.*, 1992](#); [Lapp & Castranova, 1993](#)).

The precise role of chronic inflammation in the development of cancer is uncertain, but considerable evidence shows that chronic inflammation may have a multifaceted role in this process. Activated cells in the lung are known to release various reactive intermediates, most notably those derived from oxygen. A sustained excess of oxidant activity is known to deplete antioxidant defences gradually. Clear differences in these lung defence mechanisms exist between humans and rats, and evidence shows that humans in general are relatively deficient in some of these mechanisms compared with rats ([Hatch *et al.*, 1985](#)). ROS within cells may directly damage DNA and potentially induce mutations. Moreover, cell damage and promitotic stimuli initiated by ROS promote cell turnover and proliferation, both of which may enhance the risk for DNA replication error and/or expand a mutated or transformed cell to initiate the tumorigenic process (see Section 4.3.1).

Dosimetric correlation between lung particle burden and response

Because particle overload is the critical determinant that underlies the adverse biological response to inhaled particles, an understanding of the appropriate dosimetric expression for overload is essential for hazard evaluation. Several studies have shown that, for particles of different sizes but with the same chemical composition, the dose expressed as particle surface area is a better predictor of adverse pulmonary inflammation than particle mass ([Oberdörster et al., 1992](#); [Tran et al., 1999](#); [Bermudez et al., 2002, 2004](#)). Particle surface area is also related to pulmonary inflammation in mice ([Lison et al., 1997](#)). [Oberdörster & Yu \(1990\)](#) and [Driscoll et al. \(1996\)](#) showed that particle surface area is also a better predictor of lung tumours than particle mass in rats exposed to various poorly soluble fine or ultrafine particles.

The particle characteristics and methods used to estimate particle surface area may influence the magnitude of the observed response. For example, carbon black that has a high specific surface area (220 m²/g) was shown to induce a lower inflammatory response than that expected on the basis of total particle surface area dose ([Driscoll et al., 1996](#)). This could be due to less disaggregation of the deposited carbon black into smaller units compared with ultrafine particles, such as titanium dioxide ([Oberdörster, 1996](#)). It may also be due to the more porous surface of carbon black (carbon black has a greater internal surface than titanium dioxide), which may increase the surface area as measured by nitrogen absorption, but does not accurately measure the effective surface area in contact with the epithelial cell surface ([Tran et al., 1999](#)).

Deposition: Studies of the deposition of diesel engine exhaust, which is representative of fresh urban exhaust, showed a total deposition of inhaled particles ranging from 10 to 20% ([IARC, 1984](#)). Models for the deposition of diesel engine

exhaust particles predict that, as the median size increases from 0.10 to 0.30 µm, total deposition in rats decreases from approximately 25% to ~15%, ([IARC, 1984](#); [Schroeter et al., 2012](#)), in agreement with measured deposition ([Garcia & Kimbell, 2009](#)).

Mucociliary clearance: The rapid phase of clearance of particles from the lung following a single exposure to radiolabelled diesel particles is conventionally assumed to be due to mucociliary action, and the remainder (slow phase) to pulmonary clearance. The fraction of lung deposit cleared in the rapid phase ranged from 6% to 75% whereas the slow phase ranged from 25% to 66%. The half-time for clearance in the slow phase ranged from 60 to 80 days and was occasionally dose-dependent ([IARC, 1984](#)).

Pulmonary (alveolar) clearance: The pulmonary clearance of diesel particles is much slower than mucociliary clearance, with retention half-times ranging from 60 days to more than 1000 days. In general, half-times are greater after long exposures and for larger particles. With longer half-times, the lung burden can increase linearly over the lifetime of the animal, leading to the 'overload' phenomenon. The clearance rate of insoluble particles following prolonged exposure can be seriously impaired, leading to very long-term retention of material in the lung, usually referred to as 'sequestration'. The majority of particles that are cleared by macrophages from the pulmonary region leave via the ciliated epithelium and are excreted through the gut. However, about one-third of the particles penetrate the lymphatic system, borne by macrophages, and are filtered by the lymph nodes to form aggregates ([IARC, 1984](#)). Only the gas phase appears to have no effect on pulmonary clearance in rats or hamsters ([Reed et al., 2004](#)).

Retention: Organic compounds adsorbed on engine exhaust particles can be extracted by biological fluids, with a half-time for the slow phase of lung clearance of 18–25 days in rats ([IARC, 1984](#)). This phenomenon has been

reviewed ([Stöber & McClellan, 1997](#)) and additional data are available ([Claxton & Woodall, 2007](#)). In dogs, 37% and 59% of the [³H]benzo[a]pyrene originally coated onto diesel particles did not remain on the particles after 5.6 months ([Gerde et al., 2001](#)). The retention of several nitroarenes adsorbed onto diesel engine exhaust particles is described in the *Monographs* on those compounds in this Volume.

Metabolism: The metabolism of several components of engine exhausts has been reported previously: some PAHs ([IARC, 2010a](#)), formaldehyde ([IARC, 1982, 2006a, 2012a](#)), lead ([IARC, 1980, 2006b](#)), nitroarenes ([IARC, 1989](#)) and benzene ([IARC, 1982, 2012a](#)).

Three dogs were exposed to aerosolized [³H]benzo[a]pyrene coated onto diesel particles. Blood sampling demonstrated a bi-phasic half-life, with a first half-life for benzo[a]pyrene in the blood of 4.3 minutes and a second half-life of 1.8 minutes. Radiolabel appeared in the blood almost immediately after exposure, indicating rapid metabolism, and persisted throughout the 1-hour sampling period ([Gerde et al., 2001](#)).

Groups of eight rats were exposed to Standard Reference Material (SRM) 2975 at 0.064 or 0.64 mg/kg body weight (bw) for 6 and 24 hours. Increased levels of 8-OH-dG lesions were observed at the highest dose after 6 and 24 hours in colon, liver, and lung. Elevated levels of *OGG1* mRNA were observed after 24 hours of exposure to both doses of diesel engine exhaust particles in the lung but not in the colon or liver. Elevated levels of *haeme oxygenase 1* (*Hmox-1*) mRNA and bulky DNA were observed in the liver and lung 6 hours (bulky DNA only) and 24 hours after administration ([Danielsen et al., 2008](#)).

[³H]Benzo[a]pyrene coated onto diesel particles was instilled intratracheally into male Sprague-Dawley rats and the distribution of radioactivity was analysed at selected time-points over 3 days. About 50% of the radioactivity remained in the lungs 3 days after instillation; 30% was excreted in the faeces and the remainder

was distributed throughout the organs ([Bevan & Ruggio, 1991](#)).

Interspecies comparison of particle retention in the lung

Impairment of clearance leads to an increased retention of particles, which is the hallmark of lung overload. Thus, an understanding of interspecies differences in the mechanisms of particle retention can aid hazard evaluation and risk assessment. Differences in the patterns of particle retention of coal dust or diesel engine exhaust were observed in rats and monkeys exposed by inhalation for 7 hours a day, 5 days a week to 2 mg/m³ of coal dust and/or diesel engine exhaust particulates for 2 years. A higher volume percentage of coal dust was retained in the alveolar lumen in rats and in the interstitium in monkeys ([Nikula et al., 1997a, b](#)). A greater proportion of particles was also retained in the interstitium in humans, because the pattern of retention changed as the duration of exposure and assumed concentration of coal dust increased. In contrast, the pattern of retention in rats did not vary with increasing concentrations of diesel engine exhaust particulates from 0.35 to 7.0 mg/m³ ([Nikula et al., 2001](#)).

One class of insoluble particles—carbon black—has been identified in human lungs, although no quantitative data are available on its retention in humans. However, based on studies with other poorly soluble particulate materials, it can be assumed that the normal retention half-times of particles such as carbon black in humans is longer than that measured in rats and mice. For example, [Bailey et al. \(1985\)](#) found that the retention time in humans of inhaled, monodisperse, fused aluminosilicate particles 1 and 4 μm in diameter followed a two-component exponential with phases that had half-times of the order of tens of days and several hundred days, respectively. At 350 days after inhalation, retention of the remaining material averaged 46 ± 1% for the 1-μm particles and 55 ± 11% for the 4-μm particles. In contrast, data in rats ([Oberdörster,](#)

1995) and mice ([Kreyling, 1990](#)) demonstrated retention half-times of ~70 days and ~55 days, respectively.

Heavy exposure to particles in occupational settings may lead to high particle burdens in the human lung. By analogy to the rat, if the human lung burden exceeds ~0.5–1 mg/g of lung, it is expected that the normal retention half-time would be prolonged. Indeed, there is some evidence that workers in occupations that are associated with high particle burden in the lungs (e.g. coal mining) show increased long-term retention of particles ([Stöber *et al.*, 1965](#); [Freedman & Robinson, 1988](#)). Retention half-times of the order of years have been measured in several human studies that involved accidental exposure to radionuclides ([ICRP, 1994](#)).

Little is known about overloading in non-rodent species, including humans. The most frequently cited human data come from coal miners, who are one of the best studied occupational cohorts with regard to quantitative exposure–response relationships ([Attfield & Kuempel, 2003](#)). Coal miners have historically experienced high rates of occupational lung diseases, including increased morbidity and mortality from pneumoconiosis and chronic obstructive lung diseases ([NIOSH, 1995](#)). However, excess mortality from lung cancer has not generally been observed in coal miners ([NIOSH, 1995](#)), although, in a more recent study of German coal miners, elevated lung cancer mortality was detected in those who had developed pneumoconiosis (standardized mortality ratio, 1.57), which was independent of the effect of tobacco smoking ([Morfeld & Lampert, 2002](#); [Xiao *et al.*, 2012](#)).

Retained lung burdens have also been reported to be relatively high; an average of ~14 mg/g of lung has been observed historically in coal miners in the USA ([Kuempel *et al.*, 2001](#)) and the United Kingdom ([Tran & Buchanan, 2000](#)). This mean lung burden is comparable with retained mass lung burdens in rats that experienced overload. Because an elevated incidence

of lung cancer has not generally been observed in coal miners, and because clear differences in lung defence mechanisms exist between humans and rats (humans are deficient in some of these mechanisms compared with rats), it has been suggested that the rat may not be an appropriate model to predict lung cancer in humans. However, although the mean lung burden is relatively high in coal miners, it is actually lower than the mean lung burdens associated with the excess incidence of lung tumours in rats. For example, in rats chronically exposed to coal dust, mass lung burdens of 24 mg/g of lung tissue were associated with an 11% incidence of lung tumours (versus 0% in unexposed controls) ([Martin *et al.*, 1977](#)). In rats exposed to fine-sized titanium dioxide, lung burdens of up to ~35 mg/g were not associated with lung tumours, and increased incidences of lung tumours were observed only in rats with lung burdens greater than ~100 mg/g (approximately 16% in male and female rats, excluding keratinizing cystic squamous cell carcinomas) ([Lee *et al.*, 1985a, b, 1986](#)). In female rats that inhaled talc chronically for 2 years, 9 mg of talc/g of lung tissue were not associated with an elevated incidence of lung tumours (0/48, 0%), while an average retained burden of 29 mg/g of lung was associated with an incidence of 13 out of 50 (26%) alveolar/bronchiolar tumours ([NTP, 1993](#)).

Based on these chronic inhalation studies in rats exposed to various fine-sized, poorly soluble particles of relatively low toxicity, lung tumours were not observed in rats that had lung burdens similar to those of coal miners. Rats that developed lung tumours following chronic inhalation of these particles had retained mean mass lung burdens that were at least twice as high as those found in coal miners. Thus, the observed lung tumour response in rats and the absence of reported tumours in coal miners when both are exposed chronically to fine-sized, poorly soluble particles, such as coal dust, are somewhat consistent. The surface area of particles may be

a more appropriate dose metric for predicting response; therefore, it is useful to evaluate rat and human responses to particle surface area dose in addition to particle mass dose. In rat lungs, fine and ultrafine particles of similar composition have shown consistent dose–response relationships when the dose is expressed as particle surface area rather than as particle mass. The mean surface area dose of coal dust in the lungs of miners from studies in the USA and United Kingdom can be calculated as 0.1 m² of coal dust/g of lung tissue (assuming 7.4 m²/g of coal dust; [Vallyathan et al., 1988](#); [Tran & Buchanan, 2000](#); [Kuempel et al., 2001](#)). In rats, the lowest observed surface area doses associated with elevated incidences of lung tumours (excluding keratinising cystic squamous cell tumours) following chronic inhalation were: 0.18 m² of coal dust/g of lung tissue in female rats (assuming 7.4 m²/g coal dust), with an 11% tumour incidence versus 0% in controls ([Martin et al., 1977](#)); 0.58 m² of carbon black/g of lung tissue for female rats, with a 7.5% tumour incidence versus 0% in controls ([Nikula et al., 1995](#)); 6.9 m² of carbon black/g of lung tissue in female rats, with a 28% tumour incidence versus 0.46% in controls ([Heinrich et al., 1995](#)); 1.3 m² of ultrafine titanium dioxide/g of lung tissue in female rats, with a 19% tumour incidence versus 0.46% in controls ([Heinrich et al., 1995](#)); 1.2 m² of fine titanium dioxide/g of lung tissue, with a 16% and 17% tumour incidence in male and female rats versus 2% and 0% in male and female controls, respectively ([Lee et al., 1985a](#)); and 0.27 m² of talc/g of lung tissue in female rats, with a 26% tumour incidence versus 2% in controls ([NTP, 1993](#)). These comparisons show that the retained particle surface area dose in coal miners was lower by a factor of approximately 2–70 than that associated with elevated incidences of lung tumours in rats exposed to either fine or ultrafine poorly soluble particles. Thus, using particle surface area as the dose metric, an excess incidence of lung cancer would not necessarily be expected to be observed in coal miners

because of their relatively low particle surface area dose compared with that associated with lung tumours in rats.

These comparisons illustrate the importance of using normalized doses to compare responses across species. Furthermore, due to their faster clearance rate, rats do not attain lung burdens that are comparable with those observed in humans who work in dusty jobs (e.g. coal miners) unless they experience overloading of lung clearance.

(ii) *Relevance of mechanistic data to assess carcinogenic hazards in humans*

To evaluate the appropriateness of the rat as an experimental model to assess the carcinogenic hazards of poorly soluble particles in the lungs of humans, it is useful to evaluate the scientific evidence that allows for comparisons among species with regard to exposure, dose–response and mode of action.

Exposure–dose: Inhaled particles may present a hazard when they are deposited in sufficient quantities (dose) and interact with cells/tissues at responsive target sites along the respiratory tract. The relationship between exposure to particles and inhaled dose is described by the kinetics of particle deposition and clearance, and that retained at or within respiratory tract tissues. Inhaled and deposited particles are cleared from the normal lungs of healthy rats at a faster rate than those from humans. However, at high lung burdens, normal clearance from the rat lung can be impaired and overwhelmed, and, in time, effectively ceases. This phenomenon (termed ‘overload’) is observed with particles that are poorly soluble and are generally considered to be of low toxicity ([Morrow, 1988](#)).

Particle lung burdens observed in humans in some dusty jobs (e.g. coal mining) have sometimes approximated the overload dose in rats. At sufficient concentrations and durations of inhalation, the lungs of rats can accumulate higher levels of particles than the lung burdens seen in most workers. For ultrafine particles, the attained

mass doses associated with impaired clearance in rodents approximate those that could occur in workers. For any experimental model used for hazard assessment in humans or to evaluate dose–response relationships, it is important to evaluate doses in experimental animals that are comparable with those that may occur in humans.

Lung clearance can be impaired in humans and experimental animals for many reasons. In humans, toxic gases and particles have been shown to impair clearance by affecting normal cilia function, mucus rheology and phagocytosis. Ultrafine particles may be cleared less effectively than larger particles due to impaired phagocytosis (Renwick *et al.*, 2001, 2004; Geiser *et al.*, 2005).

Much more is known about overload in rats than in humans. Overwhelmed or impaired clearance in rats has been postulated to be a pivotal factor in the development of lung overload (Morrow, 1988). The same factors that can interfere with clearance in rats may contribute to mass dose accumulation in humans (e.g. the cytotoxicity of the material and/or ineffective phagocytosis). Overload was originally described in terms of mass- or volume-based dose. For fine and ultrafine carbon black and titanium dioxide, surface area dose has been shown to be a better predictor of impaired clearance (Oberdörster & Yu, 1990; Oberdörster *et al.*, 1992; Oberdörster, 1996; Tran *et al.*, 1999). Impaired clearance and overload are not unique to rats, and can also occur in other species, although to different degrees. For example, overload has not been observed in hamsters at concentrations at which it readily occurs in rats and mice (Bermudez *et al.*, 2002, 2004; Elder *et al.*, 2005). Although the behaviour of human lung clearance under similar circumstances is unclear, by analogy to coal workers, impairment of clearance occurs after chronic exposure and often persists long after the cessation of exposure (Freedman & Robinson, 1988).

Rats chronically exposed to sufficiently high concentrations of poorly soluble particles experience a steady reduction in their alveolar clearance rates and an accumulation of particles in the alveolar lumen and interstitium (Ferin *et al.*, 1992; Ferin, 1994; Warheit *et al.*, 1997; Bermudez *et al.*, 2002, 2004), and ultrafine particles translocate to the interstitium of rodents to a greater extent than fine particles (Ferin *et al.*, 1992; Oberdörster, 1996). In studies that compared the patterns of particle retention in the lungs of rats, monkeys and humans exposed to coal dust and/or diesel engine exhaust, the largest volume percentage of dust was observed in the alveolar lumen in rats and in the interstitium of monkeys and humans (Nikula *et al.*, 1997a, b, 2001). No data were available to compare the doses retained in the specific regions of the lung of each species. The biological significance of the interstitial/luminal distribution in the development of overload and toxic sequelae is not clear, either within a given species or among species.

Dose–response and mode of action: After continued inhalation of high concentrations of particles, rats that achieve overload may develop pulmonary fibrosis and both benign and malignant tumours (Lee *et al.*, 1985a, b, 1986; Warheit *et al.*, 1997). Oberdörster (1996, 2002) proposed that the high-dose effects observed in rats may be associated with two thresholds: (i) a pulmonary dose that results in a reduced macrophage-mediated clearance leading to overload; and (ii) a higher dose associated with overload, at which normal antioxidant defences within the lung are overwhelmed and pulmonary tumours may be induced.

As discussed above, the series of events that are involved in the biological process that begins with some particle deposition at critical target cells or tissues within the rat lung and results in rat lung tumours include: sustained inflammation, production of ROS, depletion of antioxidants and/or impairment of other defence mechanisms, cell proliferation and gene

mutations. These individual steps comprise an overall mode of action that can be used to compare the responses of rats with those of other species, including humans (see [Fig. 4.3](#)).

When the particle mass lung burden reaches a level at which overload is observed in rats (estimated to begin at ~0.5 mg/g of lung and to be fully developed at ~10 mg/g of lung; [Muhle et al., 1990b](#)), a sustained and widespread cellular inflammatory response occurs. The degree of sustained inflammation experienced by rodents (most notably rats) with high lung burdens is not observed in humans, although humans may experience sustained inflammation during certain diseases.

Interspecies extrapolation: Several studies have shown that rats, but not mice or hamsters, develop an excess incidence of lung cancer after exposure to chronic ‘overloading’ doses of inhaled poorly soluble particles. Several studies have discussed this phenomenon and the challenges it poses for the extrapolation of chronic effects in rats to the human situation ([Morrow, 1994](#); [Levy, 1995](#); [Oberdörster, 1995, 2002](#); [Watson & Valberg, 1996](#); [ILSI Risk Science Institute Workshop Participants, 2000](#); [Miller, 2000](#); [Hext et al., 2005](#)).

Uncertainty remains with regard to the detailed identification of the series of events that lead to lung cancer in rats following inhalation of poorly soluble particles (i.e. talc, carbon black and titanium dioxide). However, as shown in [Fig. 4.3](#), several important steps can be identified that are supported by a substantial rodent database. An important question that needs to be addressed is the extent to which the steps outlined in [Fig. 4.3](#) for rat lung cancer are also operative in other animal species, including humans. The majority of animal studies that have evaluated the effects of poorly soluble particles on the respiratory tract have been conducted in rats. It is therefore necessary to consider species differences, such as particle inhalability, breathing conditions, respiratory tract structure and pulmonary

defences, when extrapolating toxicological findings from rodents to humans ([Brown et al., 2005](#)).

All animal species that are routinely used in particle toxicology, as well as humans, are susceptible to the impairment of clearance of poorly soluble particles from the lungs. Impaired clearance is probably one of the first steps necessary to initiate a sequence of events that may lead to lung cancer in rats (see [Fig. 4.3](#)). Importantly, however, various animal species exhibit differences in particle-induced impairment of clearance, which can result in diverse lung burdens (expressed as mass or surface area) following exposures to the same particle concentrations. Similarly, pulmonary inflammation has been reported to be a consequence of exposures to poorly soluble particles in both experimental animals and humans. The pathophysiology of particle-induced fibrosis in humans and fibrosis and lung cancer in rats from lung overload involves chronic inflammation, hyperplasia and cell proliferation, and altered deposition and architecture of collagen.

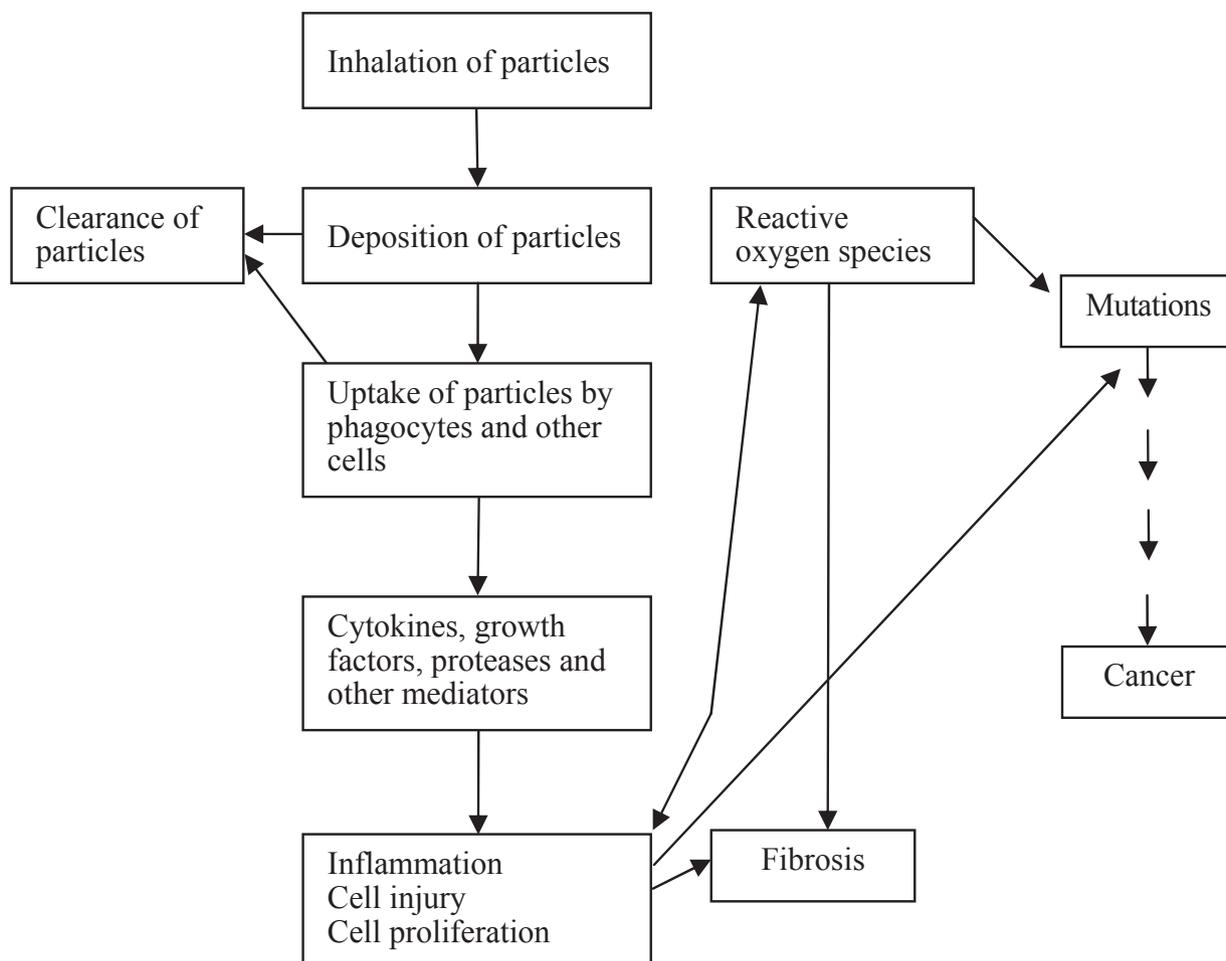
Rats and mice, in contrast to hamsters, exhibit sustained inflammation associated with particle lung burden, but lung tumours induced by poorly soluble particles have only been observed in rats. Rats have been shown to be uniquely susceptible to particle-induced lung cancer in comparison with mice and hamsters. Although some of the steps indicated in [Fig. 4.3](#) have been demonstrated in humans exposed to poorly soluble particles, it is not known to what extent humans are susceptible to particle-induced lung cancers.

(b) Gasoline engine exhaust

(i) Deposition

Limited evidence has suggested that the deposition rate of gasoline engine exhaust particles in rat lungs is 30% ([IARC, 1989](#)).

Fig. 4.3 Conceptual framework of carcinogenesis induced by poorly soluble particles in rats



The scheme represents the sequence of events and modes of action that are considered to be involved in the formation of tumours that are observed in the lungs of rats after high exposure to poorly soluble particles (see text for further details).

(ii) Clearance

A marginal increase in the half-time for pulmonary clearance of ferric oxide by rats but not hamsters was observed following exposure to gasoline engine particles (IARC, 1989).

(iii) Metabolism

The major DNA adduct derived from benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide was found in male BALB/c mice following topical application of a crude extract of gasoline engine exhaust over a period of 1–2 weeks (IARC, 1989).

Blood samples from Fischer rats and Syrian golden hamsters exposed to different dilutions of gasoline engine exhaust for 6–24 months were analysed for levels of 2-hydroxyethylvaline and 2-hydroxypropylvaline in haemoglobin. A dose-dependent increase in the level of haemoglobin adducts, corresponding to the metabolic conversion of ~5–10% of inhaled ethylene and propylene to ethylene oxide and propylene oxide, respectively, was observed (IARC, 1989).

Table 4.1 Summary of genetic and related effects of atmospheres containing engine exhaust (diesel only) in humans

End-point	Exposure	Result	Association with exposure		Reference
			Urine	Air	
Oxidative stress, inflammation (gene expression)	2-h exposure to diesel, 200 µg/m ³ PM _{2.5} , blood taken 6 h after start of exposure	↑	–	PM _{2.5} +	Peretz et al. (2007)
Oxidative stress, protein degradation, coagulation (gene expression)	2 × 1 h exposures to diesel, 300 µg/m ³ PM _{2.5} , blood taken 24 h after each exposure	↑	–	PM _{2.5} +	Pettit et al. (2012)
IL-8 gene, IL-8 and GRO-α protein	1-h exposure to diesel, 300 µg/m ³ PM ₁₀ , lavage 6 h after end of exposure	↑	–	PM ₁₀ +	Salvi et al. (2000)

GRO, growth-regulated oncogene; h, hour, IL, interleukin; PM, particulate matter; ↑, increase

4.3 Genetic and related effects

4.3.1 Humans

Various studies have identified chromosomal damage, such as micronuclei, sister chromatid exchange and chromosomal aberrations, as well as DNA adducts and oxidative damage in traffic policemen, gas station attendants and other workers exposed to traffic exhaust. Such workers are occupationally exposed to diesel and gasoline engine exhausts, but the Working Group noted that they were also exposed to ambient air that may have been especially high in engine exhaust content. Thus, the results of these studies must be viewed within that context. Only three studies involved exposure solely to engine (diesel) exhaust. Nevertheless, most of the studies found an adverse effect of exposure to engine exhaust through ambient air, and approximately half of the studies incorporated measurements of internal (urinary 1-hydroxypyrene) or external (e.g. PAHs in the air) exposure, most of which also showed an association between exposure and effect.

Fewer than half of the studies included a measure of either internal or external exposure, but the majority of these and most studies that provided exposure assessments found that

individuals with chronic exposure to engine exhaust had increased frequencies of a variety of genotoxic biomarkers, and the latter also showed an association between the measured biomarkers and exposure (see [Tables 4.1](#) and [4.2](#)). Positive associations between biomarkers of exposure and genetic effects were also observed in the majority of the studies across all three exposure categories.

(a) Diesel only

See [Table 4.1](#)

Changes in gene expression

An experimental study in which five subjects were exposed for 2 hours to 200 µg/m³ of PM_{2.5} from diesel engine exhaust (derived from a 2002 model turbocharged direct-injection 5.9-L Cummins B-series engine), blood samples were taken 6 hours after the initiation of the exposure and showed changes in the expression of genes involved in oxidative stress, inflammation, leukocyte activation and vascular homeostasis, which were associated with the levels of PM_{2.5} in the air ([Peretz et al., 2007](#)). In another experimental study ([Pettit et al., 2012](#)), 14 healthy subjects were exposed to 300 µg/m³ of PM_{2.5} from diesel engine exhaust (derived from a 5500-W Yanmar electricity generator, with a 406-ml displacement

Table 4.2 Summary of genetic and related effects of atmospheres containing engine exhaust (predominantly diesel) in humans

End-point	Exposure group	Result	Association with exposure		Reference
			Urine	Air	
DNA adducts	Bus/automobile garage workers	+	OHPy –		Hemminki et al. (1994b)
		+			Hou et al. (1995)
		+			Nielsen et al. (1996a)
		–			Schoket et al. (1999)
	Diesel vehicle drivers in a mine	–	1-NP –	Knudsen et al. (2005)	
DNA damage	Diesel vehicle drivers in a mine	+		1-NP +	Knudsen et al. (2005)
	Road tunnel construction workers	–			Villarini et al. (2008)
<i>Hprt</i> mutant frequency	Bus maintenance workers	–			Hou et al. (1995)
<i>Hprt</i> spectra		+			Österholm et al. (1995)
Urinary mutagenicity	Railroad workers	–		PM –	Schenker et al. (1992)
Urinary/faecal mutagenicity	Car mechanics	–		PM –	Willems et al. (1989)
Sister chromatid exchange	Tunnel construction workers	–			Villarini et al. (2008)
MN buccal lymph		+	OHPy +	PAH +	Schoket et al. (1999)
MN lymph	Train mechanics	+			Lu et al. (1999a)
	Road tunnel construction workers	+			Villarini et al. (2008)

HPRT, hypoxanthine-guanine phosphoribosyltransferase; MN, micronuclei; 1-NP, 1-nitropyrene; OHPy, 1-hydroxypyrene; PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; +, positive; –, negative

air-cooled engine) for 1 hour a day, during 2 days at an interval of at least 1 week (total exposure, 2 hours). Blood was drawn 24 hours after each exposure for the analysis of gene expression, which indicated that genes involved in oxidative stress, protein degradation and coagulation pathways were differentially expressed. Thus, both experimental studies found that exposure to engine exhausts caused alterations in the expression of genes involved in oxidative stress.

[Salvi et al. \(2000\)](#) exposed 15 healthy subjects to 300 µg/m³ of PM₁₀ from diesel engine exhaust (derived from an idling Volvo diesel engine) for 1 hour. Bronchial lavage was performed 6 hours after the end of the exposure, and the expression of selected gene and protein was assessed. Upregulation of the interleukin (IL)-8 gene, IL-8 protein and growth-regulated oncogene-α protein was observed and provided evidence of

an inflammatory response due to exposure to diesel engine exhaust.

(b) *Predominantly diesel*

See [Table 4.2](#)

(i) *DNA adducts*

The levels of DNA adducts determined in lymphocytes by postlabelling were elevated in bus and truck terminal workers compared with hospital mechanics who were used as a control. The highest levels of adducts were found for garage workers among the bus maintenance workers and for those who drove diesel forklifts among the terminal workers ([Hemminki et al., 1994b](#)). Another study also found elevated levels of DNA adducts (determined by postlabelling in lymphocytes) in bus maintenance workers exposed to diesel engine exhaust compared with controls ([Hou et al., 1995](#)).

Workers and mechanics in a diesel bus garage had elevated levels of DNA adducts (determined in lymphocytes by postlabelling) compared with administrative workers in the same facility. Elevated levels of hydroxyethylvaline adducts in haemoglobin were also found among the garage workers compared with the controls and were associated with the concentrations of 1-hydroxypyrene in the urine, whereas the levels of DNA adducts were not. Postlabelling was the most sensitive assay, and the authors noted that dermal exposure to oils and grease might be an important route of exposure to PAHs and other chemical classes in addition to the inhalation of diesel engine exhaust (Nielsen *et al.*, 1996a). A study of garage mechanics in another country did not find an increase in DNA adducts (determined by postlabelling in lymphocytes; Schoket *et al.*, 1999).

The level of 1-nitropyrene–DNA adducts (determined by HPLC of postlabelled lymphocytes) was not higher in shale-oil miners who drove diesel-powered excavation machines in the mine than in surface workers (Knudsen *et al.*, 2005).

(ii) DNA damage

A study of shale-oil mine workers exposed to diesel engine exhaust found that DNA damage, as measured by the comet assay in lymphocytes, was higher among smoking miners who drove diesel-powered excavation machines compared with smoking surface workers, and the levels of DNA damage were associated with the levels of 1-nitropyrene in the air. A decrease in DNA-repair capacity (as determined by the cytogenetic challenge assay; i.e. an increase in dicentrics and deletions) was also observed, and a relationship between the increase in DNA damage, the decrease in DNA-repair capacity, the levels of 1-hydroxypyrene in the urine and the levels of PAHs in the air was established (Knudsen *et al.*, 2005). A study of road tunnel construction workers in Genoa, Italy, found no

increase in DNA damage (as measured by the comet assay in lymphocytes) compared with office controls (Villarini *et al.*, 2008).

(iii) Mutagenicity

No significant difference in mutant frequency at the *Hprt* gene (assessed in lymphocytes) was found between bus maintenance workers exposed to diesel engine exhaust and controls (Hou *et al.*, 1995), whereas splice mutations were more frequent among the garage workers compared with controls (Österholm *et al.*, 1995).

No increase in urinary mutagenicity in *Salmonella typhimurium* TA98 in the presence of an exogenous metabolic activation system was found among a population of railroad workers with a wide range of exposures to diesel engine exhaust (Schenker *et al.*, 1992). Similarly, no increase in either urinary or faecal mutagenicity in *S. typhimurium* TA98 in the presence of an exogenous metabolic activation system was found among a group of car mechanics exposed to high concentrations of diesel engine exhaust compared with a group of office workers (Willems *et al.*, 1989).

(iv) Chromosomal effects

A study in Genoa, Italy, did not find an increased frequency of sister chromatid exchange in the lymphocytes of road tunnel construction workers compared with office controls (Villarini *et al.*, 2008).

Garage mechanics in Budapest, Hungary, had elevated frequencies of micronuclei in their lymphocytes, which correlated with the levels of 1-hydroxypyrene in the urine and PAHs in the air (Schoket *et al.*, 1999). Increased frequencies of micronuclei were found in diesel train attendants and wheel axle workers in Henan, China (Lu *et al.*, 1999a), as well as in road tunnel construction workers in Genoa, Italy (Villarini *et al.*, 2008).

(c) *Mixed exposures*

See [Table 4.3](#)

(i) *DNA adducts*

An early study of adducts in the DNA and haemoglobin of bus and taxi drivers in Stockholm, Sweden, found elevated levels of DNA adducts (by postlabelling in lymphocytes) in suburban bus drivers and taxi drivers (covering a mixed route) but not in city bus drivers compared with controls from a mechanical workshop in a hospital ([Hemminki et al., 1994a](#)).

Taxi drivers also had elevated levels of PAH-plasma protein adducts determined by an immunoassay. Alkylvaline levels in the haemoglobin of urban bus drivers did not differ significantly from those of the controls. A study of bus drivers in Copenhagen, Denmark, found significantly elevated levels of DNA adducts (by postlabelling in lymphocytes) in drivers in central and suburban Copenhagen compared with rural controls, and drivers in central Copenhagen had more adducts than suburban drivers ([Nielsen et al., 1996b](#)).

Traffic police in Genoa, Italy, had significantly higher levels of DNA adducts (by postlabelling in lymphocytes) compared with office workers in the same city; however, the levels of DNA adducts were not associated with the levels of benzo[*a*]pyrene in the air ([Merlo et al., 1997](#)). A similar study of traffic police in Genoa showed that the increased levels of adducts were related to seasonal variations, with higher levels occurring in the summer, suggesting a role of atmospheric transformation products, and were associated with the levels of benzo[*a*]pyrene in the air ([Peluso et al., 1998](#)). Traffic police in Prague, Czech Republic, had levels of benzo[*a*]pyrene-like DNA adducts (determined by postlabelling in lymphocytes) that correlated with the levels of carcinogenic PAHs in the air determined from personal samplers ([Topinka et al., 2007](#)).

In Milan, Italy, newspaper vendors working < 50 m from streets with high traffic flow (> 1300

vehicles/hour) did not have elevated levels of DNA adducts (by postlabelling in lymphocytes) compared with those working along streets with low traffic flow (< 1300 vehicles/hour) ([Yang et al., 1996](#)).

(ii) *DNA damage*

A study by [Kim et al. \(2004\)](#) showed that DNA damage (assessed as Olive tail moment by the comet assay in lymphocytes) was greater among automobile emission inspectors than control subjects, and the levels were associated with the urinary markers of exposure (1-hydroxypyrene and 2-naphthol). DNA damage (measured by the comet assay; both percentage of cells damaged and Olive tail moment) in lymphocytes was greater in traffic police than in resident controls in Shanghai, China, and was associated with the level of exposure to PM_{2.5} determined by personal monitors ([Li et al., 2010](#)).

An increase in urinary 1-hydroxypyrene and DNA damage (as measured by the comet assay in lymphocytes) was found in children at a school located near areas of high-density traffic in Bangkok, Thailand, compared with children at a school in the provincial area of Chonburi ([Tuntawiroon et al., 2007](#)).

Urinary excretion of 8-OH-dG, which is a biomarker of oxidative DNA damage, was increased among bus drivers from central Copenhagen, Denmark, compared with drivers from suburban/rural areas around Copenhagen ([Loft et al., 1999](#)), and was increased threefold in nonsmoking security guards who worked at an entrance gate by a road with heavy traffic following an 8-hour work shift. The latter increase in DNA damage was associated with the concentration of PAHs, PM_{2.5} and metals, but not polar organics, in the air at the site ([Wei et al., 2009](#)).

An analysis of lung tissue from nonsmokers with lung cancer found carbon particles that were morphologically similar to those of diesel engine exhaust-like particles; the mass of particles and the concentration of 8-OH-dG in the lung DNA

Table 4.3 Summary of genetic and related effects of atmospheres containing engine exhaust (mixed) in humans

End-point	Exposure group	Result	Association with exposure		Reference
			Urine	Air	
DNA adducts	Bus/taxi drivers	+			Hemminki et al. (1994a)
	Bus drivers	+			Nielsen et al. (1996b)
	Traffic police	+		B[a]P +	Peluso et al. (1998)
		+		PAH +	Topinka et al. (2007)
		+		B[a]P -	Merlo et al. (1997)
Newspaper vendors (street)	-			Yang et al. (1996)	
DNA damage	Auto-emission inspectors	+	OHPy + 2Naph +		Kim et al. (2004)
	Traffic police	+		PM _{2.5} +	Li et al. (2010)
	Children in school (high traffic density area)	+	OHPy +		Tuntawiroon et al. (2007)
8-OH-dG	Bus drivers	+			Loft et al. (1999)
	Security guards	+		PM _{2.5} + PAH + Metals +	Wei et al. (2009)
Oxidative damage	Cancer patients	+			Tokiwa et al. (2005)
	Bicycling in traffic	+		PM ₁₀ +	Vinzents et al. (2005)
	Traffic police	+			Yang et al. (2005)
Urinary mutagenicity	Airport personnel	+	OHPy +	PAH +	Cavallo et al. (2006)
	Bus drivers	+	OHPy -		Hansen et al. (2004)
Chromosomal aberrations	Traffic police	+			Tokiwa et al. (1999)
		+			Anwar & Kamal (1988)
		+			Burgaz et al. (2002)
		+			Chen et al. (1999)
		+			Fu et al. (1999)
		+			Luo & Shi (1994)
	+			Sree Devi et al. (2009)	
Taxi drivers	+	OHPy +		Burgaz et al. (2002)	
Gas station attendants	+			Santos-Mello & Cavalcante (1992)	

Table 4.3 (continued)

End-point	Exposure group	Result	Association with exposure		Reference
			Urine	Air	
Sister chromatid exchange	Petrochemical workers	+			Sobti & Bhardwaj (1993)
	Airport personnel	+	OHPy +	PAH +	Cavallo <i>et al.</i> (2006)
	Traffic police	+			Anwar & Kamal (1988)
		+			Chandrasekaran <i>et al.</i> (1996)
		+			Zhao <i>et al.</i> (1998)
		+			Sreedevi <i>et al.</i> (2006)
		+			Soogarun <i>et al.</i> (2006)
		+			Anbazhagan <i>et al.</i> (2010)
		–			Bolognesi <i>et al.</i> (1997a)
	Airport personnel	+	OHPy +	B[a]P – PAH +	Cavallo <i>et al.</i> (2006)
MN buccal	Traffic police	+			Karahalil <i>et al.</i> (1999)
		+			Hallare <i>et al.</i> (2009)
	Gas station attendants	+			Hallare <i>et al.</i> (2009)
		+			Sellappa <i>et al.</i> (2010)
	Auto mechanics	+			Karahalil <i>et al.</i> (1999)
	Taxi drivers	+			Karahalil <i>et al.</i> (1999)
	Firebreathers	–			Torres-Bugarín <i>et al.</i> (1998)
Gene expression	Auto-emission inspection workers	↑	OHPy + 2Naph +		Kim <i>et al.</i> (2004)

B[a]P, benzo[a]pyrene; MN, micronuclei; 1-NP, 1-nitropyrene; 2Naph, 2-naphthol; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; OHPy, 1-hydroxypyrene; PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; +, positive; –, negative; ↑, increase

of cancerous tissue, but not of the adjacent non-cancerous tissue, increased with the age of the patient. The lung particles were stripped of organic compounds and induced the formation of 8-OH-dG when administered to mice. The authors suggested that the 8-OH-dG found in the lung DNA of cancer patients was formed by the generation of hydroxyl radicals that were produced after inflammatory cells phagocytized non-mutagenic particles; mutation leading to lung cancer might then have been induced subsequently by unrepaired 8-OH-dG ([Tokiwa et al., 2005](#)).

An evaluation of subjects who cycled in traffic in Copenhagen, Denmark, with personal sampling monitors found an association between the concentration of ultrafine particles (PM₁₀) and purine oxidation (oxidative damage) in their lymphocytes (determined by the comet assay). However, no association was found between PM₁₀ and DNA strand breaks (also determined by the comet assay; [Vinzents et al., 2005](#)). Increased oxidative damage was found among traffic police in Henan Province, China, as assessed by the levels of superoxide dismutase and malondialdehyde in blood serum ([Yang et al., 2005](#)).

Outdoor airport workers in Rome, Italy, had higher levels of urinary 1-hydroxypyrene than airport office workers, as well as increased levels of oxidative and direct DNA damage (as measured by the comet assay) in both buccal cells and lymphocytes. An association was also found between these two biomarkers and the level of PAHs in the air ([Cavallo et al., 2006](#)).

(iii) Mutagenicity

The urine of bus drivers in central Copenhagen, Denmark, showed higher levels of mutagenicity in the presence of an exogenous metabolic activation system in *S. typhimurium* YG1021 (a frameshift strain that expresses excess nitroreductase) than mail carriers, but these were not associated with levels of urinary 1-hydroxypyrene ([Hansen et al., 2004](#)).

A unique *ex-vivo* study of tissues from lung cancer patients found that extracts of lung tissue were mutagenic in the *Salmonella* mutagenicity assay in a strain that responds to ROS. A simultaneous analysis suggested that the deposition of particles in the tissue correlated with the mutagenicity of the extracts. In addition, 1-nitropyrene, which may be indicative of exposure to diesel engine exhaust, was present in all of the tissues ([Tokiwa et al., 1999](#)).

(iv) Chromosomal effects

Increased frequencies of chromosomal aberrations have been found in the lymphocytes of traffic policemen in Cairo, Egypt ([Anwar & Kamal, 1988](#)), Shanghai District ([Luo & Shi, 1994](#)) and Henan Province ([Chen et al., 1999](#); [Fu et al., 1999](#)), China, Ankara, Turkey ([Burgaz et al., 2002](#)) and Hyderabad, India ([Sree Devi et al., 2009](#)) in comparison with controls. Elevated chromosomal aberrations frequencies have also been found among gas station attendants in Rio de Janeiro and São Paulo, Brazil ([Santos-Mello & Cavalcante, 1992](#)), petrochemical workers from scooter markets in Chandigarh, India ([Sobti & Bhardwaj, 1993](#)), taxi drivers in Ankara, Turkey ([Burgaz et al., 2002](#)), and outdoor airport workers in Rome, Italy ([Cavallo et al., 2006](#)). The increase in chromosomal aberrations in the taxi drivers in Turkey was associated with their urinary levels of 1-hydroxypyrene, and that in airport workers in Italy were associated with the levels of both urinary 1-hydroxypyrene and PAHs in the air.

Increased frequencies of sister chromatid exchange compared with controls have been found in the lymphocytes of traffic policemen in Cairo, Egypt ([Anwar & Kamal, 1988](#)), Madras, India ([Chandrasekaran et al., 1996](#)), Lanzhou, China ([Zhao et al., 1998](#)), Bangkok, Thailand ([Soogarun et al., 2006](#)), and Hyderabad ([Sreedevi et al., 2006](#)) and Chennai city ([Anbazhagan et al., 2010](#)), India. An increase in sister chromatid exchange was also found among outdoor airport workers compared with office workers in Rome,

Italy, and was associated with increased levels of urinary 1-hydroxypyrene and PAHs in the air (Cavallo *et al.*, 2006). No increase in sister chromatid exchange was found among traffic policemen in Genoa, Italy (Bolognesi *et al.*, 1997a).

Increased frequencies of micronuclei have been found in the buccal cells of engine repair workers, taxi drivers and traffic police in Ankara, Turkey (Karahalil *et al.*, 1999), gasoline station attendants and traffic police in Manila, the Philippines (Hallare *et al.*, 2009), and gasoline station attendants in Coimbatore, India (Sellappa *et al.*, 2010). Although increased frequencies of micronuclei were found in the lymphocytes of traffic policemen in Lanzhou, China (Zhao *et al.*, 1998), diesel train attendants and wheel axle workers in Henan, China (Lu *et al.*, 1999a), and road tunnel construction workers in Genoa, Italy (Villarini *et al.*, 2008), no increase was found among traffic police in Genoa, Italy (Bolognesi *et al.*, 1997b).

No increase in micronuclei in either buccal cells or lymphocytes was found among outdoor airport workers compared with office workers in Rome, Italy (Cavallo *et al.*, 2006), or in buccal-cell micronuclei among 'firebreathers' who used diesel fuel (Torres-Bugarín *et al.*, 1998).

(v) Changes in gene expression

Examination of changes in global gene expression in the lymphocytes and proteomic changes in the blood plasma of automobile emission inspectors in Seoul, Republic of Korea, revealed the upregulation of genes and proteins involved in oxidative stress. The upregulated genes included *integrin-linked kinase*, *CYP2F1*, *CYP2D6*, *IL-1 receptor-associated kinase* and *antioxidant protein 2*. Two proteins in particular were upregulated (transthyretin and sarcolectin) and one was downregulated (haptoglobin-1). The changes in both gene and protein expression were associated with the levels of 1-hydroxypyrene and 2-naphthol in the urine (Kim *et al.*, 2004).

4.3.2 Experimental systems

(a) Introduction and brief summary of IARC Monographs Volume 46

Before the publication of Volume 46 (IARC, 1989), several comprehensive reviews had provided excellent summaries regarding the genotoxic activity of emissions released by diesel- and gasoline-driven internal combustion engines (e.g. Claxton, 1983; Lewtas *et al.*, 1981; Lewtas, 1983; Lewtas & Williams, 1986). IARC (1989) noted that the soluble organic fraction of PM from mobile-source emissions readily induced mutations in the *S. typhimurium* reverse mutation assay. *S. typhimurium* TA98 and TA100 were the most responsive strains, and the mutagenic activity was generally higher in the absence of a mammalian metabolic activation system (e.g. from induced rat liver). Chemical fractionation of PM extracts and analysis of the mutagenicity of these fractions with the nitroreductase-deficient *S. typhimurium* strain TA98 led to the recognition that nitroarenes are major contributors to the mutagenic activity of diesel engine exhaust particulates. Examination of the factors that modify the mutagenicity of mobile-source emissions revealed that fuel formulation, type and degree of combustion, ambient environmental conditions and the methods of sample collection can all influence the mutagenic activity of the soluble organic fraction of mobile-source PM. Briefly, the mutagenic activity of the soluble organic fractions from mobile-source PM samples was shown to increase with increasing aromatic content of the fuel, and to vary across engine types and between runs with the same engine. The mutagenic activity of organic extracts from gasoline-derived PM was found to be similar to that of diesel engine exhaust particles. However, when differences in PM emission levels between gasoline and diesel vehicles were taken into account, the mutagenic activity of diesel PM (i.e. per unit mass or per mile driven) was found to be much greater than that of gasoline PM.

[IARC \(1989\)](#) included an overview of several comprehensive studies by Lewtas and colleagues. These authors examined the mutagenic activity of exhausts from several diesel and spark-ignition (gasoline) engines in numerous *in vitro* assay systems including the *Salmonella* reverse mutation assay, the WP2 mutagenicity assay in *Escherichia coli*, the thymidine kinase (*Tk*^{+/-}) gene mutation assay in L5178Y mouse lymphoma cells, the ouabain-resistance mutagenicity assay in BALB/c 3T3 cells, the *Hprt* gene mutation assay in Chinese hamster ovary cells, the mitotic recombination assay in *Saccharomyces cerevisiae* strain D3, the DNA strand-break assay (assessed by means of alkaline elution) in Syrian hamster embryo cells, the test for unscheduled DNA synthesis in primary rat hepatocytes, sister chromatid exchange and chromosomal aberration assays in Chinese hamster ovary cells and the chromosomal aberration assay in cultured human lymphocytes. The *Salmonella* mutagenicity results showed that mutagenic activity per unit extractable organic matter (EOM) varied across engine types with no appreciable difference between gasoline- and diesel-derived PM samples; three out of four diesel-derived samples were more active in the presence of exogenous metabolic activation. [IARC \(1989\)](#) also noted that particle emission levels (per mile driven) from diesel vehicles can be 100-fold higher than those of gasoline vehicles, yielding mutagenic activities that are 45–800-fold higher. The results of the assays in mammalian cells indicated that organic PM extracts from both gasoline and diesel vehicles can induce DNA strand breaks, gene mutations and chromosomal damage. However, the test outcome and the magnitude of the response were dependent on engine type and the end-point examined. Several samples induced significant increases in *Tk*^{+/-} mutations in mouse lymphoma cells; the diesel-derived material was more potent in the absence of exogenous metabolic activation and gasoline-derived samples giving a stronger response in the presence of metabolic activation.

In the presence of metabolic activation, the gasoline samples were more potent than the diesel samples. A similar pattern was observed for the ouabain-resistance assay. PM extracts from two out of four diesel engines induced significant increases in *Hprt* mutations in Chinese hamster ovary cells, whereas gasoline-derived samples did not. DNA-damage assays indicated that one gasoline emission sample elicited a significant increase in the number of DNA strand breaks in Syrian hamster embryo cells. Only one diesel sample induced a significant increase in unscheduled DNA synthesis in primary rat hepatocytes. Assays for chromosomal effects indicated that both diesel- and gasoline-derived samples induced a significant increase in sister chromatid exchange; this effect diminished in the presence of exogenous metabolic activation. Only two diesel samples were examined for their ability to induce chromosomal aberrations: one induced a significant increase in aberrations in Chinese hamster ovary cells and the other had a similar effect in cultured human lymphocytes. In the absence of exogenous metabolic activation, two out of three extracts of diesel-derived PM induced a significant increase in mitotic recombination in *Saccharomyces cerevisiae*, while the third sample was weakly positive. The sole extract of gasoline-derived PM that was tested failed to induce mitotic recombination in this assay. Additional analyses showed that diesel PM extracts did not induce gene mutations or gene conversion at the *Trp-6* and *Ilv1-92* loci in *Saccharomyces cerevisiae* strain D7.

[IARC \(1989\)](#) also summarized the results of *in-vivo* assays to assess the mutagenicity of diesel engine emissions. Whole emissions and gaseous emission fractions (i.e. filtered exhaust) induced chromosomal damage and stamen-hair mutations in *Tradescantia*, but failed to induce sex-linked recessive lethal mutations in *Drosophila melanogaster*. Experiments with mice exposed *in vivo* indicated that whole emissions failed to induce chromosomal damage or

sister chromatid exchange in bone marrow, or specific locus mutations. In addition, the dominant lethal and heritable translocation assays in mice showed that, under the conditions of the test, whole emissions did not induce a significant increase in mutation or chromosomal damage. However, extractable organic compounds from diesel engine exhaust particles did induce significant increases in chromosomal damage in mouse bone marrow. *In-vivo* experiments with Syrian or Chinese hamsters indicated that whole emissions increase the frequency of sister chromatid exchange in lung cells, but did not induce sister chromatid exchange in fetal liver or chromosomal damage in bone marrow. In contrast, extractable organic compounds induced a weak increase in chromosomal damage in bone marrow, and significant increases in sister chromatid exchange in lung and fetal liver cells.

These studies indicated that the extractable organic fraction of particulate material released by both diesel and gasoline engine emissions can induce a variety of genetic effects (e.g. DNA damage, gene mutations, sister chromatid exchange, chromosomal aberrations and mitotic recombination) in a wide range of experimental systems *in vitro* and *in vivo* (e.g. bacteria, yeast, cultured mammalian cells and experimental animals). Although the magnitude of the induced effects varied with assay type (i.e. *in vitro* versus *in vivo*), end-point (i.e. strand breaks, mutations and chromosomal damage), engine type (i.e. gasoline or diesel), exposure conditions (i.e. whole exhaust, PM extracts or filtered exhaust) and metabolic capacity of the experimental system (i.e. in the presence or absence of exogenous activation *in vitro*), several general statements can be made.

First, the soluble organic fraction of diesel-derived PM generally induced genotoxic effects *in vitro* (e.g. mutations in *Salmonella*) in the absence of exogenous metabolic activation. Second, chemical fractionation of diesel engine exhaust particulate extracts indicated

that the most potent activity was associated with the moderately polar and highly polar fractions, and, moreover, that nitroarenes such as mono- and dinitro-PAHs often accounted for a substantial proportion of the observed genotoxic responses. Third, the soluble organic fraction of gasoline-derived PM was also genotoxic, but, in this case, exogenous metabolic activation *in vitro* generally enhanced the magnitude of the response. Fourth, fractionation of extracts from gasoline-derived PM showed that the mutagenic activity was associated with the PAH-containing neutral aromatic fraction, and catalytic treatment of gasoline exhausts significantly reduced the emission of mutagenic material.

Since [IARC \(1989\)](#), more than 200 scientific papers and reports (e.g. from the Health Effects Institute and the Society of Automotive Engineers) have used a wide range of experimental systems to examine the genetic and related effects of exposure to diesel and gasoline engine emissions. These findings are summarized below.

Studies investigating the genotoxicity of engine emissions can be conducted on diluted exhaust, exhaust PM, filtered exhaust (i.e. the gaseous and volatile portions), PM extracts or semi-volatile organic concentrates adsorbed onto a solid matrix (e.g. XAD resin) or a chilled surface. The most sophisticated *in-vivo* studies are inhalation experiments, during which animals are exposed to diluted engine emissions. These studies require complex installations to collect, dilute and deliver emissions effectively to the experimental animals. Moreover, highly technical equipment is required to control engine speed and load (i.e. test cycle), to monitor diluted emissions for particle concentration and size distribution and to determine the concentration of selected exhaust gases. Exposures can be 'whole-body' or 'nose-only'. Doses delivered via inhalation exposure are generally expressed in terms of milligrams of PM per cubic metre of air in the exposure chamber, with additional

information on the duration and frequency of the exposure. Other *in-vivo* studies may involve the delivery of suspensions of exhaust PM or PM extracts to the pulmonary system via intratracheal instillation, or the delivery of PM or PM extracts via oral gavage, dietary intake, topical application or intraperitoneal injection. Intratracheal, intraperitoneal or dietary doses are generally expressed as total milligrams of PM delivered/consumed, or as milligrams of PM ingested per kilogram of body weight (mg/kg bw).

The majority of *in vitro* assessments of effects elicited by engine emissions involve exposures of cells suspended in liquid medium, cells attached to solid culture surfaces (e.g. polystyrene) or cells embedded in agar. More recently, it has become possible to maintain cultured cells, including primary human cells or three-dimensional constructs of pulmonary tissue (e.g. EpiAirway), on semi-permeable membranes and expose the cells at an air-liquid interface ([Aufderheide & Mohr, 1999, 2000](#); [Aufderheide et al., 2003](#); [Bakand et al., 2006](#)). Such systems (e.g. VitroCell® or Cultex®) have been employed to examine the toxicity of engine emissions, but rarely to determine genetic and related effects ([Knebel et al., 2002](#); [Seagrave et al., 2007](#); [Tsukue et al., 2010a](#)). Thus, most *in vitro* assessments involve exposures to collected PM, organic extracts of PM, semi-volatile organic concentrates or, in rare instances, gaseous emissions bubbled through liquid culture medium. Collection of PM can present a substantial technical challenge, and most studies collect PM on glassfibre filters during the filtration of diluted exhaust. In some instances, bulk PM is collected by means of devices such as cascade impactors. In either case, the preparation of PM extracts generally involves Soxhlet extraction, sonication extraction or pressurized fluid extraction with solvents such as dichloromethane, acetone, hexane, ethanol, methanol or solvent mixtures. Extracts are generally exchanged with a bioassay-compatible

solvent such as dimethyl sulfoxide (DMSO) before testing. Semi-volatile organic concentrates are generally collected by passing filtered exhaust over a solid adsorbent matrix (e.g. XAD resin) followed by solvent elution, concentration and exchange with DMSO. [The Working Group noted that sample collection and processing can introduce alterations in samples of engine emissions, which may modulate/affect the genetic and related effects observed in experimental systems.]

The units of concentration employed for *in vitro* assessments of genetic and related effects induced by engine emissions vary depending on the nature of the test substance and the experimental system. Exposure concentrations for PM suspensions are generally expressed as mass of PM (in micrograms or milligrams) per assay unit (e.g. per agar plate or per millilitre of culture medium). Those for organic PM extracts are often given as micrograms of EOM or microlitres of extract per assay unit. Measures of EOM per unit mass of PM can then be used to convert these concentrations into equivalent milligrams of PM per assay unit. In addition, measures of engine work, engine run-time, fuel consumption or distance travelled can be used to convert exposure concentrations to equivalent amounts of engine work in kilowatt-hours (kW-h) or horse power-hour (hp-h), equivalent volume of fuel consumed, equivalent hour of engine run-time or equivalent distance travelled. Concentrations for aqueous extracts of PM are generally expressed as milligrams of PM equivalent per assay unit, again with the possibility of converting to units of engine work, engine run-time, fuel consumed or distance travelled. Concentrations of semi-volatile organic concentrates collected by adsorption on solid resins (e.g. XAD polystyrene copolymer) are generally expressed as micrograms of EOM per assay unit.

The information in the following sections and accompanying tables is primarily organized by route of exposure (i.e. inhalation, intratracheal instillation, topical treatment, oral

administration, *in vitro* treatment of cultured cells or isolated DNA), and secondarily by end-point (i.e. gene mutation, chromosomal damage, DNA damage and DNA strand breaks).

(b) *Diesel engine emissions*

(i) *Effects observed in vivo in experimental animals*

Diesel engine exhaust particulate matter

The results of relevant studies published since 1989 are described below, and are summarized in [Table 4.4](#).

The induction by emissions from a four-cylinder light-duty direct injection diesel engine of mutations at the *lacI* and *guanine phosphoribosyl transferase (gpt)* loci was assessed in transgenic BigBlue® rats and *gpt* delta mice, respectively ([Sato et al., 2000](#); [Hashimoto et al., 2007](#)). *LacI* mutations, stable DNA adducts and oxidative DNA damage were studied in the lungs of rats following whole-body exposure to diesel engine exhaust at 1 or 6 mg/m³ of PM for 4 weeks (12 hours per day, 7 days a week); a significant increase in *lacI* mutation frequency was observed with the higher dose, and significant increases in aromatic DNA adducts (measured by [³²P]-postlabelling) and oxidative DNA lesions (measured as 8-OH-dG/deoxyguanosine by HPLC with electrochemical detection) were found with both exposure levels. Analyses of the *lacI* mutations revealed a high frequency of AT→GC and GC→AT transitions and GC→TA transversions. *Gpt* mutations were examined in the lungs of mice following whole-body exposure to diesel engine exhaust at 1 or 3 mg/m³ of PM for 4, 12 and 24 weeks (12 hours per day, 7 days a week); significant increases were found in *gpt* mutant frequency following all exposures, with a peak response at 12 weeks. Analyses of the *gpt* mutations showed a high frequency of GC→TA transversions, reaching 77% at 24 weeks.

Changes in gene expression in the lungs of Fischer 344 rats and the induction of stable DNA

adducts in *Nrf2* knock-out mice were examined after exposure to diesel engine exhaust ([Sato et al., 1999](#); [Aoki et al., 2001](#)). The rats were exposed to 6 mg/m³ of PM for 4 weeks (12 hours per day, 7 days a week), and a significant upregulation of the proto-oncogene *Araf* was noted. The *Nrf2* knock-out mice (both *Nrf2*^{-/-} and *Nrf2*^{+/-}) were exposed by whole-body inhalation to 3 mg/m³ of PM for 4 weeks (12 hours per day, 7 days a week), and a significant increase in stable DNA adducts (measured by [³²P]-postlabelling) was observed, with the highest levels in *Nrf2*-null (-/-) animals.

The induction of DNA damage was also assessed in rats exposed to diluted diesel engine emissions. [Gallagher et al. \(1993\)](#) examined the frequency of stable DNA adducts in the lungs of CDdl(WI)Br rats exposed to 7.5 mg/m³ of soot for 24 months. A major nuclease-P1-sensitive adduct was detected in lung DNA, which was suspected to be derived from nitro-PAHs. [Gallagher et al. \(1994\)](#) assessed the frequency of stable adducts in the lungs of Wistar rats exposed to diesel engine exhaust (particle concentration, 7.5 mg/m³) for 2, 6 and 24 months (18 hours per day, 5 days a week), and found no significant increase in PAH-derived adducts, but a modest increase in adducts assumed to originate from nitro-PAHs. [Iwai et al. \(2000\)](#) studied the frequency of stable DNA adducts and oxidative DNA damage (i.e. 8-OH-dG) in the lungs of Fischer 344 rats exposed to 3.5 mg/m³ of PM for 1, 3, 6, 9 and 12 months (17 hours per day overnight, 3 days a week), and observed an increase in oxidative damage that reached a plateau after 9 months. The amount of bulky DNA adducts (measured by [³²P]-postlabelling/HPLC) peaked after 1 month and then declined.

The induction of heritable mutations was studied in C57BL mice exposed to diluted emissions from a single-cylinder research engine ([Hedenskog et al., 1997](#)). Mutations in the offspring of male mice were scored as effects on pre- and post-meiotic sperm at the hyper-variable expanded simple tandem repeat loci, *PcI*

Table 4.4 Summary of studies in animals exposed *in vivo* to diesel emissions or diesel exhaust particulate matter

Engine type and specifications	Run conditions	Test material collection and processing	Animal model	Exposure regime	End-points examined	Results	Reference
Inhalation exposure							
Isuzu A4JB1 2.7-L, light-duty 4-cylinder, direct injection engine	Steady-state 1500 rpm, 10 kg/m; EDYC dynamometer	Constant-volume dilution tunnel	Male BigBlue® (F344) rats (5 wks)	Whole-body exposure for 12 h/d, 7 d/wk for 4 wks to 1 or 6 mg/m ³	<i>LacI</i> mutations, stable lung DNA adducts by [³² P]- postlabelling, oxidative DNA damage (e.g. 8-OH-dG) by HPLC	Significant increase in <i>LacI</i> mutations at 6 mg/m ³ ; significant increase in total DNA adducts and oxidative damage at 1 and 6 mg/m ³ ; major mutations: A→G and G→A transitions, G→T transversions	Sato et al. (2000)
Isuzu 4JG2, 3.1-L, light-duty, 4-cylinder, direct injection engine	Steady-state 1500 rpm, 10 kg/m; EDYC dynamometer	Constant-volume dilution tunnel, DEP collected on glassfibre filters; range of particle size, 10–470 nm, with mass peak at 110 nm	<i>Gpt</i> delta transgenic mice (7 wks) (C57BL/6J background)	Whole-body exposure for 12 h/d, 7 d/wk to 1 or 3 mg/m ³ for 4, 12 or 24 wks; intratracheal instillation of DEP (0.125, 0.25, 0.5 mg) or DEP extract (benzene/ ethanol; 0.05, 0.1, 0.2 mg) in 50 µL PBS	<i>Gpt</i> transgene mutations in lung 3 d after exposure by inhalation or 14 days after intratracheal instillation	Significant increase in <i>Gpt</i> mutations for each of the exposure scenarios; response after inhalation peaked at 12 wks; dose- related increase following treatment with DEP or DEP extract (potency of DEP extract twice that of DEP); G→A transitions predominant, G→T transversions were induced by both DEP and DEP extract.	Hashimoto et al. (2007)
Hydra IDI single-cylinder diesel research engine	Steady-state 1800 rpm, 1 kg/m.	Constant-volume dilution tunnel, 1:10 dilution of exhaust	Male C57BL mice (8–10 wks)	Whole-body exposure for 8 h/d for 14 days, with a 2-days break after 5 d; exposed males then mated with unexposed females	Heritable mutations (in pre- and postmeiotic sperm) at murine mini-satellite loci <i>Pc1</i> and <i>Pc2</i>	Small sample size; no significant increase in mutation frequency	Hedenskog et al. (1997)

Table 4.4 (continued)

Engine type and specifications	Run conditions	Test material collection and processing	Animal model	Exposure regime	End-points examined	Results	Reference
Isuzu A4JB1 2.7-L, light-duty 4-cylinder, direct injection engine	Steady-state 1500 rpm, 10 kg/m; EDYC dynamometer	Constant-volume dilution tunnel	<i>Nrf2</i> knock-out mice (null ^{-/-} and heterozygotes ^{+/-}) (7 months)	Whole-body exposure for 12 h/d, 7 d/wk for 4 wks to 3 mg/m ³	Stable lung DNA adducts by [³² P]-postlabelling	Significant increase in levels of DNA adducts; highest in <i>Nrf2</i> null (-/-) mice	Aoki et al. (2001)
Volkswagen 1.6-L, 40-kW diesel engine	US FTP-72	Exhaust diluted 1:9 with clean air; cyclone removal of particles < 1 µm; MMAD, 0.3 µm	Female Wistar rats (7 wks)	Whole-body exposure to 7.5 mg/m ³ for 18 h/d, 5 d/wk for 2, 6 or 24 months	Stable DNA adducts in lung (by [³² P]-postlabelling)	No significant elevation in PAH-derived adducts; modest increase in adducts suspected to be nitro-PAH-derived	Gallagher et al. (1994)
Two 1988 model LH6 General Motors 6.2-L, V-8 engines	US FTP-72	Constant-volume dilution tunnel	Male and female F344//N rats (8.5 wks)	Whole-body exposure for 16 h/d, 5 d/wk for up to 24 months to 2.4–2.5 or 6.2–6.5 mg/m ³	Stable DNA adducts in lung and isolated alveolar type-II cells by [³² P]-post-labelling, chromosomal aberrations in circulating lymphocytes	At 3 months, dose-related increase in total lung adducts; significant 4-fold increase in adducts in type-II cells; no increase in chromosomal aberrations	Mauderly et al. (1994)
Light-duty diesel engine [details not provided]	Steady-state 1050 rpm, 80% load	Constant-volume dilution tunnel	Female F344 rats (8 wks)	Whole-body exposure for 17 h/d (at night), 3 d/wk for 1, 3, 6, 9 or 12 months to 3.5 mg/m ³	Oxidative DNA damage (i.e. 8-OH-dG by HPLC) and stable lung DNA adducts by [³² P]-postlabelling	Increase in oxidative DNA damage, reaching plateau at 9 mo; bulky DNA adducts peaked after the first month, then declined	Iwai et al. (2000)
US 2007-compliant heavy-duty engine (selected in ACES Phase I)	16-h duty cycle developed for ACES study	Constant-pressure dilution tunnel, 1:5 initial dilution rate	Wistar Han rats and C57BL/6 mice (8 wks)	Whole-body exposure for 16 h/d, 5 d/wk for up to 3 mo; target NO ₂ concentrations of 0.1, 0.8 or 4.2 ppm	MN in reticulocytes and normochromatic erythrocytes, DNA strand breaks in the lung, 8-OH-dG in serum	No significant increase in MN frequency, strand breaks or 8-OH-dG	Khalek et al. (2009) , Bemis et al. (2012) , Hallberg et al. (2012) , McDonald et al. (2012)

Table 4.4 (continued)

Engine type and specifications	Run conditions	Test material collection and processing	Animal model	Exposure regime	End-points examined	Results	Reference
Heavy-duty 6-cylinder 9.2-L engine, with and without urea (32.5% in water); SCR (details not provided)	Steady-state 1320 rpm, 840 nm [84 kg/m]	Partial-dilution type dilution tunnel	Male F344 rats (7 wks)	Whole-body exposure for 6 h/d for 1, 3 or 7 days to 1:29, 1:290 or 1:580 dilutions of exhaust	Oxidative stress measured as free 8-OH-dG in serum; expression of <i>Il-1β</i> , <i>Hmox-1</i> , <i>Tnf-α</i> and <i>Cyp1A1</i> in lung	<i>Conventional engine</i> : significant dose-related increase in 8-OH-dG, significant increase in <i>Cyp1A1</i> expression (1 d) <i>SCR engine</i> : slight increase in 8-OH-dG (1 day 3 d); significant increase in <i>Cyp1A1</i> (7 d), <i>Hmox-1</i> (1 day 3 d) and <i>Tnf-α</i> (1, 3, 7d)	Tsukue et al. (2010b)
Isuzu A4JB1 2.7-L, light-duty 4-cylinder, direct injection engine	Steady-state 1500 rpm, 10 kg/m; EDYC dynamometer	Constant-volume dilution tunnel	Male F344 rats (5 wks)	Whole-body exposure for 12 h/d, 7 d/wk for 4 wks to 6 mg/m ³	Gene expression in lung (by cDNA micro-array and Northern blot)	Significant upregulation of the proto-oncogene <i>A-Raf</i> and of PCNA mRNA	Sato et al. (1999)
Intratracheal or intranasal instillation							
Isuzu A4JB1 2.7-L, light-duty 4-cylinder, direct injection engine	Steady-state 2000 rpm, 6 kg/m; EDYC dynamometer	Constant-volume dilution tunnel, DEP collected on glassfibre filters	Male ICR mice (6 wks)	Single intratracheal instillation of 0.1–0.6 mg under anaesthesia	Oxidative DNA damage (i.e. 8-OH-dG by HPLC) in the lung	Significant increase in oxidative damage 12 h after exposure to 0.1–0.4 mg; damage peaked 2 d after exposure	Sagai et al. (1993) , Nagashima et al. (1995)
Isuzu A4JB1 2.7-L, light-duty 4-cylinder, direct injection engine	Steady-state 1500 rpm, 10 kg/m; EDYC dynamometer	Constant-volume dilution tunnel, DEP collected on glassfibre filters; MMAD, 0.4 μ m	Male ICR mice (4 wks)	Ten weekly intratracheal instillations with 0.1 mg DEP suspended in PBS; comparison with hexane/benzene/methanol-washed DEP	Oxidative DNA damage (i.e. 8-OH-dG by HPLC) in lung 24 h after final treatment	Significant increase in oxidative damage; no significant difference between DEP and washed DEP	Sagai et al. (1993) , Ichinose et al. (1997b)

Table 4.4 (continued)

Engine type and specifications	Run conditions	Test material collection and processing	Animal model	Exposure regime	End-points examined	Results	Reference
Isuzu A4JB1 2.7-L, light-duty 4-cylinder, direct injection engine	Steady-state 1500 rpm, 10 kg/m; EDYC dynamometer	Constant-volume dilution tunnel, DEP collected on glassfibre filters; MMAD, 0.4 µm	Male ICR mice (4 wks)	Ten weekly intratracheal instillations with 0.05, 0.1 or 0.2 mg DEP suspended in PBS	Oxidative DNA damage (i.e. 8-OH-dG by HPLC) in lung 24 h after final treatment	Dose-related increase in oxidative DNA damage with 0.1 and 0.2 mg DEP/wk	Sagai et al. (1993) , Ichinose et al. (1997a)
Mercedes-Benz MB1620, 210-hp bus engine, with Euro-III emission profile	Not specified	PM-retention device [details not given]	Male BALB/c mice (8 wks)	Daily intranasal instillation of 30 µg DEP in saline on 5 d/wk for 30 or 60 d	Expression of <i>Muc5ac</i> mRNA in lung (by real-time RT-PCR)	Significant increase in <i>Muc5ac</i> gene expression at 60 d	Yoshizaki et al. (2010)
Isuzu A4JB1 2.7-L, light-duty 4-cylinder, direct injection engine	Steady-state 1500 rpm, 10 kg/m; EDYC dynamometer	Constant-volume dilution tunnel, DEP collected on glassfibre filters, suspended in saline	Female BALB/c mice (5–6 wks)	Daily intranasal instillation of 30 µg DEP for 5 d, followed by daily 1 h inhalation exposures to 6 mg/m ³ for 3 d	Expression of <i>Ym1</i> and <i>Ym2</i> in lung (by qualitative RT-PCR)	Significant increase in expression of <i>Ym1</i> and <i>Ym2</i>	Song et al. (2008)
2002 Cummins 5.9-L diesel engine	Steady-state operation at 75% load	DEP from outflow duct [details not given]	<i>Gclm</i> wild-type, <i>Gclm</i> ^{-/+} , <i>Gclm</i> ^{-/-} mice (8–12 wks)	Single intranasal instillation of 200 µg DEP	Expression of <i>Tnf-α</i> , and <i>Il-6</i> (by quantitative real-time RT-PCR) 6 h after exposure	Significant increase in <i>Tnf-α</i> and <i>Il-6</i> expression; <i>Gclm</i> ^{-/-} mice most sensitive to the effects of DEP	Weldy et al. (2011)
Topical application							
Nissan Datsun 220C engine, Volkswagen (VW) Rabbit engine (turbo-charged)	HWFET, steady-state; particle emission rate, 0.33 and 0.18 g/mile for Nissan and VW, respectively	Constant-volume dilution tunnel; DEP collected on Teflon-coated glassfibre filters; Soxhlet extraction with dichloromethane	Female C-57 mice (6–8 wks)	Topical application of 120, 50 and 20 mg/mouse DEP extract at 0, 6, 30 and 54 h	Stable DNA adducts in skin, liver and lung by [³² P]-postlabelling and HPLC	Significant induction of DNA adducts in all tissues 24 h after exposure; in skin, the main adduct derived from Nissan exhaust was formed by chrysene; in lung, <i>anti</i> -BPDE adducts constituted up to 67% of total adducts	Lewtas et al. (1981) , Gallagher et al. (1990) , Savelle et al. (1995)

Table 4.4 (continued)

Engine type and specifications	Run conditions	Test material collection and processing	Animal model	Exposure regime	End-points examined	Results	Reference
Two 1988 model LH6 General Motors 6.2-L, V-8 engines	US FTP-72	DEP collected on glassfibre filters; sonication; extraction with dichloromethane	Male CD1 mice (4 wks)	Single topical application of 20 mg-equivalent DEP in 100 µL acetone or five daily applications of 20 mg	Stable DNA adducts in skin, lung and heart by [³² P]-postlabelling 1, 3, 14, 42 and 77 days after the single dose, and 1, 3, 14, 42 and 70 days after repeated doses	Significant dose-related increases in adduct frequency in skin and lung, with rapid decline after 1 and 3 d, respectively; no adducts found in heart DNA	Mauderly et al. (1994) , Randerath et al. (1995)
Intraperitoneal injection							
Six diesel engines, 2 heavy-duty and 4 medium-duty	Acceleration conditions	PM collected on glassfibre filters; extraction with dichloromethane	Male Kunming mice (18–20 g)	Two daily intraperitoneal injections of 4, 20 or 100 mg-equivalent DEP extract in DMSO	MN frequency in PCEs 6 h after second treatment	Significant dose-related increase in MN	Song & Ye (1995)
Unspecified diesel engine	Idling, with periodic acceleration to maximum speed	PM collected on glassfibre filters; extract obtained by sonication in dichloromethane; fractionated on silica gel: five fractions with increasing polarity	Male Swiss mice (18–20 g)	Two intraperitoneal injections (24 h apart) of 4, 12 or 36 mg-equivalent DEP extract in DMSO	MN frequency in PCEs 6 h after second treatment	Significant dose-related increase in MN; stronger responses for base, PAH and polar fractions	Lu et al. (1999b)

ACES, advanced collaborative emissions study; *anti*-BPDE, (±)-*r-r-7,t-8*-dihydroxy-*t-9,10*-epoxy-*7,8,9,10*-tetrahydrobenzo[*a*]pyrene; *Cyp*, cytochrome P450 gene; d, day(s); DEP, diesel exhaust particles; DMSO, dimethyl sulfoxide; FTP, Federal Test Procedure; *Gclm*, gene encoding glutamate cysteine ligase (modifier subunit); h, hour; *Hmox*, haeme oxygenase gene; hp, horse power; HPLC, high-performance liquid chromatography; HWFET, highway fuel economy test; *Il*, interleukin gene; MMAD, mass median aerodynamic diameter; MN, micronucleus/micronuclei; mo, month; NO₂, nitrogen dioxide; 8-OH-dG, 8-oxo-2'-deoxyguanosine; PAH, polycyclic aromatic hydrocarbons; PBS, phosphate buffered solution; PCE, polychromatic erythrocytes; PCNA, proliferating cell nuclear antigen; PM, particulate matter; rpm, revolutions per minute; RT-PCR, reverse transcription-polymerase chain reaction; SCR, selective catalytic reduction; *Tnf-α*, tumour necrosis factor-α gene; wk, week

and *Pc2*. No increase in the frequency of heritable mutations was found.

Two comprehensive *in-vivo* inhalation studies of diesel engine emissions were sponsored by the Health Effects Institute. The earlier study assessed DNA damage in rats chronically exposed to diesel engine exhaust ([Bond et al., 1990](#); [Belinsky et al., 1995](#); [Randerath et al., 1995](#)) as part of a larger cancer bioassay conducted by the Inhalation Toxicology Research Institute ([Mauderly et al., 1994](#)). The second study, referred to as the Advanced Collaborative Emissions Study (still ongoing), examined the genetic and related effects elicited in rats and mice by chronic and subchronic inhalation of emissions from a US 2007-compliant heavy-duty diesel engine ([Bemis et al., 2012](#); [Hallberg et al., 2012](#); [McDonald et al., 2012](#)). The earlier chronic inhalation study assessed the frequency of bulky DNA adducts in the lung and isolated alveolar type II cells, as well as chromosomal damage (i.e. aberrations or micronuclei) in the circulating lymphocytes of rats exposed to diluted diesel engine emissions containing 2.5 or 6.5 mg/m³ of particles for up to 24 months (16 hours per day, 5 days a week). Analyses of adduct formation after 3 months of exposure showed a significant dose-related increase in the level of total DNA adducts in alveolar cells at the high dose and the frequency of total adducts in lung tissues. However, adduct frequencies in exposed animals examined at 6, 12, 18 and 23 months did not always exceed those in the controls. A marked increase in adduct frequency was also recorded at 18 months after exposure to the lower dose. The frequency of chromosomal aberrations in circulating lymphocytes and micronuclei in cultured bi-nucleated lymphocytes were not increased in exposed animals compared with controls.

The Advanced Collaborative Emissions Study is an ongoing *in-vivo* investigation of the toxicological effects in rats and mice chronically exposed to diluted emissions from a US 2007-compliant heavy-duty diesel engine (i.e.

maximum PM emission rate, 10 mg/hp-h). The first phase of the study involved a detailed comparison of four heavy-duty engines, and the selection of an engine for biological testing. Regulated and unregulated emissions from four test engines – a Caterpillar C13, a Cummins ISX, a Detroit Diesel DDC Series 60 and a Volvo Powertrain Mack MP7 – were assessed in four test cycles ([Khalek et al., 2009](#)). All engines were equipped with a water-cooled exhaust gas re-circulation or a clean gas induction system to reduce nitrogen oxides, catalysed diesel particulate filters (DPF) or a diesel-oxidation catalyst placed in front of a catalysed DPF. The engine selected for the toxicological assessments showed emission rates of 1.2 mg/hp-h of PM and 0.91 g/hp-h of nitrogen dioxide, and exposures lasted for up to 30 months.

The results of the 1- and 3-month exposures (16 hours per day overnight, 5 days a week) have now been published ([Bemis et al., 2012](#); [Hallberg et al., 2012](#); [McDonald et al., 2012](#)). Chromosomal damage (i.e. micronuclei) in peripheral blood, DNA strand breaks in lung tissue and a marker of oxidative damage (free 8-OH-dG) were examined in the serum from rats and mice exposed to diluted engine emissions. Due to the low concentration of PM, the concentration of nitrogen dioxide in the exhaust was used to set the doses (i.e. 0.1, 0.8 and 4.2 ppm). The 1- and 3-month exposures to diluted emissions did not induce a significant treatment-related increase in the overall frequency of micronuclei in rats or mice. However, significant effects were observed on the frequency of micronuclei in normochromatic erythrocytes of male mice, in reticulocytes and normochromatic erythrocytes of both male and female mice with duration of exposure, and in rat reticulocytes. However, these statistically significant effects were weak, and the analyses did not show a significant interaction between the outcome of treatment and the duration of exposure ([Bemis et al., 2012](#)). In contrast, the 1- and 3-month exposures to diluted diesel engine

emissions did not induce significant increases in DNA strand breaks in the lung or concentrations of 8-OH-dG in the serum, although duration of exposure had some effect on the serum levels of 8-OH-dG in both rats and mice, which was not statistically significant ([Hallberg et al., 2012](#)).

The effects of emissions from a selective catalytic reduction (SCR) engine (including the use of urea to remove nitrogen oxides) were compared with those of emissions from a heavy-duty engine in male Fischer rats. [Tsukue et al. \(2010b\)](#) assessed oxidative stress (measured as 8-OH-dG in serum) and the pulmonary expression of genes involved in xenobiotic metabolism (*Cyp1A1*), oxidative stress (*Hmox-1*) and inflammation (tumour necrosis factor, *Tnf- α*) in rats exposed to diluted emissions from the SCR engine system and from a conventional engine for 6 hours per day for 1, 3 or 7 days. The results revealed a significant dose-related increase in serum concentrations of 8-OH-dG for emissions from the conventional engine, but only slight increases for those from the SCR engine. Expression of *Cyp1A1* was significantly increased at day 1 for conventional engine emissions and at 7 days for SCR engine emissions; however, the expression of *Hmox-1* and *Tnf- α* was significantly increased only for emissions from the SCR engine.

Several studies investigated the induction of DNA damage after intratracheal instillation of diesel engine exhaust particulates from a four-cylinder light-duty direct injection engine. [Nagashima et al. \(1995\)](#) assessed the induction of oxidative DNA damage (i.e. 8-OH-dG) in ICR mice that received a single instillation of 0.1–0.3 mg of PM; a significant increase in oxidative damage in lung DNA was observed 12 hours after exposure, with a peak after 2 days. [Ichinose et al. \(1997a, b\)](#) determined oxidative damage in ICR mice 24 hours after the last of 10 weekly intratracheal instillations of diesel engine exhaust particles, and compared the level of damage induced by untreated with that of hexane-washed diesel engine exhaust particles

suspended in phosphate-buffered solution. A significant increase in oxidative damage was observed in lung DNA, but no appreciable difference was found between the two preparations. Following instillations of 0.05, 0.1 and 0.2 mg of diesel engine exhaust particles, a dose-related increase in the level of oxidative damage in lung DNA was found.

The Working Group reviewed several studies that examined changes in the expression of inflammatory genes in the lungs of mice exposed to diesel engine exhaust particles by intranasal instillation. [Yoshizaki et al. \(2010\)](#) noted significant increases in the expression of the *Muc5ac* gene, which encodes the mucin-5ac protein and is controlled by nuclear factor- κ B, in the lung of BALB/c mice that received nasal instillations of 30 μ g of diesel engine exhaust particles daily on 5 days a week for 60 days. This exposure level corresponded to an average 24-hour pulmonary deposition of PM from ambient air in Sao Paulo, Brazil. Increased numbers of leukocytes were found in the BAL fluid of these mice, which is indicative of respiratory tract inflammation. In BALB/c mice, [Song et al. \(2008\)](#) noted that intranasal exposure for 5 days followed by inhalation exposure for 3 days to diesel exhaust particles from a light-duty diesel engine induced a significant increase in the pulmonary mRNA expression of *Ym1* and *Ym2*, i.e. genes involved in inflammation-related responses to air pollutants. [Weldy et al. \(2011\)](#) reported that a single intranasal exposure to diesel engine exhaust particles (200 μ g) of mice heterozygous ($^{+/-}$) for or deficient ($^{-/-}$) in a glutamate–cysteine ligase modifier subunit (a factor involved in glutathione synthesis) resulted in significant increases in the inflammatory cytokines *Tnfa* and *Il6* in the BAL fluid from these mice.

The frequency of stable DNA adducts was examined in experimental animals topically exposed to organic extracts of diesel PM ([Gallagher et al., 1990, 1993](#); [Savela et al., 1995](#)). Dichloromethane extracts of diesel PM collected

from several different types of vehicle were applied to the shaved dorsal skin of C57 mice. Adduct formation was measured by [³²P]-postlabelling in DNA isolated from the skin, lung and liver 24 hours after the last of four topical applications (200 µL each) given at 0, 6, 30 and 54 hours, or after a single application. Significant increases in adduct levels were observed in all tissues examined. The adduct profiles included both chrysene- and benzo[a]pyrene-diol epoxide-derived adducts, and the latter accounted for a substantial portion of the adducts observed in the lung. The Inhalation Toxicology Research Institute study also assessed the formation of stable DNA adducts after topical application of diesel PM extract, and found a significant dose-related increase in the number of adducts in both the skin and lung, which rapidly declined 5 days after exposure (Randerath *et al.*, 1985, 1995).

The induction of micronuclei was examined in bone marrow following the intraperitoneal injection of diesel engine exhaust particles into mice. Song & Ye (1995) found significant dose-related increases in the frequency of micronuclei in the polychromatic erythrocytes of Kunming mice 6 hours after administration of two daily injections of exhaust particulate extracts derived from six different diesel engines. Lu *et al.* (1999b) also found a significant dose-related increase in the frequency of micronuclei in the polychromatic erythrocytes of Swiss mice 6 hours after the second of two intraperitoneal injections (given at 24-hour intervals) of 0.1 mL of a diesel engine exhaust particulate extract containing 4, 12 or 36 mg/mL of particles. After fractionation of the extract, the strongest response in the micronucleus assay was associated with the basic, PAH-containing and polar fractions.

Standard reference materials

Two standard reference materials (SRMs) of diesel engine exhaust particulates are available from the US National Institute of Standards

and Technology. SRM 1650 was formulated and issued in 1985 and contains PM collected from several direct injection diesel engines. Although not based on any particular engine, SRM 1650 is generally thought to be representative of particulate emissions from heavy-duty diesel engines from the mid-1980s. A new standard, SRM 2975, was formulated and issued in 2000 and contains PM collected from an engine equipped with a filtration system designed for use with a diesel-powered forklift. Extraction of SRM 2975 with dichloromethane produces SRM 1975. Certificates of analysis for each of these SRMs, which contain certified concentrations for numerous PAHs and nitro-PAHs, are available online at <http://www.nist.gov/srm/index.cfm>. Several *in-vivo* studies determined the genetic and related effects of these SRMs, and the results are summarized in Table 4.5.

Risom *et al.* (2003a) examined oxidative lesions (i.e. 8-OH-dG) and changes in the expression of the DNA-repair gene *Ogg1* in lung tissue collected from BALB/CJ mice 1, 3 or 22 hours after a single 90-minute exposure by inhalation (nose-only) to SRM 1650 at 20 or 80 mg/m³, or after four consecutive daily exposures for 90 minutes to 5 or 20 mg/m³ SRM 1650. Oxidative DNA damage was increased after the single 90-minute exposure, and *Ogg1* was significantly upregulated after four consecutive daily exposures. Haeme oxygenase (*Hmox-1*) mRNA was upregulated after both schedules of exposure. Additional analyses showed a significant increase in DNA strand breakage in cells from BAL fluid after the repeated exposures. Dybdahl *et al.* (2004) used the same exposure regimen to examine oxidative lesions and bulky DNA adducts in lung tissues and DNA strand breaks in BAL cells collected from exposed BALB/CJ mice. Mutations in the *cII* transgene were also measured in Muta[®]Mouse 28 days after exposure. The results showed increased DNA strand breakage in BAL cells and increased oxidative damage and

bulky adducts in lung tissue, but no significant increase in *cII* mutations.

[Saber et al. \(2005\)](#) found a significant increase in the induction of DNA strand breaks in BAL cells of TNF-knock-out mice exposed by inhalation (nose-only) to 20 mg/m³ of SRM 2975 for 90 minutes a day for 4 days, suggesting that a TNF-mediated inflammatory response is not required for the induction of DNA damage in BAL cells. Additional analyses showed a significant increase in *Il-6* gene expression in exposed TNF-knock-out animals. In a follow-up study, the expression of several inflammatory cytokines was investigated in wild-type and TNF-knock-out mice exposed by inhalation (nose-only) to SRM 1650 or SRM 2975 (20 or 80 mg/m³, 90 minutes). The results in wild-type animals showed early (< 6 hours) post-exposure increases in the expression of *Il-6*, *monocyte chemoattractant protein-1* and *neutrophil chemotactic factor Kc*, and late (1 day) increases in *Tnf* expression; in the knock-out mice, the expression of *neutrophil chemotactic factor Kc*, *monocyte chemoattractant protein-1* and *Il-6* increased in the absence of TNF ([Saber et al., 2006](#)).

The effects of SRM 2975 were examined in C57/Bl/6 mice exposed *in utero* through maternal inhalation ([Hougaard et al., 2008](#); [Ritz et al., 2011](#)). Pregnant mice were exposed to SRM 2975 at 19.1 mg/m³ for 1 hour per day on gestational days 7–19. DNA strand breaks (measured in the comet assay) and the levels of mRNA expression of the *Ogg1* and *excision repair cross-complementing group 1 (Ercc1)* genes were determined in the liver of offspring on postnatal day 2. The results showed modest, non-significant increases in DNA strand breaks and no difference in gene expression. When the offspring (F1) were reared to maturity and mated with control CBA mice, pedigree analysis of the F2 descendants was used to determine the rates of extended simple tandem repeat germ-line mutation rates at the hyper-variable alleles *Ms6-hm* and *Hm2*. A significant, twofold elevation in mutation frequency was

observed in prenatally exposed F1 males. Thus, repeated daily exposures to diesel engine exhaust particles *in utero* resulted in increased rates of germ-line mutation in exposed males ([Ritz et al., 2011](#)).

The induction of DNA strand breaks, DNA adduct frequency, the expression of DNA-repair genes and the frequency of *cII* transgene mutations was studied in BigBlue[®] rats exposed to SRM 1650 in the diet (*ad libitum*). [Müller et al. \(2004\)](#) examined lung tissue following 21 days of exposure to 0.2–80 mg/kg of diet of PM and noted significant increases in DNA strand breaks (measured in the comet assay), oxidative DNA damage (in the comet assay including endonuclease III and formamidopyrimidine DNA-glycosylase) and bulky DNA adducts (by [³²P]-postlabelling). No significant increase in *cII* transgene mutations or the expression of *Ogg1* (involved in the repair of 8-OH-dG) or *Ercc1* was observed. Using the same exposure protocol, [Dybdahl et al. \(2003\)](#) assessed the same end-points in the liver and colon. Significant increases in DNA strand breaks and bulky adducts were found in both organs, and an increase in *Ercc1* gene expression in the liver only. No significant increases were observed in endonuclease III-enhanced DNA strand breaks, directly measured levels of 8-OH-dG in the liver, colon cells or urine or *cII* transgene mutations. [Risom et al. \(2003b\)](#) examined effects in the liver and colon following 21 days of exposure to 0.8 mg/kg of diet of PM with or without an elevated level of sucrose. Significant increases were found in bulky DNA adducts in the colon and liver, and in DNA strand breaks in the colon but not in the liver. The elevated sucrose diet in the absence of diesel engine exhaust particles also increased the number of bulky adducts in the colon and, to a lesser extent, in the liver. Significant increases were noted in the hepatic expression of the *N-methylpurine DNA glycosylase* gene and *Ogg1*. No effect was seen on the levels of oxidative DNA damage or on mutation frequency in the *cII* transgene in either tissue. In

Table 4.5 Summary of studies in animals exposed *in vivo* to diesel particulate standard reference materials (SRMs)

SRM	Animal model	Exposure regimen	End-points examined	Results	Reference
Inhalation exposure					
SRM 1650	Female BALB/CJ mice (8 wks)	Single 1.5-h head-only exposure to 20 or 80 mg/m ³ or daily 1.5-h exposures to 5 or 20 mg/m ³ on 4 consecutive days	Oxidative DNA damage (8-OH-dG by HPLC-EC) and expression of <i>Ogg1</i> and <i>HO-1</i> genes in lung tissue collected 1, 3 or 22 h after exposure; DNA strand breaks in BAL cells (by comet assay)	Significant increase in 8-OH-dG after the single high dose; <i>Ogg1</i> significantly upregulated after repeated exposures; <i>HO-1</i> expression increased at both single doses; significant increase in DNA strand breaks in BAL cells after repeated exposures	Risom et al. (2003a)
SRM 1650	Female BALB/CJ mice and MutaMouse (8 wks)	Single 1.5-h head-only exposure to 20 or 80 mg/m ³ or daily 1.5-h exposures to 5 or 20 mg/m ³ on 4 consecutive days	DNA strand breaks (by comet assay) in BAL cells, oxidative DNA damage (8-OH-dG by HPLC-EC) and bulky adducts (by [³² P]-postlabelling) in lung collected 1, 3 or 22 h after exposure; transgene <i>cII</i> mutations in lung 4 wks after exposure	Increase in DNA strand breaks in BAL cells and in bulky adducts in lung after repeated exposures; increased oxidative DNA damage after single exposure; no increase in <i>cII</i> mutations	Dybdahl et al. (2004)
SRM 2975 and 1650	Female BALBcj mice (8 wks), female and male C57xCBA mice (7–8 wks), female B6 (<i>Tnf</i> ^{-/-}) and C57BL/6J (<i>Tnf</i> ^{+/+}) mice (9–11 wks)	Single 1.5-h head-only exposure to 20 or 80 mg/m ³ SRM 1650 (BALBcj mice), 80 mg/m ³ SRM 2975 (C57xCBA mice) or 20 or 4 × 20 mg/m ³ SRM 2975 (<i>Tnf</i> ^{-/-} and <i>Tnf</i> ^{+/+} mice); repeat exposure was for 1.5 h on 4 consecutive days	Expression in lung tissue of <i>Tnf</i> , <i>Mip2</i> , <i>Kc</i> , <i>Il6</i> and <i>Mcp1</i> (by real-time RT-PCR)	Significant increases in <i>Tnf</i> expression late (1 day after exposure to the higher dose; significant increases in expression of <i>Il6</i> , <i>Mcp1</i> and <i>Kc</i> < 6 h after exposure; expression of <i>Kc</i> , <i>Mcp1</i> and <i>Il6</i> increased in <i>Tnf</i> ^{-/-} animals	Saber et al. (2006)
SRM 2975	Exposure <i>in utero</i> via maternal inhalation (pregnant C57Bl/6 mice); at 19 wks, offspring mated with unexposed CBA/J mice to generate F2	Exposure to 19.1 mg/m ³ for 1 h/d on gestational days 7–19 in 18-L inhalation chamber with a flow of 20 L/min; peak mass concentration, 292 nm; MMAD, 240 nm	F2 germ-line mutation rates at ESTR loci from full pedigrees	Significant 2-fold increase in mutation frequency for F1 males (but not females) exposed <i>in utero</i>	Hougaard et al. (2008) , Ritz et al. (2011)

Table 4.5 (continued)

SRM	Animal model	Exposure regimen	End-points examined	Results	Reference
SRM 2975	Exposure <i>in utero</i> via maternal inhalation (pregnant C57Bl/6 mice)	Exposure to 19.1 mg/m ³ for 1 h/d on gestational days 7–19 in 18-L inhalation chamber with flow of 20 L/min; peak mass concentration, 292 nm; MMAD, 240 nm	In offspring at postnatal day 2, damage in liver DNA: strand breaks (by comet assay) and oxidative damage via expression of <i>Ogg1</i> , <i>HO-1</i> and <i>Ercc1</i>	No increase in DNA strand breaks or gene expression	Hougaard et al. (2008)
Intratracheal instillation					
SRM 1650	Male Sprague-Dawley rats (150–170 g); primary tracheal epithelial (RTE) cells collected 24 h after exposure	Three consecutive daily instillations of 7.5–75 mg/kg bw DEP	Frequency of transformed foci in RTE cells 5 wks after exposure and isolation	Significant dose-dependent increase in transformation efficiency at 15, 30 and 75 mg/kg bw DEP	Ensell et al. (1998)
Oral administration					
SRM 1650	Male BigBlue [®] (Fischer) rats (8 wk)	DEP fed at 0.2–80 mg/kg of diet (<i>ad libitum</i>) for 21 d	Oxidative DNA damage (8-OH-dG by HPLC-EC), DNA strand breaks and oxidative lesions (by comet assay with ENDOIII), expression of <i>Ogg1</i> and <i>Ercc1</i> genes, <i>cII</i> transgene mutation frequency, stable adducts (by [³² P]-postlabelling) in liver and colon	Significant increase in DNA strand breaks and bulky adducts in liver and colon; increased expression of <i>Ercc1</i> in liver, and <i>Ogg1</i> in liver and colon; no increase in ENDOIII sites, oxidative damage or <i>cII</i> transgene mutations	Dybdahl et al. (2003)
SRM 1650	Male BigBlue [®] (Fischer) rats (8 wks)	DEP fed at 0.2–80 mg/kg of diet (<i>ad libitum</i>) for 21 d	DNA strand breaks and oxidative lesions (by comet assay including ENDOIII and FPG), stable DNA adducts (by [³² P]-postlabelling), transgene (<i>cII</i>) mutation frequency, and expression of <i>Ogg1</i> and <i>Ercc1</i> genes in lung	Significant increase in DNA strand breaks, oxidized bases and stable DNA adducts; no increase in <i>cII</i> mutations; no change in expression of <i>Ogg1</i> or <i>Ercc1</i>	Müller et al. (2004)

Table 4.5 (continued)

SRM	Animal model	Exposure regimen	End-points examined	Results	Reference
SRM 1650	Male BigBlue® (Fischer) rats (8 wks)	DEP fed at 0.8 mg/kg of diet (<i>ad libitum</i>), with or without elevated sucrose, for 21 d	Oxidative DNA damage (8-OH-dG by HPLC-EC), DNA strand breaks and oxidative lesions (by comet assay including ENDOIII and FPG), expression of <i>Ogg1</i> , <i>Mpg</i> and <i>Ercc1</i> genes, <i>cII</i> transgene mutation frequency, stable adducts (by [³² P]-postlabelling) in liver and colon	DNA adducts and strand breaks significantly increased in colon; adducts significantly increased in liver; no increase in oxidative damage or <i>cII</i> mutations in liver or colon; significant increase in expression of <i>Mpg</i> and <i>Ogg1</i> in liver; no effect of elevated sucrose in diet	Risom et al. (2003b)
SRM 1650	<i>Ogg1</i> -null and wild-type C57Bl/6 mice (10–13 wks)	DEP fed at 0.8 or 8 mg/kg of diet (<i>ad libitum</i>) for 21 d	DNA strand breaks and oxidative lesions (by comet assay with ENDOIII and FPG) and oxidative DNA damage (8-OH-dG by HPLC-EC) in colon, liver and lung	Increase in FPG sites in liver and lung in <i>Ogg1</i> -null mice relative to wild-type; increased DNA strand breaks in liver; no relation with exposure to DEP for any end-point in either strain	Risom et al. (2007)
SRM 2975	Male F344 rats (9 wks)	Single administration (by gavage) of 0.064 or 0.64 mg/kg bw DEP in saline	Oxidative DNA damage (8-OH-dG by HPLC-EC/UV), bulky DNA adducts (by [³² P]-post-labelling) and <i>Ogg1</i> gene expression in colon, liver and lung 6 and 24 h after exposure	Increased oxidative damage in all tissues at the higher dose; increased levels of bulky adducts in liver and lung at both doses (colon not tested); increased expression of <i>Ogg1</i> in lung (but not in colon or liver) 24 h after exposure	Danielsen et al. (2008)

BAL, bronchoalveolar lavage fluid; bw, body weight; d, day; DEP, diesel exhaust particles; ENDOIII, endonuclease-III; *Ercc*, excision repair cross-complementing gene; ESTR, expanded simple tandem repeat; FPG, formamidopyrimidine-DNA glycosylase; h, hour; *HO*, haeme oxygenase gene; HPLC-EC, high-performance liquid chromatography–electrochemical detection; *Il*, interleukin gene; *Kc*, chemokine gene; min, minute; *Mcp*, monocyte chemoattractant protein-1 gene; *Mip*, major intrinsic protein gene; MMAD, mass median aerodynamic diameter; *Ogg*, 8-oxoguanine glycosylase gene; 8-OH-dG, 8-oxo-2'-deoxyguanosine; RT-PCR, reverse transcription-polymerase chain reaction; SRM, standard reference material; *Tnf*, tumour necrosis factor gene; UV, ultraviolet; wk, week

a later study, [Risom et al. \(2007\)](#) assessed DNA strand breaks and oxidative damage in Ogg1-deficient (*Ogg1*^{-/-}) and wild-type C57Bl/6 mice following 21 days of dietary exposure to diesel engine exhaust particles (SRM 1650) at 0.8 or 8 mg/kg of diet. An increase in formamidopyrimidine DNA-glycosylase-sensitive sites was observed in the liver and lung of Ogg1-deficient mice compared with wild-type mice, but no association with exposure to diesel engine exhaust particles was found for any end-point in either strain.

[Danielsen et al. \(2008\)](#) assessed oxidative DNA damage, bulky DNA adducts and *Ogg1* expression in the colon, liver and lung of Fischer 344 rats 6 and 24 hours after a single oral administration of SRM 2975 (0.064 and 0.64 mg/kg bw) by gavage, and found increased oxidative DNA damage in all tissues, increased levels of bulky DNA adducts in the liver and lung (colon not investigated) and increased expression of *Ogg1* in the lung, but not in the colon or liver, 24 hours after exposure.

A significant increase in the frequency of transformed foci was observed in primary rat tracheal epithelial cells collected from Sprague-Dawley rats 24 hours after an intratracheal instillation of SRM 1650 (7.5, 15, 30 or 75 mg/kg bw) after 5 weeks of cell culture ([Ensell et al., 1998](#)).

(ii) Effects observed *in vitro*

A wide range of *in vitro* systems have assessed the genetic and related effects of diesel engine emissions, diesel engine emission particulates and/or organic extracts of diesel engine emission particulates, the most common of which are the *S. typhimurium* reverse mutation test and the single-cell gel electrophoresis (comet) assay. Many studies have also investigated genotoxic effects in cultured mammalian cells (e.g. Chinese hamster ovary, Chinese hamster V79, human A549 lymphocytes, MCF-7 and BEAS-2B), and several studies have examined the induction of

DNA damage in isolated calf-thymus DNA *in vitro*.

(iii) Effects in cultured mammalian cells

See [Table 4.6](#)

The single-cell gel electrophoresis (comet) assay was used to assess the ability of diesel engine emissions, diesel engine emission particulates or diesel engine emission particulate extracts to induce DNA strand breaks. [Oh & Chung \(2006\)](#) treated human A549 adenocarcinoma cells and Chinese hamster ovary cells with a crude dichloromethane extract or extract fractions of diesel emission particulates derived from a 1995 commercial heavy-duty diesel vehicle. The crude extract induced a significant increase in DNA strand breaks in both the absence and presence of exogenous metabolic activation. After fractionation of the crude extract, the slightly polar fraction containing nitro-PAHs, ketones and quinones induced the highest number of strand breaks. [Song et al. \(2006, 2007\)](#) exposed rat L-929 fibroblasts to dichloromethane extracts or extract fractions of diesel emission particulates from a heavy-duty diesel engine with no catalytic treatment of the exhaust and observed statistically significant concentration-dependent increases in DNA strand breaks. Each of the fractions was more potent than the crude extract itself. [Song et al. \(2007\)](#) examined diesel engine emission particulates from different diesel-ethanol blends and indicated that particulates from fuels with 0 and 20% v/v ethanol yielded higher responses in the comet assay than those obtained from blends with 5, 10 or 15% ethanol.

[Totlandsdal et al. \(2010\)](#) exposed human BEAS-2B bronchial epithelial cells to diesel emission particulates (25–400 µg/mL for 2 hours) from a light-duty engine and found a significant dose-related increase in DNA strand breaks, with a significant additional increase after treatment with formamidopyrimidine-DNA glycosylase, a DNA-repair enzyme that removes oxidized purines. Additional analyses showed induction

Table 4.6 Summary of studies of the effects of diesel emissions in cultured mammalian cells *in vitro*

Test conditions	Exposure system	End-point(s) examined	Results	Reference
Commercial 1995 diesel truck, direct injection engine; 1000 rpm, no load; dilution tunnel; DEP collected on Teflon®-coated filters; extraction with dichloromethane	Human A549 alveolar adenocarcinoma cells and Chinese hamster ovary (CHO-K1) cells exposed to crude DEP extract or extract fractions (F1–F7, obtained by acid-base partitioning) for 4 or 24 h	DNA strand breaks in A549 cells (by comet assay, with/without a CYP enzyme inhibitor) after the 24-h exposure; MN formation in CHO-K1 cells (by CBMN, with/without rat-liver metabolic activation) after the 4-h exposure	Crude extract induced significant increase in DNA strand breaks and MN; no change with metabolic activation; fraction 4 (PAHs and alkyl-PAHs) induced maximal MN frequency, higher with metabolic activation; fraction 5 (nitro-PAHs, ketones and quinones) induced maximum strand breaks, in the absence of the CYP enzyme inhibitor	Oh & Chung (2006)
Heavy-duty, 6-cylinder, 5.79-L engine; ECE R49–13 test mode; DEP collected; dichloromethane Soxhlet extraction; extract fractionated into six fractions	Rat fibrocytes exposed to DEP extract [duration of exposure not specified]	DNA strand breaks (by alkaline comet assay)	All samples induced significant concentration-related increases in DNA strand breaks; fractions were significantly more potent than crude extract	Song et al. (2006)
Heavy-duty, 6-cylinder, 5.79-L engine; ECE R49–13 test mode; run on diesel fuel or diesel fuel containing 5, 10, 15 or 20% ethanol by volume; PM collected on glassfibre filters; Soxhlet extract in dichloromethane	L-929 rat fibrocytes exposed to 0.125–1.0 mg/mL DEP extract for 24 h	DNA strand breaks (by alkaline comet assay)	All samples induced significant concentration-related increases in DNA strand breaks; DEP extracts from the fuels with 10 and 15% ethanol yielded a lower response; the 20% blend was as active as the unblended fuel	Song et al. (2007)
Two light-duty diesel engines (complying with 1996 Euro2 and 2005 Euro4 emission standards, resp.); fuels: ULSD reference, RME20, AFME20; DEP collected on quartz filters; SRM 2975 tested also	Human A549 alveolar adenocarcinoma cells exposed to 0.78–100 µg/mL DEP for 3 h	DNA strand breaks (by comet assay and FPG-assisted comet assay)	Concentration-related increase in DNA strand breaks and FPG-sensitive sites in all samples, including SRM 2975; modest increase for DEP from Euro4 engine; response with RME20 lower than that with ULSD	Hemmingsen et al. (2011)

Table 4.6 (continued)

Test conditions	Exposure system	End-point(s) examined	Results	Reference
DEP collected from a Deutz 4-cylinder 2.2-L diesel engine run at 500 rpm (no load)	Human BEAS-2B bronchial epithelial cells (SV40-transformed) treated with 25–400 µg/mL DEP for 2 or 4 h	DNA strand breaks (by comet assay and FPG-assisted comet assay) and level of phosphorylated p38 protein (by western blot)	Significant concentration-related increase in DNA damage and p38 phosphorylation at 2 and 4 h; at 2 h, significant elevation in FPG-sensitive sites; strand breaks were induced at concentrations above those required to elicit inflammatory responses and changes in phosphorylated p38	Totlandsdal et al. (2010)
Kubota 1.12-L D1105-T diesel engine, EPA Tier I ^a (EU stage II); ISO-C1 engine cycle ^b ; DEP collected via a high-volume cascade impactor, polyurethane foam and Teflon [®] -coated membrane; ultrasonic methanol extraction; fuels: European ULSD, HVO and RME	Mouse RAW264.7 macrophage cells exposed to 5, 50, 150 or 300 µg/mL DEP extract for 24 h	DNA strand breaks (by comet assay)	Significant increase in DNA strand breaks at highest two doses; aftertreatment with DOC/POC significantly reduced response with RME fuel	Jalava et al. (2010)
SRM 1650 suspended in culture medium; sonication	Human A549 alveolar adenocarcinoma cells exposed to 10–500 µg/mL DEP for 2, 5 or 24 h	DNA strand breaks (by comet assay)	Significant increase in DNA strand breaks at 100 and 500 µg/mL DEP at all time-points	Dybdahl et al. (2004)
SRM 1650, extracted with dichloromethane; both extract and washed particles tested	Human A549 alveolar adenocarcinoma cells and THP-1 human monocytes exposed to DEP suspension, extract or washed particles for 48 h	DNA strand breaks (by comet assay)	Significant increase in DNA strand breaks by DEP suspension and extract	Don Porto Carero et al. (2001)
Light-duty diesel car (1988, 2-L engine; no DOC); fuels: EN97, RD1, RD2; EU91/441/EEC and 94/12/EC test runs; DEP bulk sampling via collection on Teflon [®] -coated glassfibre filters, Soxhlet extraction in dichloromethane	Human MCF-7 mammary carcinoma cells exposed to 17–167 µg/mL DEP extract for 3–48 h; compared with extract of SRM 1650	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling)	Strong concentration-dependent increase in DNA adducts after 12 h of exposure; strongest response with RD2 (Swedish Class-1 fuel); PAH-derived adduct levels not explained by the sum of the adduct-forming PAHs detected	Kuljukka et al. (1998) , Kuljukka-Rabb et al. (2001)

Table 4.6 (continued)

Test conditions	Exposure system	End-point(s) examined	Results	Reference
SRM 1650a, SRM 1587, DEP from light-duty diesel car (1988, 2-L engine; no DOC); fuels: EN97, RD1, RD2; modified ECE/EUDC test procedure; DEP Soxhlet-extracted with dichloromethane	Human BEAS-2B bronchial epithelial cells exposed to 37–47 µg/mL DEP extracts, 15–150 µg/mL SRM 1650a or 1–10 µL/mL SRM 1587 for up to 48 h	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling)	Concentration-related increases in bulky adducts in all samples; agreement with concentrations of adduct-forming PAHs	Pohjola et al. (2003a)
SRM 1975; dichloromethane extract of SRM 1975	Human MCF-10A mammary epithelial cells exposed to 400 µg [concentration not given] DEP extract for 24 or 48 h in the presence/absence of B[a]P	Frequency of stable, bulky DNA adducts (by ³³ P-postlabelling with HPLC), oxidative DNA damage (8-OH-dG by flow cytometry with fluorochrome label)	No effects of exposure to DEP alone; significant stimulation of adduct formation and 8-OH-dG induction by B[a]P after co-exposure	Courter et al. (2007)
SRM 2975	Human-hamster hybrid A _L cells (fusion of human fibroblasts and Chinese hamster ovary cells) exposed to 2.5–100 µg/mL DEP for 24 h	Frequency of mutations at the <i>CD59</i> surface-antigen locus (positive selection assay)	Concentration-related increase in <i>CD59</i> mutations, significant for treatments with ≥ 50 µg/mL DEP	Bao et al. (2007)
SRM 1650b	Pulmonary epithelial cells from FE1 MutaMouse exposed <i>in vitro</i> to DEP at 37.5 or 75 µg/mL, for 8 exposure periods of 72 h	Mutations at the transgenic <i>cII</i> locus	Concentration-related increase in <i>cII</i> mutant frequency, significant at 75 µg/mL DEP	Jacobsen et al. (2008)
General Motors 5.7-L diesel engine; FTP urban driving cycle; DEP collected by high-volume sampling from dilution tunnel onto glassfibre filters; extracted with DMSO or with DPL	Chinese hamster V79 and Chinese hamster ovary (CH) cells exposed to DEP extracts, or to dispersions of extracted DEP (34, 68 or 136 µg/mL)	MN frequency	Significant concentration-related increase in MN in V79 and CHO cells by DMSO extract, and in CHO cells by sediment after extraction with DPL	Gu et al. (1992)
Heavy-duty, 11.15-L, direct injection, 6-cylinder engine and light-duty, 1.82-L, 4-cylinder engine, operating with torque 35.4 and 4 kg/m, respectively; DEP collected on Teflon®-coated filters, extracted with dichloromethane	Cultured human lymphocytes from 8 nonsmoking donors exposed to 50–400 µg/mL DEP extract for 44 h	MN by cytokinesis-blocked MN assay including kinetochore labelling to assess aneuploidy	DEP extract from low-duty engine induced a significant increase in MN in cells from 6 of 8, and in kinetochore-positive MN in cells from 4 of 8 donors; DEP extract from heavy-duty engine induced significant increase in MN and kinetochore-positive MN in cells from one donor	Odagiri et al. (1994)

Table 4.6 (continued)

Test conditions	Exposure system	End-point(s) examined	Results	Reference
Diesel engine operating on the US FTP urban driving cycle [further details not given]; DEP collected on glassfibre filters and dispersed in DMSO or DPPC, or extracted with dichloromethane; separation into supernatant and sediment	Chinese hamster V79 cells exposed to DEP dispersions or extract (33, 67 or 133 µg/mL) for 5 h	SCE frequency	Significant increase in SCE after exposure to DEP extract and dispersion; activity contained in the supernatant of extracted samples and in the sediment of dispersed samples; all at 133 µg/mL only	Keane et al. (1991)
DEP from an unspecified light-duty vehicle, collected on glassfibre filters, sonication/ Soxhlet extraction in dichloromethane	Cultured lymphocytes from a single nonsmoking donor, exposed to DEP extract for 4 h	SCE frequency	Significant concentration-related increase in SCE	Barale et al. (1993)
SEM 1650, extracted with dichloromethane, followed by acetone:methanol (1:1)	Primary tracheal epithelial cells from male Sprague-Dawley rats exposed to DEP or DEP extract (3–200 µg/mL) for 4 h	Frequency of transformed foci 5 wks after exposure	No increase in frequency of transformed foci	Ensell et al. (1998)
Isuzu 2.7-L (4JB1), light-duty, 4-cylinder, direct injection engine, torque 10 kg/m, 1500 rpm; DEP collected on glassfibre filters, extraction with dichloromethane (Soxhlet)	Rat alveolar type II epithelial cells (SV40T2-transformed) exposed to DEP extract fractions (dichloromethane-soluble, hexane-soluble and hexane-insoluble) at 30 µg/mL for 6 h	Global gene expression (by the Affymetrix Rat Expression Array 230A)	Significant differential upregulation of genes involved in xenobiotic metabolism (5–98-fold), oxidative stress (7.5–19.5-fold), cell cycle/apoptosis (6–124-fold), antioxidant response (50–144-fold)	Omura et al. (2009)
Isuzu 2.7-L (4JB1), light-duty, 4-cylinder, direct injection engine; DEP collected by scraping surface of dilution tunnel tubing; sonication/extraction with dichloromethane	Rat alveolar type II epithelial cells (SV40T2-transformed) exposed to DEP extract at 30 µg/mL for 6 h	Global gene expression (by Motorola CodeLink Bioarray gene-expression microarrays)	Significant upregulation of genes involved xenobiotic metabolism (2.2–5.2-fold), oxidative stress (18–82-fold) and cell cycle/apoptosis (2.1–13.2-fold)	Koike et al. (2004)
Heavy-duty, 6-cylinder, 9.2-L engine, torque 745 Nm [74.5 kg/m], 1000 rpm with or without continuously regenerating diesel-particulate filter; whole exhaust	Human A549 adenocarcinoma cells exposed at air–liquid interface (Vitrocell® system) to whole exhaust at 1:10 or 1:100 dilution for 60 min, 8.3 mL/min	Global gene expression (by Agilent human oligonucleotide microarray); expression of selected genes via quantitative real-time RT-PCR	Significant increase in expression of <i>haeme oxygenase-1</i> (<i>HMOX-1</i>) gene for conventional engine; significant increase in expression of <i>interleukin-1β</i> gene for engine with particulate filter	Tsukue et al. (2010a)
Heavy-duty 11-L, 6-cylinder diesel engine (model year 2000); steady-state: 1000–2200 rpm at 10, 50 and 100% of maximum power; DEP collected on glassfibre filters	J774A.1 murine macrophages exposed to 10, 50 or 100 µg/mL DEP suspension in PBS for 12 h	Expression of <i>p53</i> (by p53-luciferase reporter assay) and <i>Bax</i> (by semiquantitative RT-PCR)	Dose-related increases in <i>Bax</i> expression and <i>p53</i> activity, both significant at 50 and 100 µg/mL	Yun et al. (2009)

Table 4.6 (continued)

Test conditions	Exposure system	End-point(s) examined	Results	Reference
Isuzu 2.7-L (4JB1), light-duty, 4-cylinder, direct injection engine, torque 10 kg/m; DEP collected on glassfibre filters, sonication/extraction with methanol	Human microvascular endothelial cells exposed to 5, 15 or 25µg/mL DEP extract	Global gene-expression profiling (by Illumina BeadChips for 23 000 transcripts); expression of selected genes (by quantitative real-time RT-PCR)	Approximately 10-fold increase in <i>HMOX-1</i> ; upregulated genes functionally enriched for the electrophile response element, inflammation, immune response, cell adhesion and apoptosis	Gong et al. (2007)
SRM 2975	Human BEAS-2 bronchial cells exposed to 10 µg/cm ² for 1, 4, 8 or 16 h	Gene expression (by quantitative real-time RT-PCR)	Significant upregulation of <i>p21</i> mRNA and p21 protein at 1 and 4 h, and of <i>COX-2</i> mRNA and <i>COX-2</i> protein at 4 and 8 h (peak), lower at 16 h	Cao et al. (2010)
Deutz 4-cylinder diesel engine, partial (25%) load, bag-house collection of DEP	Human BEAS-2 bronchial cells and primary human bronchial epithelial (HBE) cells exposed to 10–100 µg/mL for 2, 6, 18 or 24 h; HBE cells exposed at air-liquid interface	Gene expression (by luciferase reporter-construct and quantitative real-time RT-PCR)	Significant time- and dose-dependent upregulation of <i>matrix metalloproteinase-1</i> gene in BEAS-2 cells, and in HBE at 24 h only	Li et al. (2009)
SRM 1975 (dichloromethane extract of SRM 2975)	Mouse Hepa1c1c7 hepatoma cells exposed to DEP extract (10–70 µg/mL) for 24 h	Phosphorylation of p53, p38 and JNK	No increase in phosphorylated p53; significant dose-related increases in phosphorylated MAPKs, p38 and JNK	Landvik et al. (2007)
Isuzu 2.7-L (4JB1), light-duty, 4-cylinder, direct injection engine [no details given on collection of DEP]	Primary human bronchial epithelial cells exposed to DEP at air-liquid interface (apical side, 10 µg/cm ² , 2 h)	MicroRNA expression via microRNA microarray	Significant change in expression of microRNAs involved in inflammatory response pathways and tumorigenesis	Jardim et al. (2009)

^a The first federal standards for new off-road diesel engines over 37 kW (50 hp); adopted in 1994, phased-in from 1996 to 2000 ^b Also known as ISO 8178 (8 steady-state modes with various loads and speed)

AFME20, fuel containing 20% animal fat methyl ester; B[a]P, benzo[a]pyrene; CBMN, cytokinesis-block micronucleus assay; COX-2, cyclooxygenase-2; CYP, cytochrome P450; DEP, diesel exhaust particles; DMSO, dimethyl sulfoxide; DOC, diesel oxidation catalyst; DPL, dipalmitoyl lecithin; DPPC, dipalmitoyl phosphatidyl choline (DPL and DPPC are primary components of pulmonary surfactant); ECE/EUDC, European urban driving cycle; FPG, formamidopyrimidine DNA-glycosylase; FTP, Federal Test Procedure; h, hour; HPLC, high-performance liquid chromatography; HVO, hydro-treated vegetable oil; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; min, minute; MN, micronucleus/micronuclei; 8-OH-dG, 8-oxo-2'-deoxyguanosine; PAH, polycyclic aromatic hydrocarbon; PBS, phosphate buffered solution; POC, particle oxidation catalyst; RME20, fuel containing 20% rapeseed methyl ester; rpm, revolutions per minute; RT-PCR, reverse transcriptase-polymerase chain reaction; SCE, sister chromatid exchange; SRM, standard reference material; ULSD, ultra-low sulfur diesel

of an inflammatory response, and increases in the level of phosphorylated p38 at concentrations below those that induced strand breaks. [Jalava *et al.* \(2010\)](#) treated mouse RAW264.7 macrophages with diesel emission particulates (15, 50, 150 or 300 µg/mL for 24 hours) from a light-duty engine (EPA Tier 1/EU stage II) operated with three different fuels (i.e. conventional diesel, hydro-treated fresh vegetable oil and rapeseed methyl ester), with and without a catalyst. All samples, except those derived from conventional diesel, induced a concentration-dependent increase in DNA strand breaks, which was significant at the highest two doses. Treatment of the exhaust with a combined diesel-oxidation and particle-oxidation catalyst slightly reduced the response to the samples derived from the rapeseed methyl ester-driven engine. [Hemmingsen *et al.* \(2011\)](#) exposed human A549 alveolar epithelial cells to diesel PM (0.78–100 µg/mL) from two light-duty engines that met Euro-2 (~1996) and Euro-4 (~2005) emission standards. All samples induced concentration-related increases in DNA strand breaks and formamidopyrimidine–DNA glycosylase-sensitive sites, which were lowest with samples from the Euro-4 engine. A comparison of different fuels showed that PM generated from a 20% v/v blend of low-sulfur diesel and biodiesel (i.e. rapeseed methyl ester or animal fat methyl ester) was less genotoxic than that from the reference low-sulfur diesel. The observed responses in the comet assay were similar to those elicited by samples of SRM 2975.

[Dybdahl *et al.* \(2004\)](#) exposed human A549 cells for 2, 5 or 14 hours to SRM 1650 (10, 50, 100 or 500 µg/mL) suspended in culture medium. DNA strand breaks were significantly increased at the highest two concentrations. When human A549 alveolar cells and human THP-1 monocytes were exposed for 48 hours to an SRM 1650 suspension, an SRM 1650 extract or a suspension of solvent-washed SRM 1650 (16, 160 or 1600 ng/mL), significant increases in DNA strand breaks were observed in cells exposed to high doses of the

extract or the suspension of SRM 1650. Solvent-washed material did not produce an effect ([Don Porto Carero *et al.*, 2001](#)).

[Kuljukka *et al.* \(1998\)](#) and [Kuljukka-Rabb *et al.* \(2001\)](#) assessed the frequency of bulky DNA adducts in human MCF-7 mammary cells exposed for 3–48 hours to dichloromethane extracts of diesel PM produced by a 1988 light-duty diesel engine operated with three different European fuels. Strong concentration-related increases in DNA adduct levels were seen after 24 and 48 hours of exposure, and the strongest response was observed with PM from the engine run on Swedish Class 1 diesel fuel. Use of low-sulfur standard diesel fuel did not enhance the levels of adducts. The formation of bulky adducts could not be correlated with the amounts of specific strong and weak adduct-forming PAHs in the PM extracts. Using the same exposure protocol in human BEAS-2B bronchial epithelial cells, [Pohjola *et al.* \(2003a\)](#) found that all samples induced concentration-related increases in bulky adducts in agreement with the levels of adduct-forming PAHs. [Courter *et al.* \(2007\)](#) measured stable DNA adducts (by [³²P]-postlabelling/HPLC) and oxidative DNA lesions (i.e. 8-OH-dG) in human MCF-10A breast epithelial cells exposed for 24 or 48 hours to SRM 1975, alone or in combination with benzo[*a*]pyrene or dibenzo[*a,l*]pyrene. No effects were seen after treatment with SRM 1975 alone. The diesel particulates strongly reduced adduct formation and stimulated 8-OH-dG induction produced by benzo[*a*]pyrene, but had no effect on those produced by dibenzo[*a,l*]pyrene.

[Jacobsen *et al.* \(2008\)](#) assessed mutation frequency at the *cII* locus in transgenic Muta^oMouse FE1 cells exposed to SRM 1650b (37.5 or 75 µg/mL) during eight 72-hour cycles, and found a concentration-dependent increase in mutant frequency, which was statistically significant at the highest concentration. [Bao *et al.* \(2007, 2009\)](#) exposed human–hamster hybrid A₁ cells to SRM 2975 for 24 hours or to

SRM 1975 for 30 minutes, alone or in combination with ultraviolet light. Exposure to SRM 2975 induced a concentration-related increase in CD59 mutations, with a significant response at 50 and 100 µg/mL. The short exposure to SRM 1975 alone did not elicit an increase in CD59 mutants, but caused a twofold increase in ultraviolet light-induced mutant frequency.

[Oh & Chung \(2006\)](#) (described above) noted that a crude extract of diesel emission particles from a heavy-duty vehicle induced a significant increase in the frequency of micronuclei in Chinese hamster ovary cells, in both the absence and presence of exogenous metabolic activation. After fractionation, the fraction containing PAHs and alkyl-PAHs induced the strongest increase in micronuclei, and the response was enhanced by exogenous metabolic activation. In the study by [Bao et al. \(2009\)](#), the short exposure to SRM 1975 alone did not have an effect on the frequency of micronuclei, but caused a twofold increase in ultraviolet light-induced micronucleus formation.

[Gu et al. \(1992\)](#) exposed Chinese hamster V79 lung fibroblasts to a suspension of diesel engine emission particles, DMSO or saline extracts of diesel engine emission particles or diesel engine emission particles washed with DMSO or saline. The DMSO extract induced a concentration-related increase in micronucleus frequency. [Liu et al. \(2005\)](#) showed that extracts of PM generated by three 1998/2000 diesel engines induced a weak increase in micronuclei in Chinese hamster V79 cells. [Odagiri et al. \(1994\)](#) assessed the frequency of micronuclei and kinetochore-positive micronuclei in cultured human lymphocytes exposed for 4 hours to organic extracts of diesel emission particles from a heavy-duty and a light-duty engine. Extracts of light-duty particles induced a significant increase in micronuclei in cells from six out of eight donors, and an increase in kinetochore-positive micronuclei in cells from four out of eight donors. Extracts of diesel emission particles from the heavy-duty engine induced a

significant increase in the frequency of micronuclei and in kinetochore-positive micronuclei in cells from only one donor.

Exposure to extracts of diesel engine emission particles or diesel engine emission particles dispersed in a pulmonary surfactant (dipalmitoyl phosphatidyl choline) for 39 hours induced a significant increase in the frequency of sister chromatid exchange in Chinese hamster V79 cells ([Keane et al., 1991](#)). [Barale et al. \(1993\)](#) exposed cultured human lymphocytes (from a single donor) to a dichloromethane extract of diesel emission particles from a light-duty vehicle, and found a significant concentration-related increase in sister chromatid exchange.

[Ensell et al. \(1998\)](#) treated primary rat tracheal epithelial cells with an organic extract of SRM 1650 *in vitro* and found no significant increase in transformed foci 5 weeks after exposure.

[The Working Group noted that a large number of studies reported changes in the expression of genes related to inflammation, apoptosis, cell cycle, xenobiotic metabolism, antioxidant response, invasion, migration and metastasis of transformed cells, and oxidative stress in mammalian cells exposed to diesel engine exhaust, diesel engine exhaust particles or extracts of diesel engine exhaust particles. The results of several selected publications are briefly summarized below.]

Changes in global gene expression were investigated in rat alveolar type II cells exposed to organic fractions of diesel exhaust particles from a light-duty engine. Large increases were noted in the expression of genes involved in xenobiotic metabolism, oxidative stress, antioxidant response, cell-cycle control, cell proliferation and apoptosis. [Omura et al. \(2009\)](#) exposed rat SV40T2 alveolar epithelial cells for 6 hours to various extract fractions of diesel engine exhaust particles that were soluble in dichloromethane and soluble/insoluble in *n*-hexane and found a 144-fold increase in the expression of *Hmox1*, a 98-fold increase in the expression of *Cyp1A1*, a

50-fold increase in *sulforedoxin 1 homologue*, a 13-fold increase in *Gsta3*, an 11-fold increase in *NQO1* and a five- to sixfold increase in *IL7* across the different fractions. Using the same assay system, [Koike et al. \(2004\)](#) noted a greater than twofold increase in the expression of more than 50 genes, including *Hmox1* (82-fold increase), *Gsta* (27-fold), *heat-shock protein 70-1* (18-fold) and *Akr1* (fivefold). Subsequent western blot analyses showed significant dose-related increases in the Hmox-1 protein 12 and 24 hours after exposure; this increase in protein expression was reduced by treatment with the antioxidant *N*-acetyl-L-cysteine.

Induction of apoptosis was determined in murine J774A.1 macrophages exposed for 24 hours to a suspension of diesel engine exhaust particles in saline. A significant dose-related increase was noted in the activity of p53 protein and in the expression of the *Bax* gene, a pro-apoptotic endogenous target of p53-dependent transcriptional activation ([Yun et al., 2009](#)).

Changes in global gene expression were seen in human microvascular endothelial cells exposed for 4 hours to diesel engine exhaust particulate extract. Significant upregulation of genes functionally enriched for the expression of the electrophile-responsive element, immune response, cell adhesion and apoptosis was observed. Subsequent analysis of selected genes by quantitative reverse transcription–polymerase chain reaction revealed a substantial increase in oxidative stress (i.e. *Hmox1*) ([Gong et al., 2007](#); [Lee et al., 2012](#)).

The transcription of *cyclin-dependent kinase inhibitor 1* (or *p21*), a negative regulator of the cell cycle, was significantly upregulated in human BEAS-2B bronchial cells exposed to SRM 2975 for 4 or 8 hours. Moreover, this increase was independent of p53 and Sp1, and involved activation by *STAT3*, a transcription factor associated with oxidative stress ([Cao et al., 2010](#)). In an earlier study, the same research group reported that SRM 2975 induced increased expression of

cyclooxygenase-2, a gene that plays an important role in inflammatory responses to external stimuli such as oxidative stress ([Cao et al., 2007](#)).

Significant upregulation of the *matrix metalloproteinase-1* gene was observed following exposure to diesel engine exhaust particles in human BEAS-2B bronchial epithelial cells and human primary bronchial epithelial cells obtained by brushing the airways during bronchoscopy. *Matrix metalloproteinase-1* is associated with inflammation, as well as invasion, migration and metastasis of transformed cells, and is known to be involved in pathological processes linked to malignant and non-malignant respiratory diseases, such as asthma, chronic obstructive pulmonary disease and bronchial carcinoma ([Li et al., 2009](#)).

The Working Group reviewed two studies that use a system to expose cells to diesel engine exhaust at an air–liquid interface. Human A549 adenocarcinoma cells were exposed in such a system to diluted (1:10, 1:100) emissions from a heavy-duty engine with a normal muffler or with a continuously regenerating DPF. A significant increase in oxidative stress was observed (i.e. increased expression of *Hmox1*) for emissions from the conventional engine only. However, significant increases in pro-inflammatory processes (i.e. *IL1 β* gene expression) were only seen with the continuously regenerating DPF with a high dilution of the exhaust ([Tsukue et al., 2010a](#)). [The Working Group noted that the latter effect was probably the result of increased levels of nitrogen oxides with the continuously regenerating DPF.]

[Jardim et al. \(2009\)](#) exposed primary human bronchial epithelial cells obtained from cytological brushing at bronchoscopy to diesel engine exhaust particles at an air–liquid interface and noted significant, exposure-related changes in the expression of microRNAs associated with inflammatory and tumorigenic responses.

[Landvik et al. \(2007\)](#) assessed various responses of mouse Hepa1c1c7 hepatoma cells to

exposure to SRM 1975, and found an increase in the number of necrotic cells, but little or no effect on apoptosis. In addition, slightly enhanced p53 phosphorylation, and a significant concentration-related increase in the phosphorylation of mitogen-activated protein kinases, p38 and c-Jun N-terminal kinases was noted.

(iv) *Effects in vitro in DNA solutions*

Techniques such as [³²P]-postlabelling or HPLC have been used to investigate the formation of bulky DNA adducts in calf-thymus DNA exposed to diesel engine exhaust particulate extracts in the presence of exogenous metabolic activation systems, such as post-mitochondrial supernatant from Aroclor-induced rat liver, to induce the oxidative metabolism of aromatic hydrocarbons (i.e. PAHs) or to provide enzymes, such as xanthine oxidase, that catalyse the reductive metabolism of compounds such as nitro-PAHs. The results of these studies are summarized in [Table 4.7](#).

The frequency and profile of bulky DNA adducts were studied in calf-thymus DNA after treatment with extracts of diesel engine exhaust particles in the presence of Aroclor-induced rat liver post-mitochondrial supernatant or xanthine oxidase ([Jahnke et al., 1990](#); [Gallagher et al., 1991, 1993](#); [King et al., 1994](#); [Savela et al., 1995](#); [Kuljukka et al., 1998](#); [Pohjola et al., 2003b](#)). The induction of bulky adducts by dichloromethane extracts of diesel exhaust particles from a series of light- and heavy-duty vehicles was also investigated ([Albert et al., 1983](#); [Gallagher et al., 1991, 1993](#); [King et al., 1994](#)). The results showed a high frequency of stable adducts that did not co-chromatograph with known PAH-diol epoxide-DNA adduct standards. Treatment in the presence of xanthine oxidase indicated that reductive metabolism contributed to the formation of unidentified diesel engine exhaust particle-derived nitro-PAH adducts. In addition, reductive metabolism yielded a nuclease P1-sensitive adduct (analysed by [³²P]-postlabelling) that was distinct from

adducts induced by post-mitochondrial supernatant-catalysed oxidative metabolism. [Savela et al. \(1995\)](#) confirmed a high frequency of adducts after exposure to diesel exhaust particulate extracts from two light-duty vehicles; however, although benzo[a]pyrene- and chrysene-derived adducts were detected, the major spots did not co-chromatograph with authentic standards for these two adducts, or for adducts formed from three isomeric benzofluoranthenes.

Studies that examined extracts of diesel exhaust particles from light-duty vehicles running on three different European fuels reported higher levels of adducts in the presence of xanthine oxidase than in that of post-mitochondrial supernatant. [Kuljukka et al. \(1998\)](#) found that the potency of the extract (expressed as adduct frequency per microgram of EOM) was reduced for low-sulfur fuels and that this reduction was associated with a reduction in PM and PAH emissions per distance travelled. [Pohjola et al. \(2003b\)](#) noted that diesel engine exhaust particulate extracts yielded higher levels of adducts than gasoline PM extracts, and that the difference was greatest under reductive conditions. These results were consistent with the notion that nitro-PAHs contribute to the formation of bulky adducts induced by exposure to diesel engine exhaust particulate extracts. [Topinka et al. \(2012\)](#) examined the induction of stable adducts in calf-thymus DNA exposed, in the presence of a rat liver post-mitochondrial supernatant, to extracts of diesel exhaust particles from two engines run on diesel fuel, rapeseed oil or rapeseed oil methyl ester (B100). Depending on the test cycle used, diesel emissions contained similar or substantially higher amounts of total PAHs than rapeseed oil emissions, while the amounts of carcinogenic PAHs were comparable or significantly higher in the emissions generated from rapeseed oil. In the absence of exogenous metabolic activation, the levels of some unidentified adducts were increased under certain conditions (e.g. diesel engine test cycles), but the

Table 4.7 Summary of studies on the formation of adducts and oxidative damage in calf-thymus DNA exposed to extracts of diesel exhaust particles (DEP)

Test conditions	Exposure system	End-point(s) examined	Results	References
VW Rabbit light-duty diesel vehicle [no details given]; DEP collected and Soxhlet-extraction with dichloromethane	Incubation of calf-thymus DNA with DEP extract (100 µg/mL) for 4 h with Aroclor-induced rat-liver S9 or xanthine oxidase (to induce nitro-reduction)	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling, TLC and HPLC)	High frequency of adducts, but those derived from DEP did not elute with any PAH or nitro-PAH adduct standards; reductive metabolism may have contributed to the formation of nitro-PAH adducts	King et al. (1994)
Three light-duty diesel vehicles (Mercedes, VW Rabbit, Nissan), 1 heavy-duty diesel vehicle (Caterpillar) and 1 gasoline-fuelled vehicle (Ford Van); DEP collected and Soxhlet-extraction with dichloromethane [details in Lewtas et al. (1981)]	Incubation of calf-thymus DNA with DEP extract (100 µg/mL) for 1.5 h with Aroclor-induced rat-liver S9 or xanthine oxidase	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling; enrichment by nuclease P1 digestion and extraction with butanol)	All DEP samples produced adducts with S9; gasoline sample only yielded adducts without S9; reductive metabolism (with xanthine oxidase) produced a major nuclease P1-sensitive adduct distinct from S9-induced adducts; reductive metabolism may have contributed to the formation of nitro-PAH adducts	Gallagher et al. (1991)
Light-duty diesel engines (Datsun, Oldsmobile, VW Rabbit); HWFET driving cycle; diesel fuel No. 2; DEP collected on Teflon-coated glassfibre filters, extraction with dichloromethane	Incubation of calf-thymus DNA with DEP extract (100 µg/mL) for 1.5 h with Aroclor-induced rat-liver S9 or xanthine oxidase (under anaerobic conditions)	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling)	A major adduct formed in DEP-treated human lymphocytes co-migrated with a benzo(a)pyrene-derived adduct; reductive metabolism yielded a major nuclease P1-sensitive adduct that appeared to be derived from a nitro-PAH	Albert et al. (1983) , Gallagher et al. (1993)
Light-duty diesel car (1988, 2-L engine, no DOC); fuels: EN97, RD1, RD2; EU91/441/EEC and 94/12/EC test runs; DEP bulk sampling via collection on Teflon-coated glassfibre filters, extraction in dichloromethane	Calf-thymus DNA exposed to 8 or 17 µg DEP extract [no details given], without or with rat-liver S9 or xanthine oxidase	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling)	Higher adduct levels with than without S9 or xanthine oxidase; highest levels for EN97 (high-sulfur fuel); up to 80% fewer adducts with low-sulfur RD1 and RD2 fuels; reduced PM and PAH (per km) for low-sulfur fuels	Kuljukka et al. (1998)

Table 4.7 (continued)

Test conditions	Exposure system	End-point(s) examined	Results	References
Light-duty diesel car (1988 Toyota, 2-L engine, no DOC); fuels: EN97, RD1, RD2; test runs: EU91/441/EEC and 94/12/EC; DEP collected on glassfibre filters, Soxhlet extraction in dichloromethane; gasoline-derived PM and SRM 1650 also tested	Incubation of calf-thymus DNA with 150 µg/mL PM extract for 4 h, with or without Aroclor-induced rat-liver S9 or xanthine oxidase	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling)	Higher levels of adducts under reductive conditions, consistent with higher level of nitro-PAHs; yield of PAH-derived adducts (with S9) from gasoline-derived PM and DEP not significantly different (RD1/RD2 only); DEP far more potent in 'adducts per km'	Pohjola et al. (2003b)
Light-duty diesel vehicles (Nissan Datsun 220C engine, VW Rabbit turbocharged engine); HWFET driving cycle; diesel fuel No. 2; DEP collected on Teflon-coated glassfibre filters, Soxhlet extraction in dichloromethane	Incubation of calf-thymus DNA with 100 µg/mL DEP extract for 1.5 h with Aroclor-induced rat-liver S9	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling and HPLC)	Both DEP extracts yielded a high frequency of adducts; the main DNA adduct did not co-migrate with B[a]P, B[b]F, B[j]F, B[k]F or chrysene adduct standards; chrysene adducts were identified	Savela et al. (1995)
Two direct injection, aftercooled diesel engines: Cummins ISBe4 (4.5 L; 2003; tested on WHSC and ESC) and Zetor 1505 (4.2 L; 2007; tested on NRSC); fuels: European diesel EN 590, RME (B100) and RSO; DEP collected by on high-volume sampler on Teflon-coated filters, extraction with dichloromethane	Incubation of calf-thymus DNA with DEP extract, corresponding to 0.3 or 3 m ³ Cummins engine exhaust and 0.1 or 1 m ³ Zetor engine exhaust, for 24 h, with or without rat-liver S9	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling)	Significant concentration-related increase in adduct levels for all samples; several-fold higher response with S9; adduct-forming potency per mg PM similar for the two engines, and similar across fuel types (diesel higher for WHSC); similar potency trend per kW-h	Topinka et al. (2012)
Isuzu A4JB1 2.7-L, light-duty 4-cylinder, direct injection engine; steady-state 2000 rpm, 6 kg/m; constant-volume dilution tunnel; DEP collected on glassfibre filters	Incubation of DEP (5, 10 or 20 mg) with calf thymus DNA for 15–120 min	Oxidative DNA damage (8-OH-dG by HPLC)	Concentration-related increase in oxidative DNA damage reaching a maximum after approximately 60 min	Nagashima et al. (1995)
Light-duty diesel engine [details not provided]; steady-state, 1050 rpm, 80% load	Incubation of DEP (10 mg/mL) with calf thymus DNA (2 mg/mL) for 0.5–3 h	Oxidative DNA damage (i.e. 8-OH-dG by HPLC, after DNA isolation with pronase/ethanol)	No significant increase in oxidative DNA damage	Iwai et al. (2000)

B[a]P, benzo[a]pyrene; B[b]F, benzo[b]fluoranthene; B[j]F, benzo[j]fluoranthene; B[k]F, benzo[k]fluoranthene; DOC, diesel oxidation catalyst; ESC, European steady-state cycle; h, hour; HPLC, high-performance liquid chromatography; HWFET, US highway fuel economy test; kW-h, kilowatt-hour; min, minute; NRSC, non-road steady-state cycle; 8-OH-dG, 8-oxo-2'-deoxyguanosine; PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; RME, rapeseed methyl ester; rpm, revolutions per minute; RSO, rapeseed oil; S9, metabolic activation system; SRM, standard reference material; TLC, thin-layer chromatography; VW, Volkswagen; WHSC, world harmonized steady-state cycle

adduct frequency per milligram of diesel exhaust particles was generally higher in the presence of such activation.

The formation of oxidative DNA lesions (i.e. 8-OH-dG) was studied in calf-thymus DNA following incubation with a suspension of diesel exhaust particles from light-duty engines. One study showed a concentration-dependent increase in oxidative damage, which reached a maximum after 60 minutes of exposure ([Nagashima et al., 1995](#)), while another did not report any change in the number of oxidative DNA lesions following incubations for 0.5–3 hours ([Iwai et al., 2000](#)).

(v) *Effects in vitro in the Salmonella reverse mutation assay*

The *Salmonella* reverse mutation test is the most common tool used to investigate the mutagenic activity of complex environmental matrices and extracts or concentrates of complex matrices (e.g., drinking-water, soil, urban air particulates and diesel engine exhaust particulates). Several *Salmonella* strains are routinely used to examine different types of mutation. The most common strains, TA98 and TA100, reveal frameshift and base-pair substitution mutations, respectively. Other common strains include TA97, used to detect frameshift mutations, and TA102 and TA104, used to assess base-pair substitution mutations containing an AT-rich allele. Detailed information on these strains is available ([Maron & Ames, 1983](#); [Mortelmans & Zeiger, 2000](#)). Several metabolically modified versions of TA98 and TA100 are frequently used to examine extracts of PM from combustion engines. The nitroreductase-deficient strain TA98NR is resistant to the effects of nitroarenes, and the *O*-acetyltransferase-deficient strain TA98/1,8DNP₆ is resistant to some of the highly mutagenic dinitropyrenes ([Blumer et al., 1980](#); [McCoy et al., 1981, 1983](#); [Speck et al., 1981](#)). More recently developed metabolically enhanced strains, derived from TA98 and TA100, contain higher levels of the

classical bacterial nitroreductase (i.e., YG1021 and YG1026) or *O*-acetyltransferase (i.e., YG1024 and YG1029) ([Watanabe et al., 1989, 1990](#)). Strains YG1041 and YG1042 are derivatives of TA98 and TA100, respectively, and contain enhanced levels of both nitroreductase and *O*-acetyltransferase ([Hagiwara et al., 1993](#)).

Due to the absence of mammalian metabolism that is required to convert some mutagenic agents to DNA-reactive electrophiles, the bacterial test system is generally supplemented with a post-mitochondrial supernatant derived from rodent liver. The most common metabolic activation system included is a fraction derived from the livers of Aroclor 1254-induced Sprague-Dawley rats, but bio-activation with a combination of phenobarbital and β -naphthoflavone is also common. Most assessments use the standard plate incorporation version of the test, whereas some assays involve preincubation of the test compound with the *Salmonella* tester strain and the metabolic activation mixture before plating. Several studies have shown that a variant of the preincubation assay, known as the micro-suspension assay, provides enhanced sensitivity to combustion products such as those present in coal tar, the urine of smokers, PM from urban air and diesel engine exhaust particles ([Kado et al., 1985, 1986](#); [Agurell & Stensman, 1992](#)).

Diesel emissions and extracts

More than 70 studies published since 1989 applied the *S. typhimurium* mutagenicity test to assess the mutagenic activity of diesel engine emissions. Most of these examined organic extracts of diesel PM collected on glassfibre filters. The concentration of the test compound in these studies is generally expressed as micrograms of EOM per plate, and the results are expressed as mutagenic potency – the slope of the initial, linear portion of the concentration–response curve (i.e. potency in the number of revertants per microgram of EOM). To facilitate comparisons between PM samples generated

under different engine conditions, fuel formulations and aftertreatment scenarios, these values are often converted to revertants per equivalent mass of diesel engine exhaust particles. Moreover, to facilitate comparative evaluations of the mutagenic hazard across different engines operated with different fuels and fuel blends, and/or different pollution control devices (e.g. diesel oxidation catalyst and/or different engine test cycles, potency values are often converted to revertants per unit of engine work (i.e. per kW-h or hp-h), revertants per unit of fuel consumed, revertants per distance travelled (e.g. mile or kilometre), revertants per hour of engine operation or revertants per cubic metre of exhaust. [Table 4.8](#) summarizes the results of recent studies that used the *S. typhimurium* mutagenicity assay to assess the mutagenic activity of diesel engine emissions.

More than 40 studies employed the *S. typhimurium* mutagenicity assay with strains TA98 and/or TA100 to assess the mutagenic activity of diesel PM extracts. The results indicated overwhelmingly that their mutagenicity, when expressed in revertants per microgram of EOM, was greater in the absence than in the presence of metabolic activation, which is consistent with earlier publications ([IARC, 1989](#)), and is also consistent with the presence of nitro-PAHs in the materials tested. It should be noted that some studies showed an increase in the mutagenicity of diesel extracts, in particular the crude extract of the semi-volatile components, in the presence of metabolic activation ([Westerholm et al., 2001](#)).

More than 20 studies employed a variety of metabolically altered *Salmonella* strains (e.g. TA98NR, TA98/1,8DNP₆, YG1024, YG1021 and YG1029) to assess the mutagenic activity of diesel engine emissions. Many reports showed substantial reductions in the mutagenic activity of extracts of diesel exhaust particulates from light-duty vehicles in *Salmonella* strains TA98NR (nitroreductase-deficient) and TA98/1,8DNP₆ (*O*-acetyltransferase-deficient) compared with

that observed in strain TA98, thus confirming the involvement of nitroarenes in the mutagenic response ([Barale et al., 1993](#); [Tahara et al., 1994](#); [Crebelli et al., 1995](#); [DeMarini et al., 2004](#)). One of these reports showed substantially reduced mutagenicity of extracts of diesel exhaust particles from a light-duty vehicle in strain TA98NR, but not in TA98/1,8DNP₆, which suggests a greater contribution of mono-nitroarenes to the mutagenic response ([Crebelli et al., 1995](#)). Other studies reported similar reductions for extracts of diesel exhaust particles from heavy-duty engines ([Hansen et al., 1994](#); [Harvey et al., 1994](#); [Bagley et al., 1998](#); [Westerholm et al., 2001](#)). For example, [Bagley et al. \(1998\)](#) noted reductions in mutagenicity of up to 69–78% with strain TA98NR and up to 73–83% with strain TA98/1,8DNP₆, relative to the levels found in TA98, for extracts of PM from a heavy-duty engine.

In contrast, increased mutagenicity of diesel PM from light- and heavy-duty engines was observed in assays with metabolically modified derivatives of TA98, such as YG1021 (enhanced production of nitroreductase), YG1024 (enhanced production of *O*-acetyltransferase) or YG1041 (enhanced production of both) ([Mikkonen et al., 1995](#); [Kuljukka et al., 1998](#); [DeMarini et al., 2004](#); [Turrio-Baldassarri et al., 2006](#)).

Effects of engine type, test cycle and fuel formulation on Salmonella mutagenicity

Variations in engine type, test cycle, fuel formulation and pollution control technology complicate the interpretation of the published results, but several general statements can be made.

The generation of diesel engine exhaust particles requires the installation of the engine or vehicle in a test stand to monitor engine speed, and the use of a dynamometer (e.g. eddy current, water brake or chassis dynamometer) to control engine load (see Section 1.1). Numerous test protocols (cycles) have been developed to simulate a range of driving conditions. Transient test

Table 4.8 Summary of studies on the mutagenicity of extracts of diesel exhaust particles in *Salmonella typhimurium*

Test conditions	Salmonella strains ^a /test version	Results	References
SRM 1650, DEP from 1988 2-L light-duty diesel car (no DOC); three fuels (EN97, RD1, RD2); European transient test procedure (ECE15) and EUDC, run in series; DEP collected on PUF and/or Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, standard plate incorporation assay, Aroclor-induced rat-liver S9	PM extracts more mutagenic than PUF extracts (the latter only positive with S9); without S9, DEP extracts more mutagenic (approximately 2–5-fold) than gasoline PM extracts, expressed per mg PM; when expressed per km, DEP extracts > 100-fold more mutagenic than gasoline PM extracts	Pohjola et al. (2003b)
DEP from a Mercedes-Benz 6.37-L, 6-cylinder and an IVECO 5.9-L, 6-cylinder diesel test engine with SCR ^b ; 13-mode ESC; 4 fuel blends (DF, RME, RSO, SMDS, 5%RME in SMDS, DF/RME/GTL blend); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Mutagenic potency values uniformly greater without S9; for the Mercedes engine, no significant difference in potency (per L exhaust gas) between DF, RME, SMDS and DF/RME/GTL blend, and RSO yielded significantly elevated potency (approximately 10-fold) and highest PM output in g/kW-h; for the IVECO engine, SCR significantly reduced mutagenic potency after 1000 h, with no difference between DF and RME, although RME associated with reduced PM emissions (g/kW-h)	Krahl et al. (2006, 2007a)
DEP from a Mercedes-Benz 6.37-L, 6-cylinder engine; 13-mode ESC; 7 fuels (two DFs, RME, GTL, 4 FAME mixtures from soya, palm and rapeseed); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Mutagenic potency (per m ³) greater without S9 and highest for DF; RME potency less than half that of DF; DEP emission rates lower (per kW-h) for all FAMEs	Krahl et al. (2005)
DEP from a Mercedes-Benz 4.25-L, 4-cylinder engine; 13-mode ESC; 4 fuels (DF, RME, LSDF, LSDF with high aromatics); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Mutagenic potency (per engine h) lowest for RME; DF 4–5-fold higher than RME, and LSDF 2–3-fold higher; no significant difference with or without S9; DEP emission rates (per kW-h) highest for DF	Krahl et al. (2003)
DEP and SVOCs from a Mercedes-Benz, 6.37-L, 6-cylinder engine; 13-mode ESC; 5 fuel blends (DF, RME, GTL, RSO, modified RSO); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction and condensates from gas phase collected at 50 °C	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	All samples yielded a positive response, and all potency values (per L exhaust gas) unchanged or reduced with addition of S9; DEP extract for RSO yielded the highest potency values (9.7–17-fold higher than DF on TA98 and 5.4–6.4-fold higher than DF in TA100); potency of modified RSO 2.8–4.4-fold higher than RSO; RSO condensate samples also yielded the highest potency values (up to 3-fold DF); few differences between DEP extracts for DF, RME and GTL, although RME significantly greater than DF in TA100 without S9	Krahl et al. (2007b, 2009a)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP and SVOCs from a Mercedes-Benz, 5.9-L, 6-cylinder engine, with and without DOC; 13-mode ESC; 4 fuels (2 DFs, RME, RME5); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction; SVOCs from condensates	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Mutagenic potency [unit not provided] uniformly higher without S9; response highest for DF reference fuel, and lowest for RME5 and RME; DOC further reduced activity of RME; no significant difference in potencies of SVOCs (per m ³), with complete elimination of activity by DOC	Krahl et al. (2009b)
DEP and SVOCs from 3 heavy-duty diesel engines: Mercedes-Benz, 6.37-L, 6-cylinder engine, MAN, 6.87-L, 6-cylinder engine, AVL single-cylinder, 1.47-L engine; 13-mode ESC, ETC, and rated power; 4 fuel types (DF, GTL RME, RME20); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction; SVOCs from condensates	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Mutagenic potency [unit not provided] uniformly higher without S9; for Mercedes engine, GTL lowest activity followed by DF, RME similar to DF and RME20 significantly elevated; for AVL and MAN engines, RME20 significantly elevated relative to DF, but RME lower than DF; for SVOCs from the MAN engine, DF potency greater than RME blends; for the Mercedes and MAN engines, PM emission rates (g/kW-h) for RME about half those of DF	Krahl et al. (2008)
DEP from a Farymann single-cylinder engine, 5 load modes (0–85%), with and without DOC; 4 fuels (DF, LSDF, RME, SME); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Mutagenic potency (per h engine operation) generally lower for RME and SME, compared with DF or LSDF; under partial load, DOC generally led to reduced mutagenicity; under heavy-duty conditions (rated power), DOC frequently led to increases in mutagenic activity	Bünger et al. (2006)
DEP from a VW 1.9-L TDI with DOC; FTP-75, MVEG-A, and modified MVEG-A cycles; DF and RME fuels; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, TA97a, TA102 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Significant positive responses for DF and RME samples in TA98 and TA100, and potency (per mg DEP) generally higher without S9; potency (per mg DEP) greater for DF compared with RME, particularly in TA98 (1.9–5.1-fold); similar pattern for potency expressed per km; potency generally higher for cycles that included a cold start (modified MVEG-A)	Bünger et al. (1998)
DEP from a Fendt tractor; 13-mode ESC; DF and RME fuels; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	All samples elicited significant positive responses; both fuels yielded more potent samples (per L exhaust) without S9; at rated power, RME potency far lower than that of DF; at idling, little difference with and without S9, and RME potency far lower than that of DF; DF 2–8-fold higher response in TA98 and 2–3-fold higher response in TA100; RME yielded higher particle emissions (g/h)	Schröder et al. (1999) , Bünger et al. (2000a)
DEP and exhaust condensate from a Mercedes-Benz Euro 3 6.37-L, 6-cylinder engine; 13-mode ESC; 4 fuels (DF, RSO, RME, GTL); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	All samples elicited significant positive responses; potency (per L exhaust) higher without S9 for TA100 only; DEP extracts from RSO and heated RSO fuels yielded highest potency samples (9.7–59 fold greater than DF for TA98 and 5.4–22.3-fold greater for TA100); potency of DEP extracts from RME also significant higher than DF; condensate samples from RSO and heated RSO also significantly elevated relative to DF (up to 13.5-fold).	Bünger et al. (2007)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP from a Farymann single-cylinder engine, 5 load modes (0–85%), without DOC; 4 fuels (DF, LSDF, RME, SME); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Mutagenic potency (per mg DEP) frequently higher without S9, and DF potency far greater (up to 10-fold) than that of RME or SME; no response in TA100 for RME and SME; potency per engine h yielded similar results and indicated that DF potency was higher at increased load and speed; PAH emissions per engine h greatest for DF and SME	Bünger et al. (2000b)
DEP from a Cummins 14-L, 6-cylinder, heavy-duty engine; 9 fuels with sulfur content 0.04–0.3%; FTP drive cycle; DEP collected on Teflon [®] -coated glassfibre filters, 30% toluene in ethanol Soxhlet extraction	TA98, microsuspension preincubation version, PB/5,6BF-induced rat-liver S9	Potency without S9 generally higher, but not for all fuels; potency per µg EOM tended to increase with decreasing sulfur content; EOM decreased with decreasing sulfur and potency per unit work (hp-h) did not vary with sulfur content	Rasmussen (1990)
Pooled gasoline PM from ‘normal emitters’ (1982 Nissan Maxima, 1994 GMC 1500 pick-up truck, 1995 Ford Explorer, 1996 Mazda Millenia) collected at 30 °F and 72 °F, a visible white-smoke emitter (1990 Mitsubishi Montero) and a visible black-smoke emitter (1976 Ford F-150 pick-up truck), and pooled DEP from current (2000) technology (1998 Mercedes Benz E300, 1999 Dodge 2500 pick-up truck, 2000 VW Beetle TDI) collected at 30 °F and 72 °F and high-emitter diesel (1991 Dodge 2500 pick-up truck) engines; CUD cycle; PM collected on Teflon [®] -coated glassfibre filters, acetone sonication extraction; SVOCs collected on PUF/XAD, acetone Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	All samples (i.e. combined PM and SVOC extracts) induced significant responses, with 10-fold range among samples; among the diesel samples, current diesel (30 °F) was the most potent (per µg EOM), with modest increases without S9; high-emitter diesel potency greater than current diesel (72 °F); gasoline samples generally more potent with S9; normal emitters at 30 °F and white-smoke emitter generally more potent; current gasoline and current diesel at 72 °F were generally the least potent; subsequent multivariate analyses showed associations between mutagenicity and nitro-PAH content (e.g. 6-nitroB[a]P, 1-NP, 7-nitroB[a]A) exhaust	Seagrave et al. (2002) ; McDonald et al. (2004a)
Two different DEP samples: 1/ scraping from exhaust pipe and cap of diesel truck idling for 30 min; 2/ collected on glassfibre filters from engine operating under US FTP; dichloromethane sonication or pulmonary surfactant extracts of DEP	TA98, preincubation version, Aroclor-induced rat-liver S9	All samples showed concentration-related responses without S9; dichloromethane extract more potent (per mg DEP) than surfactant extract	Keane et al. (1991)
DEP from a 1988 Cummins heavy-duty engine with CRT; 2-mode cycle representing 25 and 75% load at 1900 rpm; conventional DF and low-sulfur DF; and an Oldsmobile light-duty vehicle; HWFET cycle; DEP for both collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, standard plate incorporation assay, Aroclor-induced rat-liver S9	Mutagenic potency (per µg EOM) of DEP extracts similar for heavy-duty engine and light-duty vehicle; light-duty DEP extract more potent without S9	Lewtas et al. (1981) , Bagley et al. (1996) , Valberg & Watson (1999)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
PM and SVOCs from a Caterpillar heavy-duty 10-L, 4-cylinder diesel engine, with and without CDPF; transient test cycle to represent minimal conditions; PM collected on glassfibre filters, dichloromethane Soxhlet extraction; SVOCs collected on XAD	TA98, microsuspension preincubation version, unspecified S9	Dichloromethane extracts elicited significant responses with no difference with and without S9; mutagenic potency increased with CDPF, both in terms of rev/ μ g EOM (weighted average 14-fold increase) and rev/ μ g DEP (weighted average 1.5-fold increase); CDPF resulted in 90% reduction in DEP emission rate, and weighted average 70% reduction in potency per m ³ exhaust; SVOC samples more potent without S9, and CDPF reduced potency (per m ³ exhaust) by 80%; CDPF resulted in more than 98% reduction in DEP-associated PAHs (per m ³ exhaust)	Bagley et al. (1991)
DEP from 6 diesel engines: 2 heavy-duty and 4 medium-duty; PM collected on glassfibre filters, dichloromethane extraction	TA98 and TA100, standard plate incorporation assay, unspecified S9	Significant concentration-related increase in TA98 and TA100, with highest responses (per μ g EOM) in TA98 without S9; no significant difference between engines	Song & Ye (1995)
DEP from 2 light-duty (1.93-L and 2.5-L) engines with EGR; EUCD and FTP-75 cycles; LSDF and biodiesel (unspecified), with and without DPF, DOC and EGR modifications; DEP collected on Teflon [®] -coated glassfibre filters, acetone sonication extraction followed by separate acetone and benzene Soxhlet extractions	TA98 and TA100, standard plate incorporation assay, unspecified S9	Mutagenic activity (per μ g DEP) highest in TA98 without S9; DPF increased potency (per μ g DEP or per km) for the 1.93-L engine, and decreased potency for the 2.5-L engine (per μ g PM or per km), and dramatically reduced PM emissions per km; greater engine stress elicited greater mutagenic activity; biodiesel potency (per μ g DEP) lower than reference LSDF, and biodiesel emissions lower in PAHs and nitro-PAHs; reduced potency (per μ g DEP or per km) with EGR; DOC contributed to slight reductions for the 2.5-L engine (per μ g PM) and modest reductions in potency per km	Carraro et al. (1997)
DEP from a 2003 low-mileage Euro 3 2003 bus, with and without DOC or CRT, chassis dynamometer; BSC and OCC cycles; low-sulfur diesel; DEP collected on filters, organic extraction [solvent/method unspecified]	TA98, standard plate incorporation assay, with S9	Mutagenic potency (per unit PM) higher with DOC (1.8-fold) or CRT (2.2–3.4-fold); potency in rev/km higher with DOC (1.4-fold), but lower (50–70%) with CRT	Nylund et al. (2004)
DEP and SVOCs from a heavy-duty Mercedes-Benz OM 906 LA Euro 3-compliant, 6.4-L, 6-cylinder engine; low-sulfur diesel, RME, 5% RME in diesel, with and without DOC; ESC; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction; SVOC collected on chilled surface	TA98 and TA100, standard plate incorporation assay, PB/5,6BF-induced rat-liver S9	Without S9, mutagenic potency of DEP (per m ³ exhaust) modestly higher, highest for DF, and decreased for RME and 5% v/v RME; DOC contributed to modest reductions in potency of DEP extract without S9, and slight reductions with S9, and eliminated the mutagenic activity of SVOC	Westphal et al. (2012)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP from a heavy-duty, 5.785-L, 6-cylinder engine; ECE 13-mode cycle; DF and ethanol-DF blends containing 5, 10, 15 and 20% ethanol by volume; PM collected on glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, rat-liver S9 [inducer unspecified]	Significant positive responses in TA98 and TA100 at the highest concentration only; higher potency (per mg EOM) without S9, particularly for lower ethanol content; generally lower responses for increasing ethanol content; potency per kW-h indicated higher mutagenicity with increasing ethanol content; PAHs ($\mu\text{g}/\text{kW-h}$) increased with increasing ethanol content	Song et al. (2007)
DEP from a heavy-duty, 5.785-L, 6-cylinder engine; ECE 13-mode cycle; dichloromethane Soxhlet extract of PM, extract fractionated into 6 fractions	TA98 and TA100, standard plate incorporation assay, rat-liver S9 [inducer unspecified]	Crude extract elicited significant response in TA98 with S9, and in TA100 with and without S9; TA100 potency modestly higher without S9; strong direct-acting TA98 response for organic bases (e.g. amines); strong responses for fraction containing neutral aromatics	Song et al. (2006)
DEP and SVOCs from a 14.19-L heavy-duty diesel truck; FRG transient bus cycle; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction; SVOCs collected on XAD, PUF plugs or cryogenically; DEP extract fractionated on silica into 5 fractions with increasing polarity	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Crude DEP extract more potent (per km) without S9 with highest potency in TA100; highest responses in fractions containing nitro-PAHs, dinitro-PAHs and oxygenated PAHs; SVOC crude extract far less potent than DEP extract; SVOC extracts from XAD and PUF samplers more potent than cryogenic extracts; highest potency in polar fractions containing nitro-PAHs, dinitro-PAHs and oxygenated PAHs	Alsberg et al. (1985) , Westerholm et al. (1991)
DEP from two heavy-duty (11-L and 9.6-L), 6-cylinder engines; THB transient bus cycle; 10 DFs; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Range of potency values (per m^3) for different DFs; higher potency (1–2 orders of magnitude) for the 9.6-L engine, particularly without S9; multivariate analyses showed that mutagenic activity without S9 related to nitrates and 1-NP; S9-activated mutagenicity related to certain PACs; fuel density and flash- point positively correlated to mutagenic activity; fuel sulfur contributed to amount of emitted particles and genotoxicity	Westerholm et al. (1991) , Sjögren et al. (1996a, b)
DEP from an Isuzu 2.369-L, 4-cylinder engine; custom 4-stage cycle; aftertreatment with DOC, DPF or DFE; DEP collected on Teflon [®] -coated glassfibre filters, acetone sonication extraction	TA98, microsuspension preincubation version, without S9	Without aftertreatment, mutagenic potency higher at low load and DOC eliminated the mutagenic activity; DPF and DFE samples showed equivalent or higher mutagenic activity per μg EOM; DPF and DFE reduced PM emissions by 51–71%, and mutagenic activity (per m^3) by 30–62%	Bugarski et al. (2007) , Shi et al. (2010)
DEP from a light duty Isuzu (4JG2) 3.06-L, 4 cylinder engine and a Yamaha 1.0-L engine; DEP collected on glassfibre filters, sonication extraction with benzene/ethanol (3:1), fractionated on silica gel into 4 fractions with increasing polarity	TA98 without S9 and TA100 with S9 [unspecified], standard plate incorporation assay	Mutagenic potency (per mg DEP) higher for the larger engine, particularly without S9; S9-activated mutagenicity highest in less polar fraction; direct-acting mutagenicity highest in moderately polar fractions; detected PAHs accounted for less than 0.1% of the response with S9; nitro-PAHs accounted for 12% of the direct-acting response	Yang et al. (2010)

Table 4.8 (continued)

Test conditions	Salmonella strains*/test version	Results	References
Unspecified diesel engine; idling with periodic acceleration; PM collected on glassfibre filters, dichloromethane sonication extraction, fractionated on silica gel into 5 fractions with increasing polarity	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Mutagenic activity (per μg DEP) highest in TA98 without S9; no significant response in TA100; strongest responses for base, PAH and polar fractions	Lu et al. (1999b)
Exhaust from a 2.07-L light-duty diesel engine; 4 steady-state conditions	TA98 and TA100, direct exhaust exposure method, Aroclor-induced rat-liver S9	Mutagenic potency (per m^3) without S9 higher in TA100; higher potency at low load with increasing potency at higher speeds (i.e. maximal at low load, high speed)	Courtois et al. (1993)
DEP from a light-duty Nissan engine; HWFET; DEP collected on Teflon®-coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, plate incorporation and spiral assays, Aroclor-induced rat-liver S9	Mutagenic potency (per μg EOM) higher without S9, especially for TA98; spiral assay, which requires less sample, yielded stronger response, possibly due to differences in relative amounts of S9	Houk et al. (1991)
DEP from a light-duty 2.1-L, 4-cylinder diesel engine; FTP cycle; DEP collected on Teflon®-coated glassfibre filters, Soxhlet extraction with dichloromethane, methanol, acetone or acetonitrile, single solvent or sequential extraction	TA98, plate incorporation assay, without S9	Single-solvent extraction with dichloromethane yielded highest potency (per μg EOM); sequential extraction revealed that dichloromethane extracted 97% of total mutagenic activity	Montreuil et al. (1992)
DEP and SVOCs from a 4.6-L, 6-cylinder Caterpillar engine; EPA heavy-duty transient test cycle; 4 fuels (DF, RME, HySEE, HySEE50 blend); DEP collected on Teflon®-coated glassfibre filters, dichloromethane Soxhlet extraction; SVOCs collected on PUF plugs, supercritical carbon dioxide extraction	TA98 and TA100, microsuspension preincubation version, Aroclor-induced rat-liver S9	Mutagenic potency of DEP extract (per hp-h) higher without S9; HySEE potency lower than 50/50 blend with DF, which was lower than DF alone; SVOC samples from DF about 2-fold more mutagenic than HySEE; HySEE associated with considerable reductions in PM and PAH emission rates (per hp-h)	Chase et al. (2000)
DEP and SVOCs from a heavy-duty 7.6-L, 6-cylinder engine; low idling for 150 min; DEP collected on Teflon®-coated glassfibre filters, sequential sonication extraction with dichloromethane and methanol; SVOCs collected on PUF plugs and XAD extracted with supercritical carbon dioxide	TA98 and TA100, microsuspension preincubation version, Aroclor-induced rat-liver S9	Mutagenic activity of SVOC sample highest in TA100 with S9; DEP extract potency (per μg DEP) highest in TA98 with S9; total response for sampling period showed greatest response in TA100 with S9, followed by TA98 with S9; SVOC samples accounted for about 20% of TA98 and 50% of TA100 mutagenicity	Kado et al. (1996b)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP and SVOCs from a Cummins 10.8-L, 6-cylinder engine with EGR, with and without CPF; LSDF; 2 steady-state modes; DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction	TA98, microsuspension preincubation version, without S9	No difference between mutagenic potency of DEP or SVOC extracts (per µg EOM) with and without CPF; at lower load, CPF reduced DEP potency (per m ³) by 65% and SVOC potency by 73%; CPF significantly reduced PM, EOM and PAHs (per m ³); 1-NP emissions increased at high load	Suresh <i>et al.</i> (2001)
DEP from a heavy-duty Cummins 5.9-L engine; EPA heavy-duty cycle; 4 fuels (DF, 20% REE, 50% REE, REE), with or without DOC; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane sonication extraction	TA98, microsuspension preincubation version, Aroclor-induced rat-liver S9	Mutagenic potency (per µg DEP) lowest for REE and highest for DF (with and without DOC); DOC contributed to increases in mutagenic potency per µg DEP; similar potency pattern when expressed per mile: higher potency with DOC; some increase in 5- and 6-ring PAH emissions (µg per mile) for REE	Kado <i>et al.</i> (1996a)
DEP from 2 heavy-duty diesel engines: 7.3-L, 6-cylinder with SCR, and 8.9-L, 5-cylinder with EGR and DOC; transient THC bus cycle; DF and HVO; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, standard plate incorporation assay, without S9	No significant response for any sample; HVO associated with lower DEP emission rate (mg/km)	Kuronen <i>et al.</i> (2007)
DEP from 3 diesel engines: light-duty 1.686-L, 4-cylinder; heavy-duty 12.8-L, 6-cylinder with DPF and SCR; heavy-duty 10.52-L, 6-cylinder with DPF; DF and 4 plant oils (peanut, rapeseed, soya, sunflower); DEP collected on glassfibre filters, dichloromethane Soxhlet extraction	TA98, TA100 and TA Mix, fluctuation assay (Xenometrics)	All samples in the range of the negative control with no evidence of differences in activity between the fuels	Dorn & Zahoransky (2009)
DEP and SVOCs from a heavy-duty Cummins 10-L, 6-cylinder engine; 2 steady-state modes of EPA heavy-duty cycle, with and without DPF; DF and LSDF; DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction	TA98, microsuspension preincubation version, Aroclor-induced rat liver S9	Mutagenic potency similar with and without S9; very little variations in potency (per µg EOM, per µg DEP, per m ³) with fuel sulfur content; higher potency at low load; DPF associated with 9–67%, 30–57% and 54–94% reductions in potency per µg EOM, per µg DEP and per m ³ , respectively; SVOC mutagenicity eliminated by DPF	Kantola <i>et al.</i> (1992)
DEP and SVOCs from a heavy-duty Cummins 10-L, 6-cylinder, engine; 2 steady-state modes from EPA heavy-duty cycle; LSDF and DF; DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction	TA98, microsuspension preincubation version, unspecified S9	Mutagenic potency similar with and without S9; mutagenic potency (per µg DEP) for DEP extracts and SVOCs generally higher for LSDF, and higher at low load; similar pattern for potency expressed per m ³ ; PM emission per m ³ lower for LSDF; PAHs (per m ³) often elevated for LSDF	Opris <i>et al.</i> (1993)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP and SVOCs from a heavy-duty Cummins 10-L, 6-cylinder engine, with and without DOC; 3 steady-state modes from EPA heavy-duty cycle; DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction	TA98, microsuspension preincubation version, unspecified S9	Mutagenic potency similar with and without S9; DOC increased potency of DEP extract per µg EOM for 2 lower loads, and reduced potency (either per µg DEP or per m ³) for lowest load only; DOC generally reduced emission rates (per m ³) for PM, PAHs and nitro-PAHs, especially at low load	Pataky et al. (1994)
DEP and SVOCs from a heavy-duty Caterpillar 7-L engine with and without DOC; custom transient cycle; two DFs (cetane-adjusted fuel (CA) and LAF); DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction	TA98, microsuspension preincubation version, Aroclor-induced rat-liver S9	Mutagenic potency similar with and without S9; potency of CA fuel lower than that of LAF, but no difference when potency expressed per m ³ ; for CA, DOC removed 41, 51 and 66% of the mutagenic activity per µg EOM, µg DEP and per m ³ , respectively; similar reductions for LAF; SVOC mutagenicity (per µg EOM) increased with DOC, which contributed to substantial declines in PAH emissions rate (per m ³)	McClure et al. (1992)
DEP from a heavy-duty Caterpillar 6.96-L, 4-cylinder engine; 6-mode steady-state cycle; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane sonication extraction	TA98, preincubation version, unspecified S9	Mutagenic potency (per µg DEP) uniformly higher with S9; highest potency at 50% load and reduced at full load	Nussear et al. (1992)
DEP from a light-duty 2.5-L, 4-cylinder engine; 5 steady-state conditions; DEP collected on glassfibre filters, dichloromethane Soxhlet extraction	TA98, standard plate incorporation assay, Aroclor-induced rat-liver S9	Mutagenic potency (per µg EOM) higher with S9 at no or low load, and decreased with increasing load; higher direct-acting potency with increasing nitrogen oxides; PAH emission rate (per m ³) decreased with increasing load	Christensen et al. (1996)
DEP and SVOCs from a heavy-duty Cummins 10.8-L, 6-cylinder engine, different levels of EGR; 2 steady-state modes from EPA heavy-duty cycle; DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction	TA98, standard plate incorporation assay, without S9	Substantial increases in mutagenic potency of DEP extract (per m ³) with increasing EGR; increased EGR also associated with substantial decreases in SVOC mutagenicity (per m ³), especially at high load, and some increases in PAH emission rate (per m ³), especially at low load	Kreso et al. (1998)
DEP from 1986 Mercedes-Benz 3.0-L, 6-cylinder and a VW Jetta 1.5-L, 4-cylinder engines with EPTO; 4 test cycles (NYCC, HFET, CFTP, HFTP); low sulfur No. 2 diesel; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, microsuspension preincubation version, PB/5,6BF-induced rat-liver S9	All samples elicited significant positive responses; no change in TA100 with addition of S9; 3/8 samples more potent (per µg EOM) in TA98 without S9; no significant differences between samples with and without EPTO; EPTO resulted in significant (87–92%) reductions in PM for all cycles; expressed per mile, potency values with EPTO show substantial reductions (69–95%)	Rasmussen et al. (1989)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP from a small 0.27-L diesel engine, 2000 rpm, no load; DEP collected on glassfibre filters, sonication extraction with methanol, dichloromethane or benzene/ethanol (4:1)	TA98, TA100, TA98NR and TA98/1,8DNP ₆ , standard plate incorporation assay, Aroclor-induced rat-liver S9	Significant concentration-related responses for all samples, with highest potency in TA98 without S9; benzene/ethanol extract most potent followed by dichloromethane and methanol extracts; significant reductions in potency in TA98NR and TA98/1,8DNP ₆ indicated presence of nitroarenes; chemical analyses confirmed elevated concentrations of 1-NP and 1,6-DNP in the benzene/ethanol extract	Tahara et al. (1994)
DEP and SVOCs from a heavy-duty 1983 Caterpillar engine with DOC; custom 16-mode cycle representing light- and heavy-duty operations; LSDF and SME; DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction	TA98, TA100, TA98NR and TA98/1,8DNP ₆ , microsuspension preincubation version, Aroclor-induced rat-liver S9	Mutagenic potency (per kW-h) greater for LSDF compared with SME; potency far greater for DEP extracts than SVOCs, and DOC resulted in > 50% reduction in DEP and SVOC mutagenicity; potency of DEP extract from LSDF dramatically reduced in TA98NR (69–78%) and TA98-DNP (73–83%); SME emissions showed lower total PM and reduced PAHs and 1-NP relative to LSDF	Bagley et al. (1998)
PM from Volvo 240, 4-cylinder diesel and Volvo 4-cylinder spark ignition engines; PM collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction, silica fractionation into 5 fractions with increasing polarity	TA98 and TA98NR, standard plate incorporation assay, Aroclor-induced rat-liver S9	Significant concentration-related responses for all samples, with highest potency (per m ³) for crude DEP in TA98 without S9 and crude gasoline PM in TA98 with S9; potency of diesel 7-fold greater than that of gasoline (in TA98 without S9); both reduced in TA98NR; PAH-containing fraction only active with S9 and more potent for gasoline PM; most DEP extract mutagenicity in medium polarity fractions containing nitro-PAHs, dinitro-PAHs and oxygenated PAHs; active DEP fractions showed elevated 1-NP, and mutagenicity in a subfractions containing dinitro-PAHs; oxygenated PAH derivatives were a major component of mutagenic subfractions	Strandell et al. (1994)
DEP and SVOCs from a Volvo 12.1-L, 6-cylinder Euro 2 truck; THB transient bus cycle; 2 DFs; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction; SVOCs from acetone Soxhlet extract of PUF plugs	TA98, TA98NR and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	For both fuels, DEP extracts more potent than SVOC extracts (per km); potency higher without S9 and reduced in TA98NR; reference DF 4–9.6-fold more potent than experimental fuel (MK1). MK1 resulted in 98% and 88% reductions in PAHs and 1-NP, respectively, in DEP	Westerholm et al. (2001)
DEP from a 6-cylinder diesel bus; 5 modes of the 13-mode ESC, with and without DOC; DEP collected on glassfibre filters, acetone Soxhlet extraction	TA98, TA98NR and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Direct-acting mutagenicity (per m ³) reduced with DOC for all modes, and in TA98NR relative to TA98; lowest reduction at high load; TA98 potency with S9 enhanced with DOC for 3/5 modes; DOC reduced PM and PAH emission rates (per m ³) by 30 and 80%, respectively.	Hansen et al. (1994)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP from 3 light-duty vehicles (2.0-L without DOC, 2.0-L with DOC, 1.6-L without DOC); FTP drive cycle; 4 fuels (DF, LSDF); DEP collected on Teflon [®] -coated glassfibre filters, toluene Soxhlet extraction	TA98 and TA98NR, standard plate incorporation assay, without S9	Mutagenic potency (per km) reduced with DOC; low-sulfur fuels associated with large (30–90%) reductions in potency; difference between TA98NR and TA98 significant for reference fuel only; strong positive correlations between mutagenicity (per km) and selected PAH emission rates (per km) and fuel aromatic content	Rantanen et al. (1996)
DEP from an unspecified light-duty vehicle; DEP collected on glassfibre filters, dichloromethane sonication and Soxhlet extraction	TA98, TA100, TA104, TA98NR and TA98/1,8DNP ₆ , standard plate incorporation assay, without S9	Significant concentration-related increase in mutagenic activity; substantial declines in TA98NR and –1,8DNP ₆ relative to TA98	Barale et al. (1993)
DEP and SVOCs from a light-duty truck engine, 2000 rpm, 2 bar BMEP; 6 fuels (DF, LSDF, 4 fuels with different aromatic content); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extract; SVOCs from exhaust condensate	TA98, TA100, TA98NR and TA98/1,8DNP ₆ , microsuspension preincubation version, Aroclor-induced rat-liver S9	Mutagenic potency (per mg EOM) of combined DEP and SVOC extracts highest without S9; fuel change related to more than 10-fold change in mutagenicity, with higher potency for fuels enriched in aromatics; comparison across strains showed higher activity in TA100 and substantial reductions in TA98NR, the latter suggesting contributions from mononitro-PAHs	Crebelli et al. (1995)
DEP and SVOCs from a heavy-duty Cummins 10-L engine; 2 steady-state modes of EPA heavy-duty cycle, with and without DPF; DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction, Florisil fractionation into PAHs, nitro-PAHs and polar compounds	TA98, TA98NR and TA98/1,8DNP ₆ , standard plate incorporation assay, unspecified S9	Mutagenic potency uniformly higher without S9; DPF reduced the potency for low and high load by 27 and 67% (per µg EOM), 30 and 43% (per µg DEP) and 86 and 93% (per km), respectively, and eliminated SVOC mutagenicity	Gratz et al. (1991)
DEP and SVOCs from a heavy-duty Cummins 10-L, 6-cylinder engine; 2 steady-state modes from EPA heavy-duty cycle; LSDF with copper additive, with and without DFP; DEP collected on Teflon [®] -coated glassfibre filters, and SVOCs collected on XAD, dichloromethane Soxhlet extraction	TA98, TA98NR and TA98/1,8DNP ₆ , microsuspension version, Aroclor-induced rat-liver S9	Mutagenic potency similar with and without S9; mutagenic potency (per µg EOM) slightly higher for low load, and somewhat reduced with DFP, but only with elevated additive; potency (per µg DEP) substantially reduced with DPF; potency (per m ³) higher at low load, and substantially reduced with DPF; additive reduced potency, but only for high load; potency of DEP and SVOC extracts (per µg EOM) in TA98NR and TA98/1,8DNP ₆ substantially lower than that in TA98; DPF substantially reduced PAH emission rate (per m ³) for high load only; some nitro-PAHs increase with DPF, especially at low load	Harvey et al. (1994)
DEP from a 12-L, 6 cylinder Euro 3 truck, no DOC, with or without DPF; 13-mode ETC; 6 fuel blends (DF, B100, B5, B10, B20, PPO); DEP collected on Teflon [®] -coated glassfibre filters, ethanol/dichloromethane (1:1) sonication extraction	TA98 and YG1024, standard plate incorporation assay, Aroclor-induced rat-liver S9	No significant response in the presence of S9 for any sample; for TA98, significant response for B20 and PPO only; for YG1024, significant responses for B10, B100 and PPO only; maximal responses in YG1024 for B100 and PPO; biodiesel associated with reductions in PM (g/kW-h), PAHs and oxygenated PAHs (µg/kW-h)	Kooter et al. (2011)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP from a heavy-duty IVECO Euro 2 7.8-L, 6-cylinder engine; 13-mode ECE; DF and RME 20; DEP collected on Teflon [®] -coated glassfibre filters, toluene ASE extraction; SVOCs collected on PUFs, hexane/acetone (1:1) ASE extraction; fractionated on silica into 5 fractions with increasing polarity	TA98, TA100, TA98, TA98/1,8DNP ₆ and YG1041, standard plate incorporation assay, Aroclor-induced rat-liver S9	All samples elicited significant positive responses; potency (per mg EOM) showed little difference between DF and RME20 in any strain; most samples more potent with S9; without S9, responses generally lower in 1,8DNP ₆ than TA98; expression of potency per kW-h showed no difference between DF and RME20; fractionation showed 80–83% of the mutagenicity in fractions containing nitro-PAHs, dinitro-PAHs and oxygenated PAHs; RME20 emissions contained slightly lower levels of PAHs; subsequent study showed greater potency (per kW-h) in YG1041 without S9 relative to TA98	Turrio-Baldassarri et al. (2004, 2006)
SRM 2975 and DEP from a light-duty Isuzu (4JB1) 2.7-L, 4 cylinder engine, 2000 rpm; DEP collected on glassfibre filters, dichloromethane sonication extraction, fractionated on silica gel into 4 fractions with increasing polarity	TA98, TA100, TA98NR, TA98/1,8DNP ₆ , YG1021 and YG1024, plate incorporation assay, Aroclor-induced rat-liver S9	Mutagenic potency (per µg EOM) higher without S9 for SRM 2975 and with S9 for Isuzu DEP; without S9, SRM potency higher in YG1024; Isuzu extract had about 10-fold more EOM per unit DEP and much greater potency (per µg PM), particularly in YG1021; PAH-related mutagenicity over 200-fold higher and nitroarene-related mutagenicity 8–45-fold higher	DeMarini et al. (2004)
DEPs from 4 heavy-duty engines (1 8.5-L, 6-cylinder, 1 7.4-L, 6-cylinder and 2 9.6-L, 6-cylinder); 13-mode ESC; 6 fuels (DF, LSDF, 2 reformulated DFs, RME and RME30); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, TA98NR and YG1021, standard plate incorporation assay, Aroclor-induced rat-liver S9	Mutagenic potency uniformly higher without S9; DF showed the highest mutagenic potency (per µg EOM), followed by LSDF reformulated DFs and RME; when expressed per kW-h, RME potency lower than DF, but higher than other fuels (due to high EOM per unit mass); potency (per µg EOM) reduced in TA98NR and increased in YG1021, compared with TA98; good correlation between mutagenic potency per kW-h and PAH emissions per kW-h; RME potency higher than predicted by PAH content	Rantanen et al. (1993)
DEP from a heavy-duty 19-L, 6-cylinder engine; ECE steady-state cycle; DF and LSDF; DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, TA98NR and YG1021, standard plate incorporation assay, unspecified S9	Mutagenic potency (per kW-h) higher without S9, reduced in TA98 NR and increased in YG1021 (relative to TA98); 51–91% reductions in potency with reformulated low-sulfur fuel; low-sulfur fuel associated with large decrease in PM and PAH emission rates (per kW-h)	Mikkonen et al. (1995)
DEP from a light-duty 2-L engine; ECE and EUDC cycles in series; 3 fuels (DF and LSDFs; EN97, RD1, RD2); DEP collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and YG1021, standard plate incorporation assay, without S9	TA98 mutagenic potency for the LSDFs reduced by 44–48% per µg EOM, and 70–78% per km, relative to reference DF; large increases in potency in YG1021 relative to TA98; LSDF potency (per µg EOM) in YG1021 reduced > 50% relative to reference DF, and 72–85% reduction per km; PM and PAHs (per km) reduced for low-sulfur fuels	Kuljukka et al. (1998)

Table 4.8 (continued)

Test conditions	Salmonella strains ^a /test version	Results	References
DEP from a 3.5-L diesel engine; idling engine; DEP collected on glassfibre filters, sonication extraction with benzene/ethanol (3:1), fractionated on silica gel into 5 fractions with increasing polarity	YG1024, microsuspension preincubation version, without S9	Two most polar fractions accounted for almost 98% of the mutagenic activity (per mg DEP); nitro- and dinitro-PAHs accounted over 50% of mutagenic activity in fraction 4; mutagenic activity of most polar fraction from unknown compounds	Hayakawa et al. (1997)
DEP from a 1.997-L, single-cylinder engine; 7 steady-state conditions; 2 fuels (standard diesel, FT); total DEP collected on Teflon [®] -coated glassfibre filters, MOUDI size fractionated DEP, dichloromethane Soxhlet extraction	YG1024 and YG1029, standard plate incorporation assay, Aroclor-induced rat-liver S9	Mutagenic potency (per µg EOM) elevated at higher loads, no difference between fuels; effect of S9 and strain varied with load; potency per hp-h or engine h-r showed substantial reductions for FT fuel; larger particles (> 0.1µm) tended to be more mutagenic (per µg EOM) than smaller particles	McMillian et al. (2002)

^a YG1021, TA98 with plasmid pYG216, nitroreductase overproducing strain; YG1024, TA98 with plasmid pYG219, *O*-acetyltransferase overproducing strain; YG1041, TA98 with plasmid pYG233, nitroreductase and *O*-acetyltransferase overproducing strain; YG1026, TA100 with plasmid pYG216, nitroreductase overproducing strain; YG1029, TA100 with plasmid pYG219, *O*-acetyltransferase overproducing strain; YG1042, TA100 with plasmid pYG233, nitroreductase and *O*-acetyltransferase overproducing strain

^b SINOx honeycomb catalyst with reducing agent (32.5% urea solution)

ASE, accelerated solvent extraction; B[a]A, benz[a]anthracene; B[a]P, benzo[a]pyrene; BMEP, brake mean effective pressure; BSC, Braunschweig cycle; CDPF, catalysed diesel particulate filter; CFTP, cold-start Federal Test Procedure; CPF, catalysed particulate filter; CRT, continuously regenerating (particle) trap; CUD, California unified driving cycle; DEP, diesel exhaust particles; DF, diesel fuel; DFE, disposable filter element; DNP, dinitropyrene; DOC, diesel oxidation catalyst; DPF, diesel particulate filter; ECE, European driving cycle; EGR, exhaust gas recirculation; EOM, extractable organic matter; EPA, Environmental Protection Agency; EPTO, exhaust particle trap oxidizers; ESC, European stationary cycle; ETC, European transient cycle; EUDC, extra urban driving cycle; FAME, fatty acid methyl ester (biodiesel); FRG, Federal Republic of Germany; FTP, Federal Test Procedure; GTL, gas-to-liquid; h, hour; HFET, US highway fuel economy test; HFPT, hot-start Federal Test Procedure; hp, horse power; HVO, hydrotreated vegetable oil; HWFET, US highway fuel economy test; HySEE, hydrogenated soya ethyl ester; kW-h, kilowatt-hour; LAF, low-aromatics fuel; LSDF, low-sulfur diesel fuel; min, minute; MOUDI, micro-orifice uniform deposition impactor; MVEG, motor vehicles emissions group; NP, nitropyrene; NYCC, New York City drive cycle; OCC, Orange County cycle; PAC, polycyclic aromatic compound; PAH, polycyclic aromatic hydrocarbon; PB/5,6BF, phenobarbital/5,6-benzoflavone; PM, particulate matter; PPO, pure plant oil; PUF, polyurethane foam; REE, rapeseed ethyl ester; rev, revertant; RME, rapeseed oil methyl ester; rpm, revolutions per minute; RSO, rapeseed oil; S9, metabolic activation system; SCR, selective catalytic reduction; SMDS, Shell middle distillate synthesis (gas-to-liquid diesel); SME, soya bean oil methyl ester; SRM, standard reference material; SVOC, semi-volatile organic compound; TDI, turbocharged direct injection; THB, Technische Hochschule in Braunschweig; THC, total hydrocarbon; VW, Volkswagen; XAD, adsorbent resin

cycles, such as the US Federal Test Procedure 72, the European driving cycle (ECE-15), the California unified cycle and the New York City cycle of the US EPA, comprise a temporal series of load and speed changes designed to simulate representative driving conditions. Steady-state test cycles, such as the European stationary cycle, contain a series of steady-state stages with varying loads and speeds. Several studies investigated the effect of test cycle, load and/or speed on the bacterial mutagenicity of extracts of diesel engine exhaust particles.

Extracts of diesel exhaust particles emitted by a single-cylinder research engine showed higher mutagenicity (per engine-h) with a lower engine load (Bünger *et al.*, 2006). The mutagenicity of extracts (per microgram of EOM) emitted by a light-duty engine was higher under low-load conditions (Shi *et al.*, 2010), as, in general, was that of extracts (per microgram of PM) derived from two heavy-duty engines at lower load (Nussear *et al.*, 1992; Opris *et al.*, 1993). When expressed per cubic metre, the mutagenicity of extracts of exhaust particles derived from a light-duty diesel engine was generally higher at low load and increased with increasing running speed of the engine (Courtois *et al.*, 1993). The reduction in mutagenicity (per cubic metre of exhaust) of extracts by heavy-duty vehicles in strain TA98NR (compared with that in TA98) was lowest at the highest engine load, indicating that the emission rate of mutagenic nitroarenes is reduced at higher loads (Hansen *et al.*, 1994). Reductions in mutagenicity (expressed per microgram of EOM, per microgram of PM and per cubic metre of exhaust) were also noted at higher engine loads (Kantola *et al.*, 1992; Harvey *et al.*, 1994).

In an earlier study, the mutagenicity of extracts of diesel exhaust particles (per milligram of PM) released by a light-duty vehicle was reported in general to be higher for test cycles that included a cold start phase, which was probably due to incomplete combustion in a cold engine and a

lower conversion rate of the catalytic converter at lower temperatures (Bünger *et al.*, 1998).

A wide range of diesel fuels are distributed commercially for use in light- and heavy-duty engines for both on- and off-road applications (see also Section 1 of this *Monograph*). Fuels vary with respect to sulfur and aromatic contents, and are generally formulated to enhance combustion and improve engine performance. Changes in formulation, and the presence of additives to enhance combustion, are applied to improve ignition quality, expressed as cetane number. In addition, formulations are adjusted to enhance engine performance in colder climates. Most diesel fuels are derived from petroleum, with a sulfur content that varies with the intended application (e.g. on-road, locomotive and marine). In many countries (e.g. the USA, Canada, Europe and Japan), numerous applications, including those in on- and off-road heavy-duty vehicles and light-duty vehicles, require the use of low or ultralow sulfur diesels. Diesel fuels can also be derived from natural gas or coal gas, and these types are often referred to as gas-to-liquid diesel. Finally, diesel fuel can be prepared from plant or animal fats. These fuels are generally referred to as bio-diesel usually comprise alkyl esters (i.e. methyl or ethyl) of fatty acids. The most common bio-diesels (i.e. fatty acid methyl esters) are derived from rapeseed and soya bean oil. Pure plant oils or hydro-treated vegetable oils can also be used as fuel for diesel engines.

Numerous studies have examined the effect of fuel formulation on the mutagenic activity of extracts of diesel engine exhaust particles. Inter-study comparisons are often hampered by variations in engine type, test cycles, fuel sources, formulations and blending, as well as by the units in which the mutagenicity is expressed. Nevertheless, some general conclusions can be drawn regarding differences between bio-diesel and conventional diesel fuel, and the effects of changes in sulfur content or aromatic content.

Mutagenicity (expressed per unit EOM) was measured to evaluate the differences between extracts of engine exhaust particles from conventional diesel and rapeseed methyl ester or ethyl ester fuels. The mutagenicity of extracts derived from rapeseed methyl ester was lower than that of diesel fuel ([Rantanen et al., 1993](#)). Little difference was found between the mutagenic activities of extracts derived from a 20% v/v rapeseed methyl ester blend or from conventional diesel fuel ([Turrio-Baldassarri et al., 2004](#)). Mutagenicity (expressed per microgram on PM) was measured to compare conventional diesel engine emissions with those of rapeseed methyl ester and ethyl ester, and soya bean oil methyl ester bio-diesel fuels. In general, the mutagenic activity of extracts derived from engines run on one of the three types of bio-diesel was two- to 10-fold lower than those of engines run on conventional diesel fuel ([Kado et al., 1996b](#); [Bünger et al., 1998, 2000b](#)). However, expression of the mutagenicity per unit distance or per engine-hour eliminated the difference between the two fuel types. Another study reported that extracts of bio-diesel PM (unspecified source) were less mutagenic than those derived from conventional diesel fuel ([Carraro et al., 1997](#)).

Numerous studies measured mutagenicity – expressed per unit distance (i.e. per mile or kilometre), per engine-h, per cubic metre of exhaust or per unit of engine work (i.e. kW-h or hp-h) – to compare extracts of engine exhaust particles from conventional diesel fuel with those of bio-diesel PM. In many cases, the mutagenicity of bio-diesel PM was significantly lower (range, 2–8-fold) than that of PM from conventional diesel ([Rantanen et al., 1993](#); [Bagley et al., 1998](#); [Bünger et al., 2000a, b, 2006](#); [Chase et al., 2000](#); [Krahl et al., 2003, 2005](#)). Recently, modest reductions in the mutagenic activity of extracts of engine exhaust particles (expressed per kilometre) were reported for rapeseed methyl ester and 5% v/v rapeseed methyl ester in low-sulfur diesel fuel without metabolic activation, and

slight reductions with metabolic activation, relative to low-sulfur diesel. No fuel-related differences were detected in the mutagenic activity of semi-volatile organic compounds ([Westphal et al., 2012](#)). Several studies demonstrated that the use of bio-diesel was associated with considerable reductions in the emission rates (per hp-h or kW-h) of PM, PAHs, oxy-PAHs and nitro-PAHs ([Rantanen et al., 1993](#); [Carraro et al., 1997](#); [Chase et al., 2000](#); [Krahl et al., 2005, 2007a, b, 2008](#); [Kooter et al., 2011](#); [Westphal et al., 2012](#)).

However, several studies failed to show a difference between the mutagenicity of PM extracts associated with bio-diesel and conventional diesel fuel. For example, in studies with diesel exhaust particles from several light- and heavy-duty vehicles, no differences were detected between the mutagenicity (per litre of exhaust) of emissions from engines run on diesel fuel, rapeseed methyl ester, gas-to-liquid, rapeseed oil or a diesel/gas-to-liquid/ rapeseed methyl ester (3:1:1) mixture ([Krahl et al., 2006, 2007a, b, 2009a](#)). In these assays, samples from the engines run on rapeseed oil or preheated rapeseed oil produced a remarkably strong mutagenic response, and combustion of rapeseed oil was associated with an increase in the rate of PM emissions (per kW-h) (see also [Bünger et al., 2007](#)). Similarly, the mutagenicity (per microgram of PM, tested with *Salmonella* strain YG1024) of extracts of exhaust particles derived from an engine run on bio-diesel (EN14214) or a plant oil (DIN51605) was greater than that of samples derived from a normal diesel (EN590)-driven engine ([Kooter et al., 2011](#)).

Several studies investigated the effect of sulfur content of the fuel on the mutagenic activity of extracts of diesel engine exhaust particles. The mutagenicity (per microgram of PM) was generally greater for low-sulfur fuels ([Opris et al., 1993](#), [Mikkonen et al., 1995](#); [Rantanen et al., 1996](#)). One of these studies reported that the mutagenicity of the emissions (expressed as revertants/kilometre) was greater at low ambient

temperature ($-7\text{ }^{\circ}\text{C}$) than at $22\text{ }^{\circ}\text{C}$ ([Rantanen et al., 1996](#)). In an earlier study, the mutagenicity of particle- and vapour phase-associated extracts of diesel engine exhaust particles (per microgram of EOM, microgram of PM or cubic metre of exhaust) was slightly increased with a lower engine load and with lower sulfur content of the fuel ([Kantola et al., 1992](#)).

The influence of other fuel properties (e.g. aromatic content, flash-point and density) on the mutagenic activity of extracts of diesel engine exhaust particles was also investigated. Multivariate analyses of the mutagenicity (per cubic metre of exhaust) using several fuel properties as variables indicated that fuel density and flash-point were positively correlated with the mutagenic activity of the extracts of diesel engine exhaust particles ([Sjögren et al., 1996a, b](#)). The mutagenicity of extracts (per microgram of EOM) derived from engines run on four fuel types with different aromatic content increased with higher concentrations of aromatic compounds ([Crebelli et al., 1995](#)). A later study showed that the mutagenicity of extracts of engine exhaust particles (per kilometre) from conventional diesel fuel was four to tenfold higher than that of emissions from an ‘environmentally classified’ Swedish diesel fuel (MK1), which produced 88% and 98% lower emissions of particle-associated PAHs and 1-nitropyrene, and 77% and 80% lower emissions of semi-volatile PAHs and 1-nitropyrene, respectively, compared with emissions from conventional fuel ([Westerholm et al., 2001](#)). The mutagenic activity of diesel exhaust particles (per microgram of EOM) from an engine fuelled with diesel-ethanol blends (5–20% v/v) decreased with increasing ethanol content; this effect was clearly seen without metabolic activation in strain TA100, but to a much lesser extent in TA98 ([Song et al., 2007](#)).

Many of the above studies also examined the mutagenic activity of semi-volatile organic compounds in diesel exhausts collected via adsorption onto a solid matrix (e.g. XAD

resin) or through condensation onto a chilled surface, followed by solvent elution. Similarly to PM extracts, samples of semi-volatile organic compounds were generally stronger mutagens in the absence of exogenous metabolic activation, and were less mutagenic in strains TA98NR and TA98/1,8DNP₆ than in strain TA98 ([Harvey et al., 1994](#); [Bagley et al., 1998](#)). The specific mutagenic activity of the total vapour-phase component in TA100 (with or without metabolic activation) was reported to be similar to that of the total particulate component ([Kado et al., 1996b](#)). The mutagenicity of semi-volatile organic compound extracts (expressed per kilometre or kW-h) was generally much lower than that of parallel PM extracts ([Westerholm et al., 1991, 2001](#); [Bagley et al., 1998](#)).

Several studies used chemical fractionation methods to determine the physico-chemical properties and the identity of mutagens in extracts of diesel engine exhaust particles and semi-volatile organic compound concentrates. More recent studies noted increased mutagenicity (per microgram of EOM or PM) in fractions containing organic bases, neutral aromatic compounds and polar compounds ([Lu et al., 1999b](#); [Song et al., 2006](#)). The most strongly mutagenic chemical fractions were those that contained nitro-PAHs, dinitro-PAHs and oxygenated PAHs ([Westerholm et al., 1991](#); [Strandell et al., 1994](#); [Hayakawa et al., 1997](#)). These fractions may contain up to 80% of the mutagenic activity of an organic extract from a heavy-duty engine ([Turrio-Baldassarri et al., 2004, 2006](#)). Polar fractions of an extract of diesel exhaust particles from a light-duty engine accounted for 95% of the mutagenic activity; nitro-PAHs and dinitro-PAHs accounted for 53% of the activity in a moderately polar fraction, but the activity of the most polar fractions could not be attributed to any known mutagen ([Hayakawa et al., 1997](#)). In a comparative study of extracts of diesel exhaust particles from two different diesel engines, the enhanced mutagenic activity

in the more potent sample obtained after fractionation was related to high levels of PAHs and nitroarenes (DeMarini *et al.*, 2004). A micro-orifice uniform deposit impactor device was used to investigate the mutagenic activity of different sized fractions of diesel engine exhaust particles: larger particles (i.e. > 0.1 µm in diameter) tended to be more mutagenic (per microgram of EOM) than smaller particles (McMillian *et al.*, 2002).

Standard reference materials

Numerous studies have used the *Salmonella* mutagenicity assay to assess the mutagenic activity of SRMs 1650, 2975 and 1975, the results of which are summarized in Table 4.9. SRM 1650 – or its later formulation SRM 1650a or SRM 1650b – was included as one of the complex test substances in the International Programme on Chemical Safety collaborative study on complex mixtures. The results were published in a series of scientific papers (summarized in Claxton *et al.*, 1992a, b). The geometric mean mutagenic activity of SRM 1650 extracts (per microgram of PM) was generally higher in TA100 than in TA98, and the response in TA100 was stronger in the presence of metabolic activation while that in TA98 was more potent in its absence (Claxton *et al.*, 1992a). However, responses were quite variable across studies, some of which noted higher mutagenic activity in TA98 in the presence of metabolic activation (Aguirell & Stensman, 1992; Goto *et al.*, 1992). Two extractions with dichloromethane removed 80% of the mutagenic substances from SRM 1650, and the remainder was extracted in acetone (Nielsen, 1992); PAHs, nitro-PAHs and heterocyclic compounds were detected in the dichloromethane extracts (Savard *et al.*, 1992). A higher mutagenic response was observed in the preincubation/microsuspension *Salmonella* assay compared with the standard plate incorporation test (Aguirell & Stensman, 1992; Goto *et al.*, 1992).

Several studies investigated the behaviour of the complex mixture of components in extracts

of SRM 1650. By spiking with PAHs, it was shown that the mutagenic activities of the different PAH components were additive, at least at low concentrations and with sufficient metabolic activation (Boström *et al.*, 1998). Most of the mutagenic activities in extracts of SRM 1650 were associated with nitro- and dinitro-PAH fractions, and those of some of the active components were additive or less than additive in the mixture (Eide & Johnsen, 1998; Rivedal *et al.*, 2003). Negative interactions were reported between nitro-PAHs and dinitro-PAHs, and between nitro-PAHs and polar fractions in the absence of metabolic activation (Ostby *et al.*, 1997).

DeMarini *et al.* (2004) noted a stronger mutagenic response to extracts of SRM 2975 in the absence than in the presence of metabolic activation for both TA100 and TA98, and substantially reduced responses with TA98NR and TA98/1,8-DNP₆. Stronger mutagenic effects with strain YG1024 were indicative of a substantial contribution from dinitro-PAHs. Higher activity in the absence of metabolic activation, and increased mutagenicity with the nitroreductase-enhanced strains YG1021 and YG1026 had been reported previously for SRM 2975 (Hughes *et al.*, 1997). The latter study also showed that the mutagenicity activity of SRM 1650 was more PAH-dependent, while that of SRM 2975 was more nitro-PAH-dependent.

Effects of aftertreatment on *Salmonella* mutagenicity

Numerous studies investigated the ability of aftertreatment systems – e.g., SCR, exhaust gas recirculation, diesel oxidation catalysts, DPFs, catalysed particulate filters and continuously regenerating traps – to alter the mutagenicity of diesel emissions. Four main devices are used to control the release of regulated and non-regulated emissions from diesel engines. Exhaust gas recirculation systems recirculate a portion of the exhaust back into the engine cylinders; they are used to reduce the release of nitrogen oxides,

Table 4.9 Summary of studies on the mutagenicity of diesel standard reference materials in *Salmonella typhimurium*

Test material	Salmonella strains ^a /test version	Results	References
SRM 1650, sonication or Soxhlet extraction in dichloromethane	TA98, TA100, TA98NR and TA98/1,8DNP ₆ , standard plate incorporation assay and microsuspension/ preincubation version, Aroclor-1254-induced rat-liver S9	Summary of the IPCS collaborative study; mutagenicity (geometric mean value per mg DEP) in TA100 higher with than without S9; in TA98, higher response without S9; higher response with the microsuspension version: stronger signal in both TA100 and TA98 with than without S9; mutagenicity in TA98NR and TA98/1,8DNP ₆ about < 50% and 20–23% that in TA98; 1-nitropyrene accounted for up to 4% of direct-acting mutagenicity.	Claxton et al. (1992a, b)
SRM 1650, sonication extraction in dichloromethane, and sequential extractions in hexane, hexane/diethyl ether (9:1 and 1:1), diethyl ether, methanol (increasing polarity)	TA98 and TA100, standard plate incorporation assay, Aroclor-1254-induced rat-liver S9	Dichloromethane extracts much more mutagenic (per µL extract) without S9; mutagenicity of sequential extracts decreased with increasing polarity of solvent; PAHs, nitro-PAHs and heterocyclics detected in extract; no single class of compound accounted for the observed mutagenicity	Savard et al. (1992)
SRM 1650, sonication extraction in dichloromethane (twice) and acetone	TA98, TA98NR and TA100, standard plate incorporation assay, Aroclor-1254-induced rat-liver S9	Nearly all (89–96%) mutagenic activity (per mg DEP) present in the first dichloromethane extract; little contribution from subsequent extracts; mutagenicity higher with S9	Nielsen (1992)
SRM 1650, sonication extraction in dichloromethane	TA98 and TA100, standard plate incorporation assay and pre-incubation version, Aroclor-induced rat-liver S9	Mutagenicity (per mg DEP) higher with TA100 than with TA98; mutagenicity in TA100 higher without than with S9; mutagenicity in TA98 lower without S9; preincubation test results slightly higher than those of the standard plate incorporation assay	Goto et al. (1992)
SRM 1650, sonication extraction in dichloromethane	TA98, TA98NR and TA98/1,8DNP ₆ , microsuspension/ preincubation version, Aroclor-induced rat-liver S9	Mutagenicity (per µg EOM) slightly higher without S9; considerably lower mutagenicity with TA98NR and TA98/1,8DNP ₆ , compared with TA98	Bagley et al. (1992)
SRM 1650, sonication extraction in dichloromethane	TA98 and TA100; microsuspension/ preincubation version, Aroclor-1254-induced rat-liver S9	Mutagenicity (per µg EOM) higher with than without S9; preincubation increased mutagenicity by 3–15-fold relative to plate incorporation	Agurell & Stensman (1992)
SRM 1650, Soxhlet extraction in dichloromethane; extract spiked with nitro-PAHs, 1-nitropyrene, 2-nitrofluorene, 1,8-dinitropyrene	TA98, standard plate incorporation assay without S9	Significant mutagenicity; evidence of additive effects with no significant interactions between nitro-PAHs or between DEP and nitro-PAHs	Eide & Johnsen (1998)

Table 4.9 (continued)

Test material	Salmonella strains ^a /test version	Results	References
SRM 1650, Soxhlet extraction in dichloromethane, fractionation on silica into 5 fractions	TA100, standard plate incorporation assay without S9	Three fractions (i.e. nitro-PAH, dinitro-PAH and polar) were mutagenic; results suggested negative interactions (antagonism) between the nitro- and dinitro-PAH fractions, and between nitro-PAH and polar fractions	Ostby et al. (1997)
SRM 1650, Soxhlet extraction in dichloromethane, fractionation on silica with cyclohexane/ dichloromethane/ methanol (increasing polarity)	TA98, TA100, TA98NR, TA100NR and TA98/1,8DNP ₆ , S9 [assay and source of S9 unspecified]	Significant response without S9, higher in TA100 and substantially lower in TA100NR, TA98NR and TA98DNP; most of the mutagenic activity in the nitro- and dinitro-PAH fractions; results suggested that mutagenic responses of fractions are additive or less than additive	Rivedal et al. (2003)
SRM 1650, Soxhlet extraction in dichloromethane; extract spiked with benzo(a)pyrene, benz[a]anthracene, pyrene, fluoranthene	TA98, TA100, TA98NR and TA100NR, standard plate incorporation assay, unspecified S9	Mutagenic effects of components such as PAHs were additive at lower concentrations, provided sufficient S9 (10%) was present	Boström et al. (1998)
SRM 1975 (dichloromethane extract of SRM 2975)	TA98, TA100, TA98NR, TA100NR, YG1021 and YG1026, standard plate incorporation assay, Aroclor-induced rat-liver S9	Comparison of mutagenicity (per µg EOM; without S9) across strains showed the rank order YG1021 > TA98 > TA98NR > YG1026 > TA100 > TA100NR; higher activity without S9 and lower activity on NR-deficient strains	Hughes et al. (1997)

^a YG1021, TA98 with plasmid pYG216, nitroreductase-overproducing strain; YG1024, TA98 with plasmid pYG219, O-acetyltransferase-overproducing strain; YG1041, TA98 with plasmid pYG233, nitroreductase- and O-acetyltransferase-overproducing strain; YG1026, TA100 with plasmid pYG216, nitroreductase-overproducing strain; YG1029, TA100 with plasmid pYG219, O-acetyltransferase-overproducing strain; YG1042, TA100 with plasmid pYG233, nitroreductase- and O-acetyltransferase-overproducing strain; TA98NR and TA100NR have low nitroreductase activity compared with TA98 and TA100; TA98/1,8DNP₆ is deficient in a transacetylase that activates 1,8-DNP. DEP, diesel exhaust particles; EOM, extractable organic matter; IPCS, International Programme on Chemical Safety; PAH, polycyclic aromatic hydrocarbon; S9, metabolic activation system; SRM, standard reference material

but have been associated with an increased release of diesel soot. Diesel oxidation catalysts, which comprise a range of systems often containing palladium or platinum, catalyse the conversion of carbon monoxide and unburned hydrocarbons to carbon dioxide and water. DPFs are devices that reduce the release of PM from diesel engines. Numerous types of DPF include single-use disposable devices and a range of continuously regenerating traps with ceramic filters (e.g. Cordierite) that use catalytic or high-temperature combustion to regenerate the filter medium. SCR systems use a reducing agent, such as gaseous ammonia, and active catalytic components, including oxides of metals such as vanadium, molybdenum and tungsten, to reduce nitrogen oxides to nitrogen gas and water. Details regarding pollution control technologies applied to reduce emissions of PM, semi-volatile organic compounds and nitrogen oxides are presented in Section 1.1 of this *Monograph*.

[Table 4.10](#) summarizes the results of studies that have investigated the relative changes in response of the measured end-points associated with different exhaust aftertreatments, such as diesel oxidation catalysts, DPFs and SCR. In several studies, catalytic devices were shown to increase the mutagenic activity (expressed per unit of EOM from diesel PM). However, the use of devices to reduce the rates of PM emissions can lead to substantial reductions in mutagenic activity when expressed per unit of engine work, per cubic metre of exhaust, per engine-h or per kilometre travelled. No studies have addressed the relative changes in genetic and related effects in experimental systems associated with emissions from diesel engines fitted with emission control devices (i.e. diesel oxidation catalysts, continuously regenerating traps and SCR) that comply with current regulations in the USA and Europe.

Several studies reported that some pollution control devices caused an apparent increase in the mutagenicity of diesel engine exhaust in *S.*

typhimurium, but this was strongly dependent on the units by which mutagenicity was expressed, e.g. per unit EOM, per unit PM or per cubic metre of exhaust. [Bagley et al. \(1991\)](#) noted that the mutagenicity of extracts of diesel exhaust particles from a heavy-duty engine was increased fourteenfold in the presence of a catalysed DPF, when expressed as the number of revertants per microgram of soluble organic fraction. The same study noted a 70% reduction in mutagenicity when it was expressed as the number of revertants per cubic metre of exhaust. In the Transit Bus Emission Study, [Nylund et al. \(2004\)](#) reported that the use of a continuously regenerating trap increased the mutagenicity (in TA98 without metabolic activation) of extracts of diesel exhaust particles from a 2003 Euro-3 bus approximately two- to threefold (revertants per milligram of PM). When expressed as the number of revertants per kilometre, the mutagenicity was reduced by 50–75% relative to that seen with exhaust particles from an engine without a continuously regenerating trap. Similarly, other studies of heavy-duty diesel engine emissions noted that the use of a diesel oxidation catalyst contributed to increased mutagenicity expressed per microgram of PM ([Kado et al., 1996a](#)) or per microgram of EOM, respectively ([Pataky et al., 1994](#)). The mutagenicity of extracts of diesel exhaust particles (per microgram of PM or per kilometre) from a 1.93-L engine increased when a DPF was used, but considerable reductions in mutagenicity were observed in material from a 2.5-L engine under the same conditions ([Carraro et al., 1997](#)). A similar study of light-duty diesel engine emissions showed that the use of a particulate filter (DPF or disposable filter elements) moderately increased the mutagenic activity of extracts of diesel engine exhaust particles (expressed per unit EOM), while the use of a diesel oxidation catalyst eliminated the mutagenic activity ([Shi et al., 2010](#)).

In a recent study ([Westphal et al., 2012](#)), a diesel oxidation catalyst eliminated the

Table 4.10 Effect of various aftertreatment devices on the mutagenicity of diesel engine exhaust in *Salmonella typhimurium*

Engine type	Aftertreatment	End-point assessed	Exposure/mutagenicity	Effect of aftertreatment	Reference
Heavy-duty 7.3-L, 6-cylinder engine	DOC	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/mass PM, rev/mile	Increase in mutagenicity per unit PM and per mile	Kado et al. (1996a)
Euro 3 compliant 6.4-L, 6-cylinder engine	DOC	Mutagenicity in <i>Salmonella</i> ; DEP extract, SVOCs	Rev/m ³	Modest reduction in mutagenicity of DEP extract; mutagenicity of SVOCs eliminated	Westphal et al. (2012)
Single-cylinder engine	DOC	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/engine-h	Decrease in mutagenicity at partial load, increase at rated power	Bünger et al. (2006)
Heavy-duty 10-L, 6-cylinder engine	DOC	Mutagenicity in <i>Salmonella</i> ; DEP extract, SVOCs	Rev/unit EOM; rev/mass; rev/m ³	Increase in mutagenicity per µg EOM for two lower loads; reduced mutagenic effect per unit PM or per m ³ exhaust for lowest load only	Pataky et al. (1994)
Heavy-duty 7-L engine	DOC	Mutagenicity in <i>Salmonella</i> ; DEP extract, SVOCs	Rev/unit EOM, rev/mass PM, rev/m ³	Decrease in mutagenicity of DEP extract (all units); increase in mutagenicity of SVOCs per unit EOM	McClure et al. (1992)
Heavy-duty 7-L engine (1983)	DOC	Mutagenicity in <i>Salmonella</i> ; DEP extract, SVOCs	Rev/kW-h	Reduction in mutagenicity (50%)	Bagley et al. (1998)
Six-cylinder bus	DOC	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/m ³	Reduced mutagenicity	Hansen et al. (1994)
Light-duty 2-L vehicle	DOC	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/km	Reduced mutagenicity	Rantanen et al. (1996)
Light-duty 2.369-L, 4-cylinder engine	DOC, DPF or DFE	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/unit EOM, rev/m ³	DOC eliminated mutagenicity; DPF contributed to an increased mutagenic effect per unit EOM and to a 30–62% reduction in mutagenicity per m ³ exhaust	Shi et al. (2010)
Euro 3 bus (2003)	DOC or CRT	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/mass PM, rev/km	Increase in mutagenicity per unit PM with DOC or CRT; mutagenicity per km higher with DOC and reduced by 50–70% with CRT	Nylund et al. (2004)
Two light-duty (1.93-L and 2.5-L) engines	DOC or various DPFs	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/mass PM, rev/km	DPF increased mutagenicity per unit PM or per km for 1.93-L engine, and reduced the mutagenic effect for the 2.5-L engine; DOC contributed to a slight reduction in mutagenicity per unit PM for the 2.5-L engine and to a modest reduction per km	Carraro et al. (1997)

Table 4.10 (continued)

Light-duty 1.5-L, 4-cylinder engine	CRT	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/unit EOM, rev/mile	No effect on mutagenicity per unit EOM; mutagenicity per mile reduced by 65–95%	Rasmussen et al. (1989)
Heavy-duty 10-L, 4-cylinder engine	CDPF	Mutagenicity in <i>Salmonella</i> ; DEP extract, SVOCs	Rev/unit EOM, rev/mass PM, rev/m ³	Increase in DEP mutagenicity per unit EOM or per unit PM; mutagenic effects per m ³ exhaust reduced by 70% and 80% for DEP and SVOCs, respectively	Bagley et al. (1991)
Heavy-duty 10.8-L, 6-cylinder engine	CPF	Mutagenicity in <i>Salmonella</i> ; DEP extract, SVOCs	Rev/unit EOM, rev/m ³	No effect on mutagenicity per unit EOM; reduced mutagenicity per m ³ exhaust for DEP and SVOCs	Suresh et al. (2001)
Heavy-duty 10-L, 6-cylinder engine	DPF	Mutagenicity in <i>Salmonella</i> ; DEP extract	Rev/unit EOM, rev/mass PM, rev/m ³	Reduced mutagenicity (all units)	Kantola et al. (1992)
Heavy-duty 12-L, 6-cylinder engine	DPF	Mutagenicity in <i>Salmonella</i> ; DEP extract	[Unit not provided]	Slight increase in mutagenicity	Kooter et al. (2011)

CDPF, catalysed diesel particle filter; CPF, catalysed particulate filter; CRT, continuously regenerating trap; DEP, diesel exhaust particulate/particle(s); DFE, disposable filter elements; DOC, diesel oxidation catalyst; DPF, diesel-particulate filter; EOM, extractable organic material; PM, particulate matter; rev, revertant(s); SCR, selective catalytic reduction; SVOC, semi-volatile organic compound

mutagenic activity (per cubic metre) of condensate (semi-volatile organic compounds) samples of exhausts from a Euro 3-compliant heavy-duty engine operated with several types of fuel, but had only modest (without metabolic activation) or slight (with metabolic activation) effects in reducing the mutagenicity of particle extracts. A study of heavy-duty engine emissions noted that the use of a diesel oxidation catalyst reduced the mutagenicity by 41, 51 and 66% when expressed per microgram of EOM, per microgram of PM and per cubic metre of exhaust, respectively (McClure *et al.*, 1992). The use of an oxidation catalyst was shown to lead to a considerable decrease in the level of mutagenicity emitted, which was correlated with measured reductions in the emission rates of PM, PAHs and nitro-PAHs in some cases, particularly under low-load conditions (Hansen *et al.*, 1994; Pataky *et al.*, 1994).

Several studies reported that DPFs dramatically reduce the emission rate of mutagens associated with the soluble organic fraction of diesel engine exhaust particles. For example, the use of a catalysed DPF led to 90 and 70% reductions in the rate of PM emissions and mutagenicity per cubic metre, respectively (Bagley *et al.*, 1991). Similarly, the use of a DPF reduced the rates of PM emissions by 51–71% and mutagenicity per cubic metre by 30–62% (Shi *et al.*, 2010), while the use of a catalysed particle filter substantially diminished emissions of PM and PAHs, and reduced mutagenicity per cubic metre by 65% (Suresh *et al.*, 2001). Two other studies showed that the use of a DPF was associated with reductions in mutagenicity of 9–67%, 30–57% and 54–94% (Kantola *et al.*, 1992), and 27–67%, 30–43% and 86–93% (Gratz *et al.*, 1991), expressed per microgram of EOM, per microgram of PM and per cubic metre of exhaust, respectively. In a study of two light-duty engines fitted with a continuously regenerating trap, no reduction was found in the mutagenicity (expressed per microgram of EOM) of extracts of diesel exhaust particles; however,

when expressed per mile travelled, the mutagenicity was reduced by 69–95% (Rasmussen *et al.*, 1989).

Only one study investigated the effect of SCR on the mutagenicity of diesel engine emissions; this process significantly reduced the number of revertants (expressed per cubic metre of exhaust) induced by extracts of diesel exhaust particles emitted by a heavy-duty engine (Krahl *et al.*, 2006).

The application of exhaust gas recirculation in a heavy-duty engine was associated with an increase in the mutagenicity (expressed as the number of revertants per cubic metre of exhaust) of extracts of diesel exhaust particles, especially at low engine-load, and with an increase in the concentration of PAHs in the exhaust in one study (Kreso *et al.*, 1998). In contrast, other studies reported that exhaust gas recirculation reduced the mutagenicity (expressed per microgram of PM or per kilometre travelled) of extracts of diesel exhaust particles from a light-duty engine (Carraro *et al.*, 1995, 1997).

Emission control devices such as exhaust gas regulation, diesel oxidation catalysts and DPFs can strongly reduce the mutagenic activity (per cubic metre) of samples of diesel engine exhaust semi-volatile organic compounds (Gratz *et al.*, 1991; Kantola *et al.*, 1992; McClure *et al.*, 1992; Kreso *et al.*, 1998). However, in line with observations for extracts of diesel engine exhaust particles, the use of a diesel oxidation catalyst increased the mutagenic activity expressed per microgram of EOM of semi-volatile organic compounds of diesel engine exhaust (McClure *et al.*, 1992).

[The Working Group acknowledged that exhaust aftertreatment devices can alter the genetic and related effects induced by diesel engine exhaust, but recognized that confounding variables such as engine design, test cycle, fuel formulation and methods of sample processing do not allow any definitive conclusions.]

(vi) *Effects observed in vitro in other prokaryotic assays*

The results of several studies that used other bacterial assays to assess the genotoxic activity of diesel engine emissions are summarized in [Table 4.11](#).

The Mutatox assay is based on the use of a dark variant of the luminescent bacterium *Vibrio fischeri*, and the presence of mutagens results in the restoration of the photoluminescence. When used to examine semi-volatile organic compounds and PM extracts of diesel engine exhaust, the assay gave similar results to those observed in the *Salmonella* mutagenicity assay, i.e. that extracts of diesel engine exhaust particulates were more mutagenic in the absence than in the presence of metabolic activation, and that semi-volatile organic compounds were more potent in the presence than in the absence of metabolic activation. In addition, the mutagenicity of PM extracts was greater at low than at high engine load ([Lin & Chao, 2002](#)).

The induction of error-prone DNA repair (a so-called SOS response) was monitored in *E. coli* PQ37 (i.e. the SOS chromotest) and *S. typhimurium* TA1535/pSK1002 (i.e. the *umu* test) exposed to extracts of diesel engine exhaust particulates. The SOS chromotest was used to examine the genotoxicity of pressurized hot-water extracts, and peak activity was associated with an extract of medium polarity ([Kubátová et al., 2004](#)). As part of the collaborative study of the International Programme on Chemical Safety, the SOS chromotest was also used to examine extracts of SRM 1650, for which an eightfold increase in activity was found in the absence of metabolic activation ([Nylund et al., 1992](#)). The *umu* test was used in two studies of organic extracts of exhaust particulates from a diesel generator and several light-duty diesel vehicles, respectively ([Wasserkort et al., 1998](#); [Yamazaki et al., 2000](#)). The former found concentration-dependent genotoxicity in the absence of metabolic activation and a greater

response at higher engine loads. The second study also noted direct-acting genotoxicity (i.e. in the absence of metabolic activation), and confirmed stronger responses in the presence of human CYP1A2 and CYP1B1.

(c) *Gasoline (spark ignition) engine emissions*(i) *Effects observed in vivo in experimental animals*

The Working Group identified four studies that examined the genotoxic effects of emissions from spark ignition gasoline engines in experimental animals *in vivo*, the results of which are summarized in [Table 4.12](#). The first study examined changes in benzo[*a*]pyrene hydroxylation, 7-ethoxyresorufin *O*-deethylation and NADPH-cytochrome *c* reductase activity in liver, kidney and lung microsomes following whole-body inhalation exposure of rats for 1 hour twice a day, 5 days a week for 4 weeks to diluted emissions from a two-stroke motorcycle engine. The results showed significant increases in all three activities in all tissues ([Ueng et al., 1998](#)). The same study also assessed enzyme induction following intratracheal administration and noted significant increases in benzo[*a*]pyrene hydroxylation and 7-ethoxyresorufin *O*-deethylation activities in all tissues. After intraperitoneal administration, all three activities were significantly increased in the three organs, except for NADPH-cytochrome *c* reductase activity in the liver. A later study by the same authors reported that ‘head-only’ exposures of Wistar rats to two-stroke motorcycle engine exhaust induced significant upregulation of *Cyp1A1* and *Cyp1B1*, as well as *IL-1 α* , *IL-6* and *IL-11*, i.e. genes involved in xenobiotic metabolism and inflammation, respectively ([Ueng et al., 2005](#)).

Groups of 10–20 male and female mice (A/J and BALBc) and rats (SHR and Fischer 344) were exposed to both unfiltered and filtered exhaust from 4.3-L 1996 General Motors engines run on national average fuel in a simulated urban

Table 4.11 Summary of results of in-vitro analyses of diesel exhausts in miscellaneous bacterial assays

Test conditions	Exposure system	End-point(s) examined	Results	Reference
Mitsubishi 6.557-L, 6-cylinder, direct injection diesel engine; HD-FTP drive cycle and steady-state tests (9.2 and 27.5 kg/m); diesel No. 2 with methanol-containing additive; DEP collected on glassfibre filters and SVOCs on PUF and XAD-4, Soxhlet extraction in dichloromethane and dichloromethane/hexane (1:1)	Dark mutants of <i>Vibrio fischeri</i> M169 (Mutatox) for 16, 20 or 24 h with and without Aroclor-induced rat-liver S9 (preincubation, 45 min)	Reverse mutation of dark mutant to luminescent wild-type	PUF/XAD and PM extracts mutagenic with and without S9; PUF/XAD extracts more mutagenic and PM extracts less mutagenic with than without S9; PUF/XAD extracts yielded higher response at lower engine load; increasing levels of methanol additive in fuel produced stronger effect for both extracts	Lin & Chao (2002)
Bulk DEP from a diesel bus [no details given]; extractions with pressurized water (25, 50, 100, 150, 200, 250, 300 °C).	Incubation (2 h) of <i>Escherichia coli</i> PQ37 with 20 µL of DEP extract	SOS chromotest: colorimetric assessment of SOS response in <i>E. coli</i> PQ37	Maximum effect observed in fraction extracted at 150 °C (mid-polarity), possibly representing nitro-aromatics	Kubátová et al. (2004)
Yamaha EDA 4700 TE single-cylinder diesel generator, tested at different loads; DEP collected on glassfibre filters, Soxhlet extraction in dichloromethane	<i>Salmonella typhimurium</i> TA1535/pSK1002 (umuC test) for 2 h, Aroclor-induced rat-liver S9	Induction of SOS repair	Concentration-related increase in response without S9; strongest response at high load; strong correlation between response and amount of particle-bound PAHs	Wasserkort et al. (1998)
DEP from 4 diesel vehicles with 2.8-L (1993 model), 2.5-L (1996), 4.1-L (1990), 7.4-L (1989) engines; idling conditions; DEP collected on glassfibre filters, sonication extraction with benzene/ ethanol (3:1)	<i>S. typhimurium</i> TA1535/pSK1002 (umuC test) for 2 h, activation with <i>E. coli</i> expressing human CYP1A1, CYP1A2 or CYP1B1, all with NPR	Induction of SOS repair	Concentration-dependent increase in response without bioactivation; stronger effect with <i>E. coli</i> expressing CYP1B1 and CYP1A2	Yamazaki et al. (2000)
SRM 1650, Soxhlet extraction in dichloromethane	Incubation (2 h) of <i>E. coli</i> PQ37 with 0.3–153.6 µg DEP-equivalent/mL, Aroclor-induced rat-liver S9	SOS chromotest: colorimetric assessment of SOS response in <i>E. coli</i> PQ37	Significant positive response without S9 at ≥ 10 µg DEP-equivalent/mL; response with S9 approximately 8-fold lower	Nylund et al. (1992)

CYP, cytochrome P450; cyp, cytochrome; DEP, diesel exhaust particles; h, hour; HD-FTP, heavy-duty Federal Test Procedure; min, minute; NPR, nicotinamide adenine dinucleotide phosphate–cytochrome P450 reductase; PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; PUF, polyurethane foam; S9, metabolic activation system; SRM, standard reference material; SVOC, semi-volatile organic compounds; XAD-4, synthetic resin to remove phenols from aqueous solution

Table 4.12 Summary of studies of the exposure of animals to gasoline engine exhaust or gasoline exhaust particulate matter *in vivo*

Engine specifications	Fuel	Run conditions	Test material collection and processing	Animal model	Route of exposure/ exposure regime	End-point(s) examined	Results	Reference
Yamaha 50-mL, 2-stroke motorcycle engine	Mixture (60:1) of unleaded gasoline and low-smoke engine oil	Idle speed, no engine load	Exhaust pipe connected via mixing chamber to exposure compartment; exhaust particles collected on 0.5- μ m quartz filters, Soxhlet-extracted with dichloromethane/hexane (1:1)	Wistar rats, 100–120 g, male	Whole-body exposures to exhaust on 2 h/d, 5 d/wk for 4 wks; intratracheal (0.1 g/kg bw) or intraperitoneal (0.5 g/kg bw, daily for 4 days) administration of extract	Benzo[a]pyrene hydroxylation activity in liver, kidney and lung	All routes of exposure resulted in significant increases in activity in all tissues	Ueng et al. (1998)
Yamaha Cabin 50-ml, 2-stroke motorcycle engine	Mixture (60:1) of unleaded gasoline and low-smoke engine oil	Idle speed, no engine load	Exhaust pipe connected via mixing chamber (1:10 dilution) to exposure compartment	Wistar rats, 7 wks, female	Head-only exposure to 21.5 mg/m ³ PM from exhaust on 2 \times 1 h/d, 5 d/wk for 4 wk	Gene expression (semiquantitative RT-PCR)	Significant increases in expression of <i>Cyp1A1</i> and <i>Il-1α</i>	Ueng et al. (2005)
Yamaha 50-ml, 2-stroke motorcycle engine	Unleaded gasoline (95% octane)	At 150 rpm, no engine load	Stainless steel collection tube, exhaust particles collected on 0.5- μ m quartz filters; PM isolated by sonication of filters in methanol	ICR mice, 8–9 wks, male	Intratracheal instillation of 160, 200 or 240 mg/kg bw PM isolated from filters (suspended in corn oil)	MN formation in peripheral blood 24, 48 and 72 h after a single dose	Significant dose-related increases in MN frequency at 24 and 48 h after exposure; antioxidants reduced the response significantly	Cheng et al. (2004)

Table 4.12 (continued)

Engine specifications	Fuel	Run conditions	Test material collection and processing	Animal model	Route of exposure/ exposure regime	End-point(s) examined	Results	Reference
Five different gasoline-powered scooters	[Not specified]	Idling, with periodic acceleration	High-volume air sampler; PM collected on glassfibre filters	Swiss mice, 18–20 g, male	Two daily intraperitoneal injections of 0.25, 1 or 4 mg PM in DMSO	MN frequency in PCEs 6 h after second injection	Significant dose-related increases in MN for all PM samples tested	Zhou & Ye (1997)
Gasoline-powered scooters	Gasoline with 3 different lubricants [no details given]	Idling or acceleration	High-volume air sampler; PM collected on glassfibre filters; extraction by sonication in dichloromethane	Kunming mice, 18–20 g, male	Two daily intraperitoneal injections of 0.4, 2.0 or 10.0 mg-equivalent DEP extract per 20 g bw in DMSO	MN frequency in PCEs 6 h after second injection	Significant dose-related increases in MN frequency; reduction when lubricants were added to the fuel	Zhou & Ye (1998)

bw, body weight; *Cyp*, cytochrome P450 gene; d, day; DEP, diesel exhaust particle; DMSO, dimethyl sulfoxide; h, hour; *Il*, interleukin gene; MN, micronucleus; PCE, polychromatic erythrocyte; PM, particulate matter; rpm, revolutions per minute; RT-PCT, reverse transcript-polymerase chain reaction; wk, week

operating cycle. Changes in lipid peroxides (SHR rats) and DNA methylation (Fischer 344 rats and A/J mice) were significantly reversed by filtration of the particles from the exhaust ([Reed et al., 2008](#)).

[Cheng et al. \(2004\)](#) examined the induction of micronuclei in the peripheral blood of mice administered intraperitoneal injections of PM from a two-stroke motorcycle engine. The frequency of micronuclei was significantly increased 24 and 48 hours after exposure; simultaneous injection of antioxidants (e.g. ascorbate and α -tocopherol) significantly reduced the magnitude of the response.

Induction of micronuclei was also studied in mouse peripheral blood following intraperitoneal injections of PM extracts or suspended PM from gasoline-powered scooters. One study examined the effect of two daily doses of PM suspended in DMSO and another measured the response to two daily doses of PM extract. Both studies noted a significant dose-related increase in the frequency of micronuclei. In the second study, the magnitude of the response was dependent on the type of lubricant used in the fuel ([Zhou & Ye, 1997, 1998](#)).

(ii) *Effects in cultured mammalian cells*

In comparison with diesel engine emissions (see [Table 4.6](#)), relatively few studies have assessed the genotoxic effects of emissions from gasoline engines in cultured mammalian cells, the results of which are summarized in [Table 4.13](#).

The frequency of DNA strand breaks was measured in Chinese hamster V79 cells and in human A549 adenocarcinoma cells exposed to semi-volatile organic compounds and extracts of PM from gasoline-driven engine exhaust, respectively ([Liu et al., 2005](#); [Zhang et al., 2007](#)). Both studies revealed significant concentration-related increases in DNA strand breaks, and the earlier study noted stronger responses to extracts of gasoline exhaust PM compared with extracts of diesel engine exhaust particles. However, responses to

extracts of gasoline and diesel engine exhaust particles were more comparable when the results were expressed per distance driven. The other study ([Zhang et al., 2007](#)) compared the effects of emissions from a gasoline-driven engine with those from an engine run on pure methanol; use of the latter fuel did not show any of the effects described for gasoline. Both studies also noted significant concentration-related increases in the frequency of micronuclei after exposure to gasoline engine exhaust PM.

In a study of PM extracts derived from a Santana spark ignition engine run on leaded or unleaded gasoline, a significant concentration-related increase in the frequency of micronuclei was observed in Chinese hamster lung cells for both fuel types ([Yuan et al., 1999](#)).

The induction of chromosomal alterations (e.g. structural aberrations, sister chromatid exchange and numerical alterations) was studied in rodent cells exposed to extracts or suspensions of PM from gasoline-driven engines. In Chinese hamster ovary CHO-K1 cells exposed to a suspension of PM from a two-stroke motorcycle engine, a significant concentration-related increase was noted in the frequency of aberrant cells, in both the presence and absence of exogenous metabolic activation, but the response was higher in its presence. Antioxidants, such as ascorbate, α -tocopherol and *N*-acetylcysteine, significantly reduced the frequency of aberrant cells ([Cheng et al., 2004](#)). Exposure of Chinese hamster V79 and Syrian hamster kidney cells to DMSO extracts of PM from three spark ignition engines induced significant concentration-related increases in the frequency of sister chromatid exchange and C-metaphases, and in the proportion of hyperdiploid and polyploid cells ([Hadnagy & Seemayer, 1988, 1991](#)). In addition, these authors reported a concentration-related increase in transformed foci in Syrian hamster kidney cells ([Hadnagy & Seemayer, 1989](#)). Assessment of sister chromatid exchange and oxidative damage (i.e. 8-OH-dG and thymine

Table 4.13 Summary of studies of the effects of gasoline engine exhausts in cultured mammalian cells or isolated DNA *in vitro*

Source of exhaust	Exposure system	End-point(s) examined	Results	Reference
Cultured cells				
Spark ignition engines: 1995 Nissan Micra 1.3-L (120 km/h) and 2000 Mitsubishi Carisma 1.8-L (120 or 50 km/h); run on CEC-RF-02-99 gasoline; PM collected on Teflon-coated glassfibre filters, Soxhlet extraction in dichloromethane; DEP and SRM 1650 tested in parallel (see Table 4.6)	Human BEAS-2B bronchial epithelial cells treated with 200 µg/mL particulate extract for up to 48 h	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling)	Samples from both engines induced concentration-related increases in bulky adducts corresponding to levels of adduct-forming PAHs; adduct-forming potency of gasoline PM stronger than that of DEP and SRM 1650 when expressed as adducts/mg PM, but 11–31-fold lower in adducts/mg PM/km	Pohjola et al. (2003a)
Yamaha Cabin 50-mL, 2-stroke motorcycle engine; unleaded gasoline; particulates collected on glassfibre filters, Soxhlet extraction in dichloromethane/hexane (1:1)	Chinese hamster V79 lung cells treated with particulate extract (up to 500 µg/mL) for 12 or 24 h	Sister chromatid exchange (SCE) and oxidized bases (thymine glycol, 8-OH-dG, 8-OH-dA by GC-MS)	Dose-related increase in SCE, inhibited by antioxidants; dose-related increases in thymine glycol and 8-OH-dG; no change in 8-OH-dA	Kuo et al. (1998)
Three spark ignition engines; gasoline with 12.4, 1.5 or 0.03 µg/mL lead; FTP-75 and ECE driving cycles; PM collected on polyvinylchloride or glassfibre filters, reflux extraction with DMSO	Chinese hamster V79 lung cells and Syrian hamster kidney cells exposed to PM extract (up to 10 µg/mL) for 16 and 18 h, respectively	SCE, structural and numerical chromosome aberrations, C-metaphases and cell transformation	All extracts induced significant concentration-related increases in SCE, C-metaphases, polyploidy, hyperdiploidy and transformed foci	Hadnagy & Seemayer (1988, 1989, 1991)
Yamaha 50-mL, 2-stroke motorcycle engine; PM collected on quartz filters and retrieved in suspension by sonication with methanol	Chinese hamster ovary K1 cells exposed to PM (0.5, 5 or 50 µg/mL in DMSO) for 3 h, 3-methylcholanthrene-induced rat-liver S9	Chromosomal aberrations	Significant dose-related increase in frequency of aberrant cells; higher response with than without S9; antioxidants reduced the response significantly	Cheng et al. (2004)
Santana spark ignition engine; idling and medium-duty conditions; leaded and unleaded gasoline; PM collected on glassfibre filters, extracted by sonication in dichloromethane	Chinese hamster lung cells exposed to PM at 10, 30 or 90 µg/mL for 24 h	MN frequency	Significant concentration-related increase in MN frequency; no difference between leaded and unleaded gasoline	Yuan et al. (1999)

Table 4.13 (continued)

Source of exhaust	Exposure system	End-point(s) examined	Results	Reference
Five 1982–96 spark ignition (gasoline) engines and 3 1998–2000 diesel engines; CUD cycle; PM and SVOCs collected, acetone-extracted, sonicated in TWEEN-80; SRM 1650a was also tested	Chinese hamster V79 lung cells exposed for 20 h	MN frequency and DNA strand breaks (by comet assay)	Gasoline PM/SVOC and SRM 1650a induced significant concentration-related increases in strand breaks and MN frequency; strongest response for gasoline PM; effects of DEP and gasoline PM were comparable when expressed per mile driven.	Liu et al. (2005)
Spark ignition 40-passenger bus, no aftertreatment of exhaust; idling, empty-load conditions; PM collected on glassfibre filters and extracted by sonication in dichloromethane; SVOC collected in PUF and XAD-2, extracted with dichloromethane	Human A549 adenocarcinoma cells exposed to PM extract or SVOC for 2 h (comet assay) or 24 h (MN assay) at 0.025–0.4 L-equivalent/mL	MN frequency and DNA strand breaks (by comet assay)	Significant concentration-related increase in MN frequency and DNA strand breaks	Zhang et al. (2007)
1992 Yamaha Cabin 50-mL, 2-stroke engine; unleaded gasoline mixed (60:1) with low-smoke engine oil; idle speed, empty-load conditions; PM collected on glassfibre filters and extracted (Soxhlet) in dichloromethane/ hexane (1:1)	Human CL5 lung epithelial adenocarcinoma cells and human BEAS-2B bronchial epithelial cells treated with PM extract (CL5: 100 µg/mL; BEAS-2B: 1, 10 or 100 µg/mL) for 6 h	Changes in gene expression (by cDNA microarray and quantitative real-time RT-PCR)	Increased mRNA expression of genes active in xenobiotic metabolism (<i>Cyp1A1</i> , <i>Cyp1B1</i>), inflammation (<i>Il-1α</i> , <i>Il-6</i> , <i>Il-11</i>), cell growth (<i>Fgf-6</i> , <i>Fgf-9</i>), tumour progression (<i>Fra-1</i>) and cell cycle (<i>p21</i>)	Ueng et al. (1998, 2005)
1992 Yamaha Cabin 50-mL, 2-stroke engine; unleaded gasoline mixed (60:1) with low-smoke engine oil; idle speed, empty-load conditions; PM collected on glassfibre filters and extracted (Soxhlet) in dichloromethane/ hexane (1:1)	Human HepG2 hepatoma cells and human NCI-H322 lung carcinoma cells treated with 100 µg/mL for 24 h	Benzo[a]pyrene hydroxylase activity in microsomal fraction	Significant elevation in rate of benzo[a]pyrene hydroxylation	Ueng et al. (1998, 2000)

Table 4.13 (continued)

Source of exhaust	Exposure system	End-point(s) examined	Results	Reference
Yamaha Cabin 50-mL, 2-stroke engine; unleaded gasoline; PM collected on quartz filters, extracted in methanol, filtered (0.2 µm) to obtain extract without particles	Human A549 adenocarcinoma cells treated with 0.02–20 µg/mL PM extract with particles or 20 µg/mL PM extract without particles	Gene expression (by semiquantitative RT–PCR and nuclear factor-κB luciferase-reporter assay)	Concentration-related increase in expression of interleukin-8 and nuclear factor-κB	Lee et al. (2005)
DNA in solution				
Spark-ignition engines: 1995 Nissan Micra 1.3-L (120 km/h) and 2000 Mitsubishi Carisma 1.8-L (120 or 50 km/h); run on CEC-RF-02–99 gasoline; PM collected on Teflon-coated glassfibre filters, Soxhlet extraction in dichloromethane; DEP and SRM 1650 tested in parallel (see Table 4.6)	Incubation of calf-thymus DNA with 150 µg/mL PM extract for 4 h, Aroclor-induced rat-liver S9 or xanthine oxidase	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling and HPLC)	Gasoline PM extracts formed smaller amounts of adducts than DEP, with largest difference under reductive conditions; potency of gasoline-derived PM and DEP to form PAH-derived adducts not significantly different (with S9); DEP potency much higher when expressed as adducts/km	Pohjola et al. (2003b)
Gasoline-fuelled vehicle (Ford Van); HWFET driving cycle; PM collected on Teflon-coated glassfibre filters, Soxhlet extraction with dichloromethane; DEP tested in parallel (see Table 4.7)	Incubation of calf-thymus DNA with gasoline-derived PM extract (100 µg/mL) for 1.5 h, Aroclor-induced rat-liver S9 or xanthine oxidase	Frequency of stable, bulky DNA adducts (by ³² P-postlabelling)	Gasoline samples yielded adducts only without S9; more complex and diffuse adduct pattern compared with DEP; no indication of nitroarene-derived adducts in the xanthine oxidase-treated gasoline extract	Gallagher et al. (1991)

CUD, California unified driving cycle; *Cyp*, cytochrome P450 gene; DEP, diesel exhaust particles; DMSO, dimethyl sulfoxide; *Fgf*, fibroblast growth factor gene; *Fra*, folate receptor α gene; GC-MS, gas chromatography-mass spectrometry; h, hour; HPLC, high-performance liquid chromatography; HWFET, US highway fuel economy test; MN, micronucleus; 8-OH-dA, 8-oxo-2'-deoxyadenosine; 8-OH-dG, 8-oxo-2'-deoxyguanosine; PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; PUF, polyurethane foam; RT–PCR, reverse transcript-polymerase chain reaction; S9, metabolic activation system; SRM, standard reference material; SVOC, semi-volatile organic compound; XAD, adsorbent resin

glycol) in Chinese hamster V79 cells and in mouse BNL.C12 liver cells exposed to extracts of PM from a two-stroke spark ignition engine showed significant dose-related increases in both end-points ([Kuo et al., 1998](#)).

The frequency of bulky DNA adducts was determined in human BEAS-2B bronchial epithelial cells treated with extracts of PM from two spark ignition gasoline engines and from a diesel engine. All samples induced significant concentration-dependent responses that correlated with measured concentrations of PAHs. When expressed as adducts per milligram of PM, the results indicated that gasoline-derived PM was more potent than diesel-derived PM; however, when adjusted by PM emission rate (mg/km), the results showed that PM derived from standard diesel fuel was 31-fold more potent ([Pohjola et al., 2003a](#)).

A study with calf-thymus DNA showed that extracts of PM from a spark ignition gasoline engine were less potent in forming DNA adducts than extracts of diesel engine exhaust PM, particularly following incubation under reductive conditions; in particular, the diesel PM extract was far more potent when expressed per kilometre ([Pohjola et al., 2003b](#)).

Several studies assessed changes in benzo[a]pyrene hydroxylase activity and the expression of various genes in human cells exposed to extracts of PM from a two-stroke motorcycle spark ignition engine. One study showed that benzo[a]pyrene hydroxylase activity in human HepG2 hepatoma and human NCI-H322 lung carcinoma cells was significantly elevated following treatment with PM extracts ([Ueng et al., 2000](#)). A subsequent study with human CL5 pulmonary epithelial cells exposed to PM extracts derived from a motorcycle engine reported increased expression of genes involved in xenobiotic metabolism (e.g. *CYP1A1* and *CYP3A7*), inflammation (e.g. *IL-1 α* , *IL-6* and *IL-11*), tumour progression (e.g. *folate receptor alpha-1*), angiogenesis (e.g. *vascular endothelial growth factor-D*), apoptosis

(*TNFSF10*) and cell-cycle control (*P21*) ([Ueng et al., 2005](#)).

A study on engine exhausts from a two-stroke motorcycle engine noted that a PM extract of these emissions induced IL-8 production by the activation of the *nuclear factor- κ B* gene and increased oxidative stress (measured as dichloro-fluorescein-diacetate fluorescence) in human A549 adenocarcinoma cells ([Lee et al., 2005](#)).

(iii) Effects in vitro in the *Salmonella* reverse mutation assay

In comparison with the numerous reports on diesel engines, a relatively small number of studies used the *Salmonella* mutagenicity assay to examine emissions from spark ignition gasoline engines, the results of which are summarized in [Table 4.14](#).

Several studies compared the bacterial mutagenicity of PM extracts and/or semi-volatile organic compound samples from diesel and gasoline engine exhausts. Although gasoline PM extracts often displayed greater mutagenic activity in the presence of metabolic activation ([Crebelli et al., 1991](#); [Carroll et al., 2000](#); [Seagrave et al., 2002](#); [Zhang et al., 2007](#)), diesel and gasoline PM extracts show similar potency when expressed per microgram of EOM ([Liu et al., 2005](#)). When expressed per microgram of PM, extracts of diesel engine exhaust particles were far more potent than extracts of gasoline engine exhaust PM, especially in the absence of metabolic activation. Also, when expressed per mile driven, extracts of diesel engine exhaust particles were up to more than 100-fold more potent than those of gasoline engine exhaust PM ([Pohjola et al., 2003b](#)). [Liu et al. \(2005\)](#) reported that samples of semi-volatile organic compounds from gasoline-driven engine exhaust were more mutagenic than those from diesel-driven engine exhaust when mutagenicity was expressed per microgram of EOM; however, when expressed per mile, diesel semi-volatile organic compounds

Table 4.14 Summary of studies on the mutagenicity of extracts of gasoline engine exhaust particles in *Salmonella typhimurium*

Test conditions	Salmonella strains ^a /test version	Results	Reference
PM and SVOCs from 5 1982–96 spark ignition (gasoline) engines and 3 1998–2000 diesel engines; CUD cycle; acetone extracts and SRM 1650 extract	YG1024 and YG1029, microsuspension preincubation version, Aroclor-induced rat-liver S9	Highest responses in YG1024 without S9; potency of DEP and gasoline PM similar when expressed per µg extract, but much greater for diesel (4.5–17-fold) when expressed per mile; for YG1029, DEP more potent with S9; SVOC extracts yielded positive results with little difference across strains or S9 conditions; gasoline SVOC more potent per µg EOM, but diesel more potent (1.5–2.9-fold) when expressed per mile	Liu et al. (2005)
PM from two spark ignition engines: 1995 Nissan Micra (120 km/h) and 2000 Mitsubishi Carisma (120 or 50 km/h), SRM 1650, DEP from 1988 2-L light-duty diesel car (no DOC); three fuels (EN97, RD1, RD2); European transient test procedure (ECE15) and EUDC, run in series; PM collected on PUFs and/or Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, standard plate incorporation assay, Aroclor-induced rat-liver S9	Gasoline PM extracts showed similar mutagenicity with and without S9; PM extracts more mutagenic than PUF extracts, the latter were only positive with S9; without S9, DEP extracts more mutagenic (approximately 2–5-fold) than gasoline PM extracts, expressed per mg PM; when expressed per km, DEP extracts more than 100-fold more mutagenic than gasoline PM extracts.	Pohjola et al. (2003b)
Pooled gasoline PM from ‘normal emitters’ (1982 Nissan Maxima, 1994 GMC 1500 pick-up truck, 1995 Ford Explorer, 1996 Mazda Millenia) collected at 30 °F and 72 °F, a visible white-smoke emitter (1990 Mitsubishi Montero), a visible black-smoke emitter (1976 Ford F-150 pick-up truck), pooled DEP from current (2000) technology (1993 Mercedes Benz E300, 1999 Dodge 2500 pick-up truck, 2000 VW Beetle TDI) collected at 30 F and 72 °F and high-emitter diesel (1991 Dodge 2500 pick-up truck); CUD cycle; PM collected on Teflon [®] -coated glassfibre filters, acetone sonication extraction; SVOCs collected on PUF/XAD, acetone Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Gasoline samples generally more potent (per µg EOM) with S9; normal emitters at 30 °F and white-smoke emitter generally more potent; current gasoline and diesel at 72 °F were generally the least potent; multivariate analyses showed associations between mutagenicity and nitro-PAH content (e.g. 6-nitrobenzo[<i>a</i>]pyrene, 1-nitropyrene, 7-nitrobenz[<i>a</i>]anthracene) of exhaust	Seagrave et al. (2002)
PM/DEP from a 1.1-L spark ignition engine and a light-duty 2.5-L diesel engine; unspecified cycle; PM collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction, fractionation into acidic, neutral and basic compounds	TA98, TA97, TA102, TA100, TA104, TA98NR, TA100NR and TA98/1,8DNP ₆ , and <i>E. coli</i> WP2 <i>uvrA</i> , plate-incorporation assay, Aroclor-induced rat-liver S9	Potency of spark ignition PM extracts (per mg EOM) much higher with S9, and highest response for TA100; significant response in TA102 for spark ignition engine only; DEP and spark ignition PM extract fractions showed highest activity in TA98 in acidic and neutral fractions; mutagenicity of spark ignition PM higher with S9	Crebelli et al. (1991)

Table 4.14 (continued)

Test conditions	Salmonella strains ^a /test version	Results	Reference
PM from 2 light-duty spark ignition engines; FTP, ECE and EUDC cycles, with and without catalytic aftertreatment; PM collected on glassfibre filters, dichloromethane Soxhlet extraction	TA98, TA98NR and TA98/1,8DNP ₆ , standard plate incorporation assay, without S9	Mutagenic potency (per µg PM) uniformly higher without S9, with modest reductions in TA98NR and TA98/1,8DNP ₆ , relative to TA98; aftertreatment reduced mutagenicity by 61–99% (per µg) and 92–99% (per mile) and associated with reductions in PM, PAHs and 1-nitropyrene emissions rates (per mile) of 40–80%, 71–99% and 87–92%, respectively	Cooper & Shore (1989)
SVOCs and PM from a light-duty, 4-cylinder spark ignition engine; 3-stage drive cycle; fuels with various concentrations of ETBE or MTBE; PM collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction; SVOCs from exhaust condensates	TA98 and TA100, standard plate incorporation assay, unspecified S9	At rated power, potency (per L exhaust) higher without S9; no response from SVOC samples; additives reduced mutagenicity of all samples; reduction most pronounced for 10% ETBE in TA98	Westphal et al. (2010)
PM from 6 1.3–2.0-L light-duty spark ignition engines; modified MVEG cycle; 2 reformulated gasolines; PM collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and YG1021, plate-incorporation assay, unspecified S9	Direct-acting mutagenicity (per km) highest for older vehicle without fuel injection, lowest for lean burning and multiport injection; the engine without fuel injection showed higher activity in YG1021; S9 enhanced activity for engine tested at –7 °C; reformulated gasoline associated with reduced mutagenicity; higher mutagenicity per km associated with higher PM emissions	Kokko et al. (2000)
PM from a Santana spark ignition engine; idling and medium-duty cycle; PM collected on glassfibre filters, dichloromethane sonication extraction	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Significant concentration-related increase in TA98; higher response without S9; no significant response in TA100; no difference between leaded and unleaded gasoline	Yuan et al. (1999)
PM from spark ignition passenger bus, no aftertreatment; PM collected on glassfibre filters, sonication extraction with dichloromethane; SVOCs collected on PUF and XAD, extracted with dichloromethane	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Significant concentration-related increase in TA98; higher response with S9; no significant response in TA100	Zhang et al. (2007)
PM from 5 types of gasoline-powered scooters; idling with periodic acceleration; high-volume air sampler, PM collected on glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Significant concentration-related increase in TA98 without S9 for 4/5 PM extracts; significant response in TA98 with S9 for 2 samples; no significant response in TA100	Zhou & Ye (1997)

Table 4.14 (continued)

Test conditions	Salmonella strains ^a /test version	Results	Reference
PM from a gasoline-powered scooter; idling or acceleration; 3 different lubricants; high-volume air sampler, PM collected on glassfibre filters, dichloromethane sonication extraction	TA98 and TA100, standard plate incorporation assay, Aroclor-induced rat-liver S9	Significant concentration-related increase in TA98 ($\mu\text{g EOM/plate}$); no difference with and without S9; no significant response in TA100; new lubricant associated with significant decline in mutagenicity and PM emission rate	Zhou & Ye (1998)
PM from a 2-stroke spark ignition scooter engine; ISO 6460 drive cycle; 3 2-stroke engine oils, with and without exhaust treatment; PM collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98 and TA100, plate incorporation assay, Aroclor-induced rat-liver S9	Highest potency (per unit PM) in TA100 with S9; potency and PM emissions reduced for synthetic engine oils, and with use of exhaust catalyst	Sakai et al. (1999)
PM from a Yamaha 50-ml, 2-stroke motorcycle engine; PM collected on quartz filters and isolated by sonication with methanol and suspended in DMSO	TA98, TA100 and TA102, plate incorporation, 3-methylcholanthrene-induced rat-liver S9	Significant concentration-related responses in TA98, TA100 and TA102 with S9; significant reductions with addition of antioxidants	Cheng et al. (2004)
PM from a 0.5-L, 2-cylinder, 2-stroke engine; 5-mode steady-state snowmobile cycle; 2 engine oils; PM collected on glassfibre filters, dichloromethane extraction	[Strains unspecified], microsuspension preincubation version, unspecified S9	Mutagenic potency (per $\mu\text{g PM}$) increased 3–5-fold with S9, and reduced for bio-synthetic engine oil; potency per hp-h also 3–5-fold higher with S9	Carroll et al. (2000)
PM from 1 4-stroke and 2 2-stroke marine spark ignition engines; 5-mode steady-state cycle; PM collected on Teflon [®] -coated glassfibre filters, dichloromethane Soxhlet extraction	TA98, microsuspension preincubation version, unspecified S9	Mutagenic potency (per $\mu\text{g PM}$) much higher for 2-stroke engine, and spark ignition engine (higher PAHs per unit PM) more than 3-fold more potent than carburetor engine; no enhancement with S9; potency per 67-min run showed highest mutagenic activity for the carburetor engine (highest PM emission rate)	Kado et al. (2000)
PM from 1 4-stroke and 1 2-stroke marine spark ignition engines; 5-mode steady-state cycle; PM collected on glassfibre filters, dichloromethane Soxhlet extraction	TA98, microsuspension preincubation version, without S9	Mutagenic potency (per $\mu\text{g DEP}$) for 4-stroke engine 1.5–2.5-fold higher than 2-stroke; similar pattern for potency expressed per kW-h	Wasil et al. (2004)

^a YG1021, TA98 with plasmid pYG216, nitroreductase overproducing strain; YG1024, TA98 with plasmid pYG219, O-acetyltransferase overproducing strain; YG1041, TA98 with plasmid pYG233, nitroreductase and O-acetyltransferase overproducing strain; YG1026, TA100 with plasmid pYG216, nitroreductase overproducing strain; YG1029, TA100 with plasmid pYG219, O-acetyltransferase overproducing strain; YG1042, TA100 with plasmid pYG233, nitroreductase and O-acetyltransferase overproducing strain CUD, California unified driving cycle; DEP, diesel exhaust particles; DOC, diesel oxidation catalyst; ECE, European driving cycle; EOM, extractable organic matter; ETBE, ethyl *tert*-butyl ether; EUDC, extra urban driving cycle; FTP, Federal Test Procedure; hp, horse power; ISO, International Standards Organization; kW-h, kilowatt-hour; min, minute; MTBE, methyl *tert*-butyl ether; MVEG, motor vehicles emissions group; PM, particulate matter; PUF, polyurethane foam; S9, metabolic activation system; SVOC, semi-volatile organic compound; XAD, adsorbent resin

were 1.5- to threefold more mutagenic ([Liu et al., 2005](#)).

PM extracts of exhaust derived from two gasoline-driven spark ignition engines showed uniformly higher mutagenic activity in the absence of metabolic activation, and modest reductions in activity in strains TA98NR and TA98/1,8DNP₆. Catalytic aftertreatment reduced the mutagenicity by 61–99% when expressed per microgram of EOM and by 92–99% when calculated per mile driven; these reductions were associated with declines in the emission rates of PM, PAHs and nitro-PAHs ([Cooper & Shore, 1989](#)).

Changes in fuel formulation can reduce the mutagenic activity (expressed per kilometre driven) of PM emissions from gasoline-driven spark ignition engines ([Kokko et al., 2000](#)).

Higher mutagenic activity of gasoline-derived PM emissions (expressed per litre of exhaust) in the absence rather than in the presence of metabolic activation was reported in a recent study that also showed pronounced reductions in the mutagenic response with the use of fuel additives, such as ethyl-*tert*-butyl ether ([Westphal et al., 2010](#)).

Several studies examined the mutagenic activity of extracts of PM emitted by small gasoline engines. A suspension of PM from a two-stroke motorcycle engine produced a significant concentration-related mutagenic response in *S. typhimurium* TA98, TA100 and TA102, but only in the presence of exogenous metabolic activation ([Cheng et al., 2004](#)). The mutagenicity decreased after pretreatment with antioxidants, such as α -tocopherol, ascorbate and *N*-acetylcysteine.

Two studies assessed the mutagenic activity (per microgram of EOM) of an extract of PM from several two-stroke scooter engines. The first study showed no difference in mutagenicity in *S. typhimurium* TA98 in the presence or absence of metabolic activation, and noted a reduction in mutagenic activity associated with a newer lubricant in the fuel ([Zhou & Ye, 1998](#)).

The second study reported a stronger effect (per milligram of PM) in TA100 in the presence of metabolic activation, and showed reduced mutagenic activity when synthetic engine oil was used and catalytic exhaust aftertreatment was applied ([Sakai et al., 1999](#)).

Extracts of PM from exhaust of a two-stroke snowmobile engine were three- to fivefold more mutagenic (per microgram of PM or per hp-h) in the presence than in the absence of metabolic activation, but the mutagenicity was lower when a biosynthetic lubricant was used ([Carroll et al., 2000](#)).

Two studies assessed the mutagenic activity of PM extracts derived from the exhaust of two- and four-stroke outboard marine engines, with either fuel injection or a carburettor. The first study reported that the mutagenicity of PM extracts from a two-stroke engine was much higher (per microgram of PM) than that of extracts from the four-stroke engine, with no enhancement from metabolic activation ([Kado et al., 2000](#)). PM emissions from the engine fitted with a fuel injection system had a higher PAH content, but the higher PM emission rate of the engine with a carburettor contributed to the higher mutagenic activity. The second study demonstrated that the mutagenic activity (per microgram of PM or per kW-h) of PM extracts from the four-stroke engine was 1.5–2.5-fold higher than that of PM extracts from the two-stroke gasoline direct injection engine ([Wasil et al., 2004](#)).

4.4 Other data relevant to carcinogenicity

4.4.1 Diesel engine exhaust

Numerous human clinical and experimental animal studies have been conducted to investigate the non-cancer health effects of diesel engine exhaust. Selected studies are discussed below for a diverse range of health end-points, including lung function, lung inflammation, immunology

and infection, systemic inflammation and brain inflammation. More comprehensive reviews of the voluminous non-cancer health effects are available (EPA, 2002; Hesterberg *et al.*, 2009). Most of the findings discussed below are applicable to older types of diesel exhaust, which was emitted from diesel engines manufactured before 2007. However, a few of the studies reviewed here examined the toxicity of exhaust from modern diesel engines that has been referred to in Section 1.1 as 'new-technology diesel engine exhaust'. Because an understanding of the health consequences of new-technology diesel engine exhaust is important, a subsection regarding the limited studies that have been carried out has also been included below.

(a) *Lung function*

(i) *Humans*

The possible effects on the lung of chronic occupational exposures to low levels of diesel engine exhaust emissions were studied cross-sectionally in railroad engine house workers (Battigelli *et al.*, 1964), iron ore miners (Jørgensen & Svensson, 1970), potash miners (Attfield *et al.*, 1982), coal miners (Reger *et al.*, 1982), salt miners (Gamble *et al.*, 1983), coal miners exposed to oxides of nitrogen generated (in part) by diesel engine emissions underground (Robertson *et al.*, 1984) and bus garage workers (Gamble *et al.*, 1987). Effects of relatively high concentrations of automobile emissions have been described among bridge and road tunnel workers in two large cities (Speizer & Ferris, 1963; Ayres *et al.*, 1973). Changes in lung function over a 5-year period have also been studied longitudinally among coal miners working underground in mines with and without diesel engines (Ames *et al.*, 1984). Some, but not all, of the results from these studies showed decrements in lung function and an increased prevalence of respiratory symptoms in subgroups exposed to engine emissions.

Controlled studies of human exposure have reported mixed findings for the effects of diesel engine exhaust on lung function, including a general lack of statistically significant effects on lung volumes (e.g. forced vital capacity and forced expiratory volume in one second), but statistically significant effects on specific airway resistance (Nightingale *et al.*, 2000; Nordenhäll *et al.*, 2001; Mudway *et al.*, 2004; Stenfors *et al.*, 2004). Mudway *et al.* (2004) exposed 25 healthy adult volunteers for 2 hours to diluted whole diesel engine exhaust with a particulate concentration of approximately 100 µg/m³. No significant effects in standard lung function tests (forced vital capacity and forced expiratory volume in one second) were observed. Consistent with these findings, Stenfors *et al.* (2004) observed a lack of statistically significant effects on forced vital capacity and forced expiratory volume in one second among groups of 25 healthy and 15 mildly asthmatic volunteers exposed for 2 hours to whole diesel engine exhaust with a particulate concentration of 108 µg/m³. Both Mudway *et al.* (2004) and Stenfors *et al.* (2004) reported small, but significant, effects on airway resistance; Stenfors *et al.* (2004) reported similar increases in airway resistance in both healthy and asthmatic subjects (4.1 and 6.5%, respectively).

(ii) *Experimental animals*

Short-term exposure to diesel engine exhaust (28 days) led to a 35% increase in pulmonary air flow resistance in Hartley guinea-pigs (Wiester *et al.*, 1980), and increased vital capacity and total lung capacity in Sprague-Dawley rats exposed to raw exhaust (Pepelko, 1982a).

Prolonged exposure of rats to diluted diesel engine exhaust has led to impairment of lung function in some studies (Gross, 1981; Heinrich *et al.*, 1986a, b; McClellan, 1986; Maier *et al.*, 2008), but not in others (Green *et al.*, 1983). No significant impairment of lung function was reported in hamsters (Heinrich *et al.*, 1986b).

A classic pattern of restrictive lung disease was observed in cats after 124 weeks of exposure to diesel engine exhaust (weeks 1–61: dilution factor air:diesel, 18; particles, ~6 mg/m³; weeks 62–124: dilution factor air:diesel, 9; particles, ~12 mg/m³) ([Moorman et al., 1985](#)). No such effect was observed during the first 61 weeks of the study ([Pepelko et al., 1980, 1981](#); [Moorman et al., 1985](#)).

No impact on the function of the large airways, the elastic properties of the lung or tracheal mucous transport was observed in sheep exposed to diesel engine exhaust particulates ([Abraham et al., 1980](#)).

Animal and human models have demonstrated the effects of diesel engine exhaust particulates on the enhancement of immunoglobulin (Ig) E production and the promotion of inflammatory responses ([Casillas et al., 1999](#)), including asthma ([Casillas & Nel, 1997](#); [Saxon & Diaz-Sanchez, 2000](#); [Chiaverini, 2002](#); [Jang et al., 2005](#); [McCunney, 2005](#)). Experimental models in which animals were exposed to diesel engine exhaust particulates then challenged with various allergens showed significantly higher responses in treated animals than in matched controls, indicating hyper-responsiveness ([Muranaka et al., 1986](#); [Suzuki et al., 1993, 1996](#); [Fujimaki et al., 1994, 1995](#); [Birumachi et al., 2001](#); [Yamashita et al., 2001](#); [Farraj et al., 2006](#)).

Subchronic exposures of rats to new-technology diesel engine exhaust showed small but statistically significant trends in pulmonary function (e.g. lower values for forced vital capacity and decrements in diffusing capacity for carbon monoxide in the lung) and forced expiratory variables of mean mid-expiratory flow after 3 and 12 months of exposure (discussed in [McDonald et al., 2012](#)).

(b) *Alterations in the immune system, inflammation and risk of cancer*

Diesel engine exhaust and, in particular, diesel engine exhaust particulates have been linked with a variety of adverse health effects that potentially have a bearing on the immune system, including pulmonary inflammation.

(i) *Humans*

Several human clinical studies of exposures to whole diesel engine exhaust have investigated inflammatory responses in the lungs of both healthy and asthmatic volunteers ([Salvi et al., 1999, 2000](#); [Nightingale et al., 2000](#); [Nordenhäll et al., 2000, 2001](#); [Mudway et al., 2004](#); [Pourazar et al., 2004, 2005](#); [Stenfors et al., 2004](#); [Behndig et al., 2006](#)). These studies have generally reported evidence of mild pulmonary inflammatory responses to elevated short-term exposures by inhalation to diesel engine exhaust with particulate concentrations in the range of 100–300 µg/m³.

[Behndig et al. \(2006\)](#) observed responses indicative of mild bronchial inflammation (e.g. increased numbers of neutrophils and mast cells in the bronchial mucosa, and increased numbers of neutrophils, IL-8 and myeloperoxidase concentrations in bronchial lavage), but no evidence of an inflammatory response in the alveolar compartment based on differential lavage and bronchial biopsy 18 hours after exposure to 100 µg/m³ of airborne PM with a diameter < 10 µm for 2 hours. In addition, significant increases in urate and reduced glutathione were observed in alveolar lavage, but not in bronchial lavage. The authors suggested that the different inflammatory responses in the conducting airways and alveolar regions of the lung were related to the movement of glutathione and urate into the lung surface to protect against inflammation in the alveolar region.

Inflammatory responses in the lung are a hallmark of the lung overload response in rats caused by protracted, elevated exposures to

diesel engine exhaust. An increase in neutrophilic inflammation has been defined as the critical biological response to lung overload (see Section 4.2.2).

Human clinical studies demonstrated the detrimental effects of exposure to diesel exhaust particulates on asthma and allergy. Subjects challenged nasally with a dose of diesel engine particulates equivalent to 40 hours of ambient exposure in Los Angeles (0.3 mg) were found to have increased IgE isotype switching resulting in an increase in total IgE levels ([Diaz-Sanchez et al., 1994, 1997](#); [Fujieda et al., 1998](#)). Diesel engine exhaust particulates also appear to have direct effects on mast cells and basophils ([Devouassoux et al., 2002](#); [Nemmar et al., 2004](#)). BAL fluid of healthy individuals exposed to diesel engine exhaust showed increased histamine levels, indicating an acute inflammatory response ([Salvi et al., 1999](#)). In addition, dust mite-sensitive subjects challenged with dust mites had a threefold greater nasal histamine response when diesel engine exhaust particulates were co-administered with the allergen and an increased sensitivity to the onset of symptoms from exposure to dust mites ([Diaz-Sanchez et al., 1999, 2000](#)).

In a chamber study, human volunteers exposed to 200 $\mu\text{g}/\text{m}^3$ of diesel engine exhaust particulates for 2 hours showed an increase in sputum neutrophils and myeloperoxidase 4 hours after the exposure, indicating an inflammatory response in the airways ([Nightingale et al., 2000](#)). An earlier study also showed an increase in sputum neutrophils and a migration of alveolar macrophages into the air spaces ([Rudell et al., 1999](#)).

Goblet cell hyperplasia with increased metaplastic and dysplastic epithelia and an increase in leukocytes were found in 136 nonsmoking customs officers responsible for clearing diesel heavy-duty vehicles (for 8.4 hours per day, 42 hours per week) compared with a nonsmoking control group of 58 officers who worked in the

office only ([Glück et al., 2003](#)). The authors suggested that the significant goblet cell hyperplasia, together with a clear increase in leukocytes, could be taken as an indication of a chronic state of irritation of the nasal mucosa with an inflammatory response.

(ii) *Experimental systems*

[McDonald et al. \(2004a\)](#) reported evidence of diesel engine exhaust-induced lung inflammation, respiratory syncytial resistance and oxidative stress among C57BL/6 mice acutely exposed to 200 $\mu\text{g}/\text{m}^3$ of uncontrolled diesel exhaust emissions (6 hours a day for 7 days) from a single-cylinder diesel engine generator (Model YDG 5500E; Yanmar, Osaka, Japan). However, these effects were not observed among a second group of mice exposed to emissions from the same test engine operated using a catalysed ceramic trap and low-sulfur fuel ([McDonald et al., 2004b](#)). The composition of the exhaust was also significantly different, as expected based on the differences in technology.

CD-1 mice exposed to diesel engine exhaust (350, 3500 or 7000 $\mu\text{g}/\text{m}^3$ for up to 24 months) showed altered IgM, IgC and IgA antibody responses to a challenge with sheep red blood cells ([Bice et al., 1985](#)). The IgE antibody response of BDF₁ mice was increased after five intranasal inoculations of a suspension of diesel engine exhaust particles in ovalbumin solution ([Takafuji et al., 1987](#)).

Two samples of different types of diesel exhaust particulate – automobile-derived diesel exhaust particulates and standard reference material (SRM 2975) – were administered to CD-1 mice in an involuntary aspiration test for pulmonary toxicity. Chemical analysis showed that the automobile-derived sample had more than 10 times the amount of extractable organic material and less than one-sixth of the amount of elemental carbon than SRM 2975. Both diesel engine exhaust particulates produced mild acute lung injury and an increase in IL-6. The

automobile-derived diesel exhaust particulate sample independently induced macrophage influx and activation and stimulated an increase in TNF α , macrophage inhibitory protein-2 and IL-5 (Singh *et al.*, 2004).

The airways and the alveoli of Wistar rats exposed to filtered exhaust for 24 months were infiltrated by inflammatory cells, while morphological changes in their lungs were reduced (Kato *et al.*, 2000). Similar inflammatory responses were seen in the lungs of 16 1-year-old male Wistar Kyoto rats injected with diesel engine exhaust particulates in saline; the rats also had decreased heart rates and blood pressure (Nemmar *et al.*, 2007).

Increased numbers of alveolar macrophages containing diesel engine exhaust particles and type II pneumocytes, and an accumulation of inflammatory cells within the alveoli and septal walls were observed after a 24-hour exposure of Fischer 344 rats to high concentrations of diesel engine exhaust particles (6 mg/m³) (White & Garg, 1981). Macrophage aggregates were still present 6 weeks after a 2-week exposure to diesel engine exhaust particles (6 mg/m³) (Garg, 1983).

Following prolonged exposure of rats to diesel engine exhaust particles (2–5 mg/m³), particle-containing alveolar macrophages and type II cell hyperplasia were observed (Heinrich *et al.*, 1986a; Iwai *et al.*, 1986; Vallyathan *et al.*, 1986). Increases in both the number and size of macrophages and in the number of polymorphonuclear leukocytes were also observed in rats and hamsters (Chen *et al.*, 1980; Vostal *et al.*, 1982; Strom, 1984; Heinrich *et al.*, 1986a), together with elevated levels of lymphocytes (Strom, 1984; Heinrich *et al.*, 1986a).

Fischer 344 rats chronically exposed to diluted whole diesel engine exhaust showed a dose-dependent focal accumulation of soot with parallel active inflammation involving alveolar macrophages adjacent to the terminal bronchiole, progressive fibrosis, epithelial hyperplasia

and squamous metaplasia (adjacent to fibrotic foci) (Mauderly *et al.*, 1987).

Significant alveolar epithelial hyperplastic, inflammatory and septal fibrotic responses were seen in lung sections from male cynomolgus monkeys and Fischer 344 rats exposed to diesel engine exhaust for 7 hours a day, 5 days a week for 24 months (Nikula *et al.*, 1997a, b).

In guinea-pigs exposed to diesel engine exhaust for up to 8 weeks, B- and T-cell counts in lymph nodes were not altered (Dziedzic, 1981). No change was observed in the immunological function of splenic B or T-cells from Fischer 344 rats exposed for up to 24 months to diesel engine exhaust (Mentnech *et al.*, 1984).

(c) *Changes in lung morphology, biochemistry and cytology*

(i) *Humans*

No data were available to the Working Group.

(ii) *Experimental systems*

Damage to the lungs of experimental animals has been demonstrated in numerous studies (Warheit, 1989). The lung weights of mice, rats and Syrian golden hamsters chronically exposed to diesel engine exhaust particulates were significantly greater than those of controls (Heinrich *et al.*, 1986a). An increased lung:body weight ratio was also observed in guinea-pigs following an 8-week exposure (20 hours a day, 7 days a week) to emissions from a Nissan CN6–33 engine (dilution of 1:13 in clean air) (Wiester *et al.*, 1980).

Exposure of rats for 30 months to diesel engine exhaust (particles, 1–4 mg/m³) resulted in dose-dependent irregularity, shortening and loss of cilia in ciliated epithelia, particularly in the trachea and main bronchi (Ishinishi *et al.*, 1988).

Intratracheal instillations of three different doses of various formulations from groups of vehicles decreased potency measures for lactose dehydrogenase, lavage protein and cytotoxicity in all study groups; emissions from current-technology diesel engines elicited a similar response to

those from high-emitter diesel engines ([Seagrave et al., 2002](#)). In a similar experiment in Syrian hamsters, an influx of neutrophils was observed in BAL fluid with an elevation of protein and histamine, and a rapid activation of circulating platelets ([Nemmar et al., 2003](#)).

(d) *Other effects*

(i) *Humans*

No data were available to the Working Group.

(ii) *Experimental systems*

Exposure to diesel engine exhaust particles or their extracts has been reported to have no effect ([Chen & Vostal, 1981](#); [Rabovsky et al., 1984](#)) or only a moderate effect ([Lee et al., 1980](#); [Pepelko, 1982a, b](#); [Dehnen et al., 1985](#); [Chen, 1986](#)) on aryl hydrocarbon hydroxylase activity in the lung and liver of mice and rats and in the lung of hamsters. Cyp1A1 expression in murine lung has been shown to increase following exposure to diesel engine exhaust particles ([Takano et al., 2002](#)), which have also been shown to increase Cyp1B1 in rat brain microvessels, probably through AhR activation ([Jacob et al., 2011](#)). Quinone reductase, Cyp1A1 and Cyp2B1 were increased in the lung tissue of rats exposed to diesel engine exhaust particulates whereas GST-pi protein and catalase were decreased ([Rengasamy et al., 2003](#)).

Exposure of Fischer 344/Crl rats by inhalation to diesel engine exhaust doubled the rate of 1-nitropyrene metabolism in both the nasal tissue and perfused lung, and the amount of ¹⁴C that bound covalently to lung macromolecules was increased fourfold ([Bond et al., 1985](#)).

One week after instillation, significantly more residual benzo[a]pyrene was found in the lungs of A/Jax mice exposed to diesel engine exhaust for 9 months, probably because the benzo[a]pyrene had bound to the exhaust particles ([Cantrell et al., 1981](#); [Tyrer et al., 1981](#)).

4.4.2 Gasoline engine exhaust

(a) *Lung function*

(i) *Humans*

No data were available to the Working Group.

(ii) *Experimental systems*

Long-lasting functional disturbances in the lung were observed in beagle dogs after exposure to raw or irradiated gasoline engine exhaust (carbon monoxide, 114–126 mg/m³) for 68 months ([Lewis et al., 1974](#); [Gillespie, 1980](#)). In contrast, no impairment in lung function was detected in Crl:COBS CD(SD)BR rats exposed for 45 or 90 days to diluted (1:10) exhaust from a catalyst-equipped gasoline engine (particles, 11.32 ± 1.27 mg/m³; carbon monoxide, 19.5 ± 3.5 mg/m³; [Pepelko et al., 1979](#)). No change in lung function was observed in male and female mice (A/J and BALBc) or rats (SHR and Fischer 344) exposed to both unfiltered and filtered exhaust from 4.3-L 1996 General Motors engines run on national average fuel in a simulated urban operating cycle ([Reed et al., 2008](#)).

(b) *Alterations in the immune system, inflammation and risk of cancer*

(i) *Humans*

No data were available to the Working Group.

(ii) *Experimental systems*

The BAL fluid of mice exposed to gasoline engine exhaust showed elevated levels of total protein, alkaline phosphatase, γ -glutamyl phosphatase, lactose dehydrogenase, TNF- α and IL-6 but not of IL-1 β or IL-10, indicating lung damage and an inflammatory response ([Sureshkumar et al., 2005](#)). Mice exposed to gasoline engine emissions demonstrated elevated levels of plasma endothelin-1, but not systemic inflammation ([Campen et al., 2006](#)).

(c) *Changes in lung morphology, biochemistry and cytology*

(i) *Humans*

No data were available to the Working Group.

(ii) *Experimental systems*

Several studies in beagle dogs reported atypical epithelial hyperplasia in animals exposed for 68 months to raw or irradiated gasoline engine exhaust (carbon monoxide, 114 mg/m³). Increases in alveolar air space and cilia loss were observed after a long recovery period following exposure to the irradiated exhaust ([Hyde et al., 1980](#)).

(d) *Other effects*

(i) *Humans*

No data were available to the Working Group.

(ii) *Experimental systems*

[Lund et al. \(2007\)](#) examined aortas and plasma to evaluate histochemical markers, gene expression and oxidative stress following exposure to gasoline engine emissions and found transcriptional upregulation of genes associated with vascular modelling and increased markers of vascular oxidative stress.

4.5 Susceptibility in humans

The factors involved in human susceptibility to exposure to diesel engine exhaust include (i) genetic polymorphisms, (ii) vulnerable populations, (iii) underlying lung and airway disease and (iv) status of the respiratory tract microbiome.

The available data do not provide clear or consistent evidence of the influence of any particular genotype on biomarkers associated with exposure to engine exhaust, a situation that is somewhat similar to the influence of genetic polymorphisms on biomarkers or the risk for lung or urinary bladder cancer associated with cigarette smoking. Although some associations

have been found with increased risks for lung cancer from smoking among individuals who are *GSTM1* null or who have a particular *CYP1A1* polymorphism, the data are inconsistent ([IARC, 2012b](#)). No studies have been carried out on the influence of other susceptibility factors such as vulnerable populations, underlying disease and the microbiome in relation to exposure to diesel engine exhaust and the risk for lung cancer.

4.5.1 Genetic polymorphisms

See [Table 4.15](#)

Although at least five studies have found some influence of genotype on the biomarker response to exposure to engine exhaust in humans, the data are not consistent. A study of bus maintenance workers found that, among *N-acetyltransferase 2* (*NAT2*) slow acetylators, *GSTM1*-null subjects had higher DNA adduct levels (determined by postlabelling using thin-layer chromatography) in their lymphocytes than *GSTM*-positive subjects ([Hou et al., 1995](#)). However, *GSTM1* and *NAT2* status was found to have no influence on *Hprt* mutant frequency in lymphocytes. The three major *NAT2* alleles, *M1*, *M2* and *M3*, were determined by restriction analysis.

Among a group of diesel revision workers and established street vendors in an urban area of Chile, [Adonis et al. \(2003\)](#) and [Gil et al. \(2003\)](#) found no influence of the *GSTM1* gene alone on levels of urinary 1-hydroxypyrene. However, they found that subjects who had the combined *CYP1A1*2A* and *GSTM1*-null genotype had higher levels of urinary 1-hydroxypyrene than those who did not ($P = 0.055$).

The *NAT2* slow acetylators among a group of bus drivers or mail carriers who worked outdoors in Copenhagen, Denmark, had higher concentrations of urinary 1-hydroxypyrene than fast acetylators; however, this genotype did not influence the levels of urinary mutagenicity ([Hansen et al., 2004](#)). The *NAT2* phenotype was

Table 4.15 Summary of human susceptibility to exposure to atmospheres containing engine exhaust

Reference	Exposure group (exposure type)	Genotype ^a	Result	Assoc. with Exposure	
				Urine	Air
Hou et al. (1995)	Bus maintenance workers (predominantly diesel)	<i>GSTM1</i> -null <i>GSTM1</i> , <i>NAT2</i>	↑ DNA adducts N/E on <i>Hprt</i>		
Adonis et al. (2003) , Gil et al. (2003)	Truck/bus inspectors and street vendors	<i>CYP1A1*2A</i> + <i>GSTM1</i> -null	↑ OHPy		
Hansen et al. (2004)	Bus drivers or mail carriers	<i>NAT2</i> slow	N/E on urinary mutagenicity	↑ OHPy	
Topinka et al. (2007)	Police officers (mixed)	<i>CYP1A1</i> variants <i>CYP1A1</i> +, <i>GSTM1</i> +	↑ DNA adducts ↓ DNA adducts		PAHs +
Palli et al. (2001)	Traffic exposure	<i>XP2-Lys751/Gln</i> variants	↑ DNA adducts		
Nielsen et al. (1996b)	Bus drivers (mixed)	<i>GSTM1</i> , <i>NAT2</i>	N/E DNA adducts		
Knudsen et al. (2005)	Miners driving heavy-duty vehicles (predominantly diesel)	<i>GSTM1</i> , <i>GSTT1</i> , <i>GSTP1</i>	N/E on DNA adducts or DNA damage		1-NP
Villarini et al. (2008)	Road tunnel workers (predominantly diesel)	<i>CYP1A1</i> ; <i>GSTM1</i>	N/E on MN		

^a All genotypes were determined from DNA extracted from blood; the *NAT2* slow phenotype was determined from urinary metabolites, as noted in the text. CYP, cytochrome P450; GST, glutathione *S*-transferase; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; *NAT*, *N*-acetyltransferase; MN, micronuclei; N/E, no effect; OHPy, 1-hydroxypyrene; ↑, increase; ↓, decrease; +, positive

determined by analysis of the urinary metabolites of caffeine.

Among policemen in Prague, Czech Republic, those who had both *CYP1A1* and *GSTM1* polymorphic variants had the lowest levels of DNA adducts in lymphocytes determined by postlabelling/thin-layer chromatography (Topinka *et al.*, 2007). The levels of DNA adducts were also highest in subjects with variants of *CYP1A1*, independent of *GSTM1* status, and were associated with the levels of carcinogenic PAHs in the air.

Subjects in Florence, Italy, who had occupational exposure to traffic exhaust and at least one variant of the DNA nucleotide excision-repair gene, *XPD-Lys751/Gln*, had increased levels of DNA adducts in their lymphocytes (determined by postlabelling/thin-layer chromatography; Palli *et al.*, 2001).

However, *GSTM1* and *NAT2* (*M1*, *M2* and *M3* alleles) had no effect on the levels of DNA adducts (measured by postlabelling/HPLC) in the lymphocytes of Copenhagen bus drivers (Nielsen *et al.*, 1996b). Also, *GSTM1*, *GSTT1* and *GSTP1* had no effect on the levels of DNA adducts (measured by postlabelling) or DNA damage (measured by the comet assay) in the lymphocytes of shale-oil mine workers exposed to diesel engine exhaust (Knudsen *et al.*, 2005).

CYP1A1 and *GSTM1* had no influence on the observed increase in micronuclei in the lymphocytes of road tunnel construction workers relative to office controls in Genoa, Italy (Villarini *et al.*, 2008). An *in vitro* study of diesel engine exhaust extracts in the *umu* gene expression assay in *S. typhimurium* TA1535/pSK1002 found that the extract was activated by CYP1B1 and CYP1A2 but not by CYP1A1 (Yamazaki *et al.*, 2000).

4.5.2 Vulnerable populations

Children represent a population that is vulnerable to exposure to diesel engine exhaust because they spend most of their time playing outside,

have higher respiratory rates than adults and have underdeveloped lungs (Suwanwaiphatthana *et al.*, 2010). Alveolar development is arrested in the young due to underlying inflammatory disease (Bäckström *et al.*, 2011). An age-dependent theoretical model was developed to predict PM dosimetry in the lungs of children. The simulation predicted that the lung deposition of 2- μ m particles was 38% in adults but was as high as 73% in 7-month-old children (Musante & Martonen, 2000). However, it is uncertain how these events may affect susceptibility to lung cancer later in life.

4.5.3 Underlying lung disease

While there is evidence that exposure to diesel engine exhaust may exacerbate asthma and chronic obstructive disease and increase lung injury, it is not known how these chronic conditions may affect susceptibility to lung cancer from this exposure.

4.5.4 Respiratory tract microbiome

The respiratory tract is lined with microflora that expresses enzymes which may increase the metabolic activation of some components of diesel engine exhaust, e.g. nitroarenes. The composition of the microbiome is also affected by the use of antibiotics for upper respiratory tract infections. Thus, the microbiome represents a changing microenvironment that may affect susceptibility to the carcinogenic constituents of diesel engine exhaust.

4.6 Mechanistic considerations

4.6.1 Diesel engine exhaust

Diesel engine exhaust is a complex mixture comprised of both gaseous and particulate components. The gaseous phase comprises nitrogen oxides, sulfur, ozone and organic compounds, such as acetaldehyde, acrolein,

benzene, 1,3-butadiene, formaldehyde, naphthalene and PAHs and nitro-PAHs. Benzene, 1,3-butadiene, formaldehyde and benzo[*a*]pyrene are carcinogenic in experimental animals and have been classified as human carcinogens (IARC, 2010a, 2012a). Naphthalene (IARC, 2002) and acetaldehyde (IARC, 1999) have been classified as possibly carcinogenic to humans, and several other PAHs (IARC, 2010a) and nitro-PAHs (see the *Monographs* in this Volume) have been classified as probably or possibly carcinogenic to humans.

The particulate phase contains organic compounds including PAHs (IARC, 2010a) and nitro-PAHs (see the *Monographs* in this Volume), many of which have been classified by the IARC as possible or probable carcinogens. It also contains trace metals, including lead, manganese, arsenic and chromium, and those from the catalyst aftertreatment systems – vanadium, copper and iron. Arsenic and arsenic inorganic compounds and chromium VI have been classified as human carcinogens (IARC, 2012c), whereas lead (IARC, 1987) and inorganic lead compounds (IARC, 2006b) have been classified as probably or possibly carcinogenic to humans, respectively. These components are adsorbed onto carbon core particles that vary in size from coarse to fine to ultrafine nanoparticles.

(a) *Organic solvent extracts of particulates from diesel engine exhaust*

Organic solvent extracts of particulates of diesel engine exhaust contain higher-molecular-weight organic compounds, including PAHs and nitro-PAHs. Organic compounds adsorbed on particles have been evaluated for genotoxicity in *in vitro* and *in-vivo* assays, and have a broad range of activities. They are mutagenic in bacterial assays and in mammalian cells, form bulky DNA adducts, and induce unscheduled DNA synthesis, sister chromatid exchange, chromosomal aberrations and morphological cell transformation (IARC, 1989). They also induce

skin papillomas in mouse skin tumour-initiation studies and adenocarcinomas in mice after dermal application in cancer bioassays (IARC, 1989). More recent studies indicate that organic solvent extracts of diesel engine exhausts induce DNA strands breaks and oxidative damage, as well as increase the expression of genes involved in xenobiotic metabolism, oxidative damage, antioxidant responses and the cell cycle in mammalian cells in culture.

There is strong mechanistic evidence that organic solvent extracts of diesel engine exhaust particulates induce cancer in experimental animals by a genotoxic mechanism.

PAHs are biotransformed by phase I metabolic enzymes to a series of dihydrodiols, phenols, quinones and polyhydroxylated metabolites. Dihydrodiols can be metabolized further to chemically reactive intermediates (diol epoxides) that bind covalently to DNA to form DNA adducts. PAHs can undergo one-electron reduction to form radical cations that can adduct to DNA forming depurinating PAH adducts. PAH quinones can undergo redox cycling, generating ROS that damage DNA. Many of these DNA modifications have been associated with the induction of mutation and, eventually, tumour formation. Further metabolism of PAH metabolites by phase II enzymes converts many of the primary metabolites to glucuronic acid and sulfate and glutathione conjugates that are excreted in the faeces and urine. Nitro-PAHs can be reduced by nitroreductases to hydroxylamino and amino metabolites; the hydroxylamino intermediates have been shown to bind to DNA to form covalent DNA adducts. Some nitro-PAHs can undergo both oxidative and reductive metabolism, forming mixtures of metabolites and DNA adducts containing nitro, dihydrodiol or amino functionalities (IARC, 1989). The detailed mechanism(s) of the metabolic activation of PAHs have been described previously (IARC, 2010a) and in this *Monograph* (see Section 4.1). Detailed mechanism(s) of the

metabolic activation of nitro-PAHs are described in the individual *Monographs* in this Volume.

(b) *Bioavailability*

The organic compounds adsorbed onto particles need to be bioavailable to manifest their genotoxic activities. They can be removed from diesel engine exhaust particulates extremely efficiently with organic solvents, and some evidence shows that biological fluids can facilitate their bioavailability based on *in vitro* assays. Human serum and rat lung cytosol released 79–85% of the organic solvent-extractable mutagenic activity from diesel engine exhaust particles based on the results of bacterial mutation assays. Although the serum-associated mutagens were largely undetectable in a bacterial mutation bioassay, incubation of the serum with protease increased its mutagenic activity (King *et al.*, 1981). Incubation and phagocytosis of diesel engine exhaust particles by rabbit alveolar macrophages removed more than 97% of the bacterial mutagenic activity (King *et al.*, 1983). When dispersed into a simulated pulmonary surfactant, diesel engine exhaust particulates were mutagenic in bacteria and genotoxic in mammalian cells, inducing unscheduled DNA synthesis, sister chromatid exchange, micronuclei and chromosomal aberrations (Keane *et al.*, 1991; Gu *et al.*, 2005).

(c) *Gaseous phase of diesel engine exhaust*

Acetaldehyde, acrolein, benzene, 1,3-butadiene, naphthalene and formaldehyde have been detected in the gaseous phase of diesel engine exhaust, which was mutagenic to bacteria. Each of these individual compounds possesses a different mechanism of carcinogenic action, and it is not known whether these mechanisms contribute to the overall carcinogenicity of diesel engine exhaust in terms of their potential co-carcinogenic, cell-proliferative and/or tumour-promoting activities.

(d) *Particulates from diesel engine exhaust*

Diesel engine exhaust particles exhibit a variety of genotoxic effects *in vitro* and *in vivo*. After dietary exposure, they induced DNA adducts, oxidative DNA damage and DNA strand breaks in the colon, liver and lung of rats, but did not induce oxidative DNA damage in the lung or liver of mice. After intratracheal instillation, diesel engine exhaust particles caused oxidative DNA damage in the lungs of mice and transformed foci in the tracheal epithelium of rats. Exposure of mice to diesel engine exhaust particles by inhalation produced oxidative damage, adducts and strand breaks in lung DNA as well as heritable germ-cell mutations.

A major fraction of diesel engine exhaust particulates comprises nanoparticles and agglomerates (Kittelson, 1998) to which humans are exposed (Sawant *et al.*, 2008; Hesterberg *et al.*, 2010). Diesel engine exhaust particulates generate superoxide and hydroxyl radicals (Vogl & Elstner, 1989; Sagai *et al.*, 1993; Kumagai *et al.*, 1997), and increase the levels of 8-OH-dG in DNA (Møller *et al.*, 2010) *in vitro* and *in vivo*. In one seminal study, levels of 8-OH-dG were measured in the lungs of mice after intratracheal injection of particulates from diesel engine exhaust (Tokiwa *et al.*, 1999); the major contributor to the formation of 8-OH-dG was carbonaceous particles that had been stripped of organic compounds, and the smaller contributor was organic chemicals adsorbed on the particles. Diesel engine exhaust particles induce the formation of inflammatory cells (neutrophils, eosinocytes and alveolar macrophages), and it was proposed that, during phagocytosis of the particles, alveolar macrophages may contribute to oxidative damage through the formation of hydroxyl radicals that lead to the hydroxylation at the C8 position of the deoxyguanosine in DNA (Tokiwa *et al.*, 1999, 2005). After intratracheal instillation, unwashed diesel particles induced lung adenomas in mice. Furthermore,

the tumorigenic response and the formation of 8-OH-dG were directly correlated in the lungs of mice injected intratracheally with the vehicle (titanium dioxide), or washed or unwashed diesel exhaust particles ([Ichinose et al., 1997b](#)).

Microarray studies in cultured rat alveolar epithelial cells exposed to fractionated organic solvent extracts of diesel engine exhaust particles indicated the upregulation of genes involved in phase I and II metabolism, oxidative stress, antioxidant response, immune/inflammatory response, cell cycle/apoptosis and response to cell damage ([Omura et al., 2009](#)).

(e) *Whole diesel engine exhaust*

Exposure to whole diesel engine exhaust induced sister chromatid exchange in the lung cells of rodents ([IARC, 1989](#)). In more recent studies, lung tissues from mice or rats exposed to whole diesel engine exhaust showed increased levels of bulky DNA adducts, whereas increases in oxidative DNA damage and mutations were observed in exposed rats. In mice implanted with matrigel scaffolds of murine epithelial cells, exposure to whole diesel engine exhaust significantly increased mRNA expression of vascular endothelial growth factor and hypoxia-inducible factor-1, while it decreased prolyl hydroxylase 2 expression. Whole diesel engine exhaust increased inflammatory cell infiltration, enhanced the vessel volume/flow and increased capillary tube formation and sprouting, thereby inducing angiogenesis and vasculogenesis ([Xu et al., 2009](#)).

Extensive evidence has shown that chronic inhalation of high concentrations of diesel engine exhaust induces lung cancer in rats, but not in hamsters or mice. Although the particulate phase of diesel engine exhaust alone also induced lung cancer in rats, the gaseous phase did not. A particle overload mechanism was proposed for the induction of cancer in rats following high particle deposition, and the ensuing overloading of the particle clearance process carried out by

macrophages (phagocytosis of excessive quantities of particles) in the deep lung, that resulted in sequestration of the particles within the lung ([Morrow, 1988](#)). This engendered an influx of leukocytes that produced chronic pulmonary inflammatory effects, including the generation of ROS, which increased oxidative DNA damage in proliferating epithelial lung cells that eventually resulted in lung cancer ([Mauderly, 1994, 1997](#); [Watson & Valberg, 1996](#); [Mauderly, 1997](#); [Stinn et al., 2005](#)).

Additional inhalation studies in rats with titanium dioxide and carbon black revealed that, regardless of the particle type used in the studies, the rate of lung tumours increased with increasing exposure concentration of the particles ([Heinrich et al., 1995](#)). This indicated that, under the conditions of the bioassay, diesel engine exhaust was carcinogenic to rats. The conclusions from these and other inhalation studies in rats indicated that lower particle loads do not produce lung cancer because they do not trigger the compensatory inflammatory responses in the lung. Moreover, rats, unlike hamsters and mice, are sensitive to the inhalation of high particle loads that elicit significant physiological responses in the lung that eventually lead to cancer ([Hesterberg et al., 2012](#)). This high loading effect induced by particulates may be relevant to humans who are occupationally exposed. In addition, humans, in contrast to rodents, can mount an inflammatory response at levels corresponding to occupational exposure.

The species specificity of the rat lung response to particle overload, and its occurrence with other particle types, has been described extensively ([Hesterberg et al., 2012](#)). The rat model has limitations for studying the mechanisms of carcinogenicity involved in the induction of human lung cancer following the inhalation of whole diesel engine exhaust. However, some aspects of the responses observed in rats are similar to those seen in humans exposed to diesel engine exhaust

and could help to elucidate its mechanism(s) of carcinogenic action.

Human studies have indicated that some populations exposed to diesel engine exhaust excrete 1-hydroxypyrene, an indicator of exposure to PAHs, in the urine as well as several amino-PAHs (e.g. 1-aminopyrene and 3-aminobenzanthrone), which are reduction products of 1-nitropyrene and 3-nitrobenzanthrone and are considered to be specific markers of exposure to diesel exhaust (Seidel *et al.*, 2002). Increases in the levels of bulky DNA adducts were found in the peripheral blood lymphocytes of workers exposed to diesel engine exhaust (Nielsen *et al.*, 1996b). In controlled studies of human exposure to diesel engine exhaust, healthy subjects developed airway inflammation, with airway neutrophilia and lymphocytosis, as well as increases in IL-8 protein in lavage fluid, increased *IL-8* gene transcription in the bronchial mucosa and upregulation of endothelial adhesion molecules (Salvi *et al.*, 1999, 2000; Stenfors *et al.*, 2004). Exposure to diesel engine exhaust also increased IL-6 (Nordenhäll *et al.*, 2000), B lymphocytes in airway lavage fluid (Salvi *et al.*, 1999) and growth-regulated oncogene- α protein expression in the bronchial epithelium (Salvi *et al.*, 2000). It has been proposed that diesel engine exhaust particles induce oxidative stress in humans that leads to a cascade of downstream mitogen-activated protein kinase signalling pathways, the activation of which leads to the activation of nuclear factor- κ B and activator protein-1 transcription factors, which increase the levels of pro-inflammatory mediators (e.g. IL4, IL6, IL8 and, TNF α), leading to induced airway leukocyte infiltration and inflammation (Salvi *et al.*, 2000; Donaldson *et al.*, 2005; Ristovski *et al.*, 2011). Diesel engine exhaust particles induced IL-8 expression in human airway epithelial cells isolated from healthy adult human volunteers by brush biopsy of the mainstem bronchus (Tal *et al.*, 2010). Recent microarray studies of blood monocytes from young healthy subjects who

underwent inhalation exposures to clean filtered air or freshly generated and diluted diesel engine exhaust reported the upregulation of the expression of genes connected with key oxidative stress, protein degradation and coagulation pathways (Pettit *et al.*, 2012).

Diesel engine exhausts and the mechanisms by which they induce cancer in humans are complex in nature, and no single mechanism appears to predominate. Organic solvent and physiological fluid extracts of diesel engine exhaust particles and several of the individual components of these exhausts are genotoxic, and some are carcinogenic, generally through a mechanism that involves DNA mutation. These modifications include the formation of bulky DNA adducts and oxidized DNA bases. Both the organic and particulate components of diesel engine exhaust emissions can generate ROS, leading to oxidative stress. ROS can be generated from washed particles, fresh particles, arene quinones formed by photochemical or enzymatic processes, metals and the phagocytosis process, and as a result of the inflammatory process. ROS can lead directly to the formation of oxidatively modified DNA and DNA adducts from by-products of lipid peroxidation (Voulgaridou *et al.*, 2011), can cause lipid peroxidation, which generates cytotoxic aldehydes (Barrera *et al.*, 2008), and can also initiate a signalling cascade that leads to inflammation, resulting in further induction of oxidative stress, which in turn leads to cell proliferation and cancer (Milara & Cortijo, 2012). In response to the inflammatory insult, cyclooxygenase-2 is upregulated and is a potent mediator of cell proliferation (Speed & Blair, 2011).

4.6.2 Gasoline engine exhaust

(a) Condensates and organic solvent extracts of particulates from gasoline engine exhaust

The particulate phase of gasoline engine exhaust contains several carcinogenic PAHs ([IARC, 1989](#)), including benzo[*a*]pyrene, which is carcinogenic in experimental animals and is classified as a human carcinogen ([IARC, 2010a](#)). The particulate phase of gasoline engine exhaust contains other PAHs, some of which have been classified as probably or possibly carcinogenic to humans ([IARC, 2010a](#)). Organic solvent extracts of gasoline engine exhaust particulates were mutagenic in bacteria and in mammalian cells, and induced oxidative DNA damage, DNA strand breaks, sister chromatid exchange, micronuclei, chromosomal abnormalities and morphological cell transformation in mammalian cells. Gasoline engine exhaust condensate and particulate extracts of gasoline engine exhaust induced cancer in rodents. These data provide strong evidence that a genotoxic mechanism is involved in the carcinogenicity of condensates and particulate extracts of gasoline engine exhausts ([IARC, 1989](#); [Liu et al., 2005](#)).

(b) Gaseous phase of gasoline engine exhaust

The gaseous phase of gasoline engine exhaust is mutagenic to bacteria ([IARC, 1989](#)) and contains nitrogen oxides, sulfur, ozone and several carcinogenic volatile organic compounds, such as benzene, 1,3-butadiene and formaldehyde ([IARC, 1989](#)), which are carcinogenic in experimental animals and have been classified as human carcinogens ([IARC, 2012c](#)). Benzene, 1,3-butadiene and formaldehyde each act through a different mechanism of carcinogenic action and it is not known whether these mechanisms are altered in the presence of the other components of gasoline engine exhaust.

(c) Particulates from gasoline engine exhaust

After intratracheal instillation or intraperitoneal injection into mice, particles from gasoline engine exhaust induced micronucleus formation in peripheral blood cells, suggesting a genotoxic mechanism ([IARC, 1989](#)).

(d) Whole gasoline engine exhaust

Whole gasoline engine exhaust is mutagenic in bacteria and induces micronuclei in mice ([IARC, 1989](#)).

Overall, the mechanistic evidence from human and experimental studies of exposures to whole gasoline engine exhaust is too weak to formulate a mechanism of action for this exposure.

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