2-NITROFLUORENE

2-Nitrofluorene was evaluated by a previous IARC Working Group in 1988 (IARC, 1989). New data have since become available, and these have been taken into consideration in the present evaluation.

1. Exposure Data

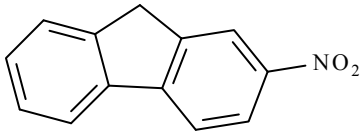
1.1 Chemical and physical data

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 607-57-8

IUPAC Systematic Name: 2-Nitrofluorene

1.1.2 Structural and molecular formulae and relative molecular mass



$C_{13}H_9NO_2$

Relative molecular mass: 211.2 g/mol

1.1.3 Chemical and physical properties of the pure substance

Description: Needles, recrystallized from 50% acetic acid or acetone (Weast, 1985); light-yellow, fluffy solid (Chemsyn Science Laboratories, 1988)

Melting-point: 156 °C (Buckingham, 1982); 158 °C (Weast, 1985)

Spectroscopy data: Ultraviolet, nuclear magnetic resonance, infrared and mass spectral data have been reported (Schuetzle & Jensen, 1985; Chemsyn Science Laboratories, 1988).

Solubility: Sparingly soluble in water (Beije & Möller, 1988a); soluble in acetone, benzene, tetrahydrofluorenone and toluene (Weast, 1985; Chemsyn Science Laboratories, 1988)

1.1.4 Technical products and impurities

2-Nitrofluorene is available for research purposes at purities of 95%, 98% or > 99%, and in radiolabelled form at purities of ≥ 98% (^{14}C) or ≥ 99% (^{3}H) (IARC, 1989).

Currently, 2-nitrofluerene (98% pure) is sold in small quantities for research purposes (Sigma-Aldrich, 2012).

1.2 Analysis

For the analytical methods of nitro-polycyclic aromatic hydrocarbons (PAHs) in general, the reader is referred to Section 1.2.2(d) of the

Monograph on Diesel and Gasoline Engine Exhausts in this Volume.

2-Nitrofluorene is present in the gas phase and in the particle phase, and polyurethane foam can be used for its collection in the gas phase (Albinet *et al.*, 2006). 2-Nitrofluorene has been detected in particles that were collected on glass and quartz fibre or Teflon-coated silica fibre membrane filters (Tokiwa *et al.*, 1990; Schauer *et al.*, 2004).

2-Nitrofluorene can be extracted from particulate matter using dichloromethane for extraction by sonification (Tokiwa *et al.*, 1990). Gas chromatography-negative ion chemical ionization-mass spectrometry was used for its analysis following pressurized-fluid extraction (Bamford *et al.*, 2003; Albinet *et al.*, 2006). This substance was also analysed after its reduction to 2-aminofluorene with titanium(III)citrate and subsequent detection by fluorescence spectrometry (Zielinska & Samy, 2006). Chemiluminescence was also used as an alternative and sensitive method of detection in place of fluorescence (Murahashi & Hayakawa, 1997).

1.3 Production and use

1.3.1 Production

No evidence was found that 2-nitrofluorene is currently produced for purposes other than laboratory use.

1.3.2 Use

2-Nitrofluorene was not reported to be used in commercial applications.

1.4 Occurrence and exposure

1.4.1 Diesel engine exhaust

2-Nitrofluorene has been identified in diesel engine exhaust emissions in several studies (Table 1.1). Two unspecified isomers of nitrofluorene were reported in exhaust emissions from three light-duty diesel passenger cars at concentrations in the range of 50–200 µg/g of particulates (Schuetzle, 1983). Emission levels from 1980–85 model light-duty diesel engines running on an urban Federal Test Procedure cycle were 90 µg/mile [56 µg/km] for the gas phase and 97 µg/mile [61 µg/km] for the particulate phase (Schuetzle & Frazier, 1986). Concentrations in particulates from a heavy-duty mining diesel engine were reported to be 0.63 µg/g when running at a 100% load and 1200 rpm and 8.8 µg/g when running at a 75% load and 1800 rpm (Draper, 1986).

The use of biodiesel and an oxidation catalyst was studied in the heavy duty transient Federal Test Procedure. In three types of heavy-duty diesel engine, different fuels were tested: neat biodiesel fuel (B100), a blend of biodiesel with normal diesel fuel (20:80 by volume; B20) and neat diesel fuel (2D). The formation of 2-nitrofluorene in the exhaust from B100 ranged widely between 6.5 and 142 ng/horse power (hp)–h. When an oxidation catalyst was added, emissions of 2-nitrofluorene were reduced in two of the engines to values of 14 and 73 ng/hp-h. When fuelled with B20 and 2D, the amounts of 2-nitrofluorene ranged from 70 to 144 ng/hp-h in two of the three engines (two 1997 models). In the third engine (a 1995 model), the level of 2-nitrofluorene was 257 ng/hp-h for 2D. After the addition of an oxidation catalyst, the production of 2-nitrofluorene increased to 478 ng/hp-h with (Sharp *et al.*, 2000). [This comparison could not be made for the blended fuel B20 due to the absence of data.]

1.4.2 Ambient air and dust

Low levels of 2-nitrofluorene were detected in most, but not all, samples of airborne particulate matter or gas-phase samples collected at urban locations in Japan, Germany and France (Tanabe *et al.*, 1986; Tokiwa *et al.*, 1990; Schauer *et al.*, 2004; Albinet *et al.*, 2006, 2007; Table 1.2), whereas no detectable quantities of 2-nitrofluorene were

Table 1.1 Levels of 2-nitrofluorene in particulate matter from diesel exhaust emissions and other sources

Reference	Source	Type of sampling	No. of samples	Concentration		
				(µg/km)	(µg/g)	(ng/bhp-h)
Nishioka *et al.* (1982)	Light-duty diesel passenger car	PM	4	–	ND-0.4	–
Schuetzle & Perez (1983)	Heavy-duty vehicle	PM				
	Idle		1	–	84	–
	High speed; zero load (2100 rpm)		1	–	62	–
	High speed; full load (2100 rpm)		1	–	1.9	–
Schuetzle (1983)	Light-duty diesel passenger car	PM	3	–	71, 78, 186[a]	–
Schuetzle & Frazier (1986)	Light-duty diesel (1980–1985 models)	GP	7	56	–	–
		PM	7	61	–	–
Draper (1986)	Heavy-duty diesel					
	100% load (1200 rpm)	PM	1	–	0.63	–
	75% load (1800 rpm)	PM	1	–	8.8	–
	Diesel exhaust particles (SRM 1650)	PM	3	–	15.1	–
Tokiwa *et al.* (1990)	City gas (methane)	PM	1	–	0.021	–
	Heavy oil	PM	1	–	0.013	–
Khalek *et al.* (2011)	Heavy-duty engines	PM				
	12 repeats of 16-h cycles over FTP transient cycle					
	2007 technology		4	–	–	3.6 ± 4.1
	2000-technology		1	–	–	65

[a] Concentrations are accurate to within ± 30–40%

bhp, break horse power; FTP, Federal Test Procedure; GP, gas phase; h, hour; ND, not detected; PM, particulate matter; rpm, revolutions per minute; SRM, Standard Reference Material

found in similar samples collected from rural locations (Schauer *et al.*, 2004; Albinet *et al.*, 2006, 2007).

1.4.3 Other sources

2-Nitrofluorene has been detected in particulate extracts of emissions from kerosene heaters, gas burners and liquefied petroleum gas burners, but no attempt was made to quantify the contents (Tokiwa *et al.*, 1985). In another study, Tokiwa *et al.* (1990) reported low values of 2-nitrofluorene in city gas and in heavy oil (see Table 1.1).

1.4.4 Exposure of the general population and in occupational settings

Zwirner-Baier & Neumann (1999) analysed blood samples for the presence of haemoglobin adducts of 2-aminofluorene, a metabolite of 2-nitrofluorene. The study comprised groups of garage workers, inhabitants of Southampton and inhabitants of small villages in the region (for details, see Scheepers *et al.*, 1999). The proportion of blood samples that contained haemoglobin adducts of 6-nitrofluorene in the three groups was 22/29, 14/20 and 10/14, respectively. The levels of adducts were all below than 0.17 pmol/g haemoglobin. The method of analysis was based on gas chromatography-negative ion chemical ionization-mass spectrometry (see Section 1.2 of

Table 1.2 Mean air concentrations of 2-nitrofluorene associated with particulate matter

Reference	Source	Type of sampling	No. of samples	Mean ± standard deviation (range) (in pg/m³)
Tanabe *et al.* (1986)	Urban (Tokyo, Japan)	PM	8	1.8; 27.2[a]
Tokiwa *et al.* (1990)	Urban (Sapporo, Japan)	PM	1	210
Schauer *et al.* (2004)	Urban (Munich, Germany)	GP	10	3.6 ± 3.0
	Rural (Munich, Germany)	GP	5	< 0.01
	Rural (alpine site)	GP	9	< 0.02
Albinet *et al.* (2006)	Rural (Maurienne Valley, France)	PM GP	13 13	0.2 (0.0–2.0) ND
Albinet *et al.* (2007)	Urban (Marseille, France)	GP + PM	12	21.4 (0.1–92.9)
	Suburban (La Penne sur Huveaune, France)	GP + PM	14	5.1 (ND–24.4)
	Rural (Plan d'Aups, France)	GP + PM	14	1.4 (ND–8.4)

[a] Not detected in six samples
GP, gas phase; ND, not detected; PM, particulate matter

the *Monograph* on Diesel and Gasoline Engine Exhausts in this Volume).

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

See Table 3.1

3.1 Mouse

Initiation/promotion studies

Groups of 20 or 25 female SENCAR mice [age unspecified] received a single topical application of 0 or 50–1500 µg of 2-nitrofluorene or 5–10 µg of 7,12-dimethylbenz[*a*]anthracene (positive control) in acetone followed by promotion with topical applications of 2 or 5 µg of 12-*O*-tetradecanoylphorbol-13-acetate twice a week for 13 or 19 weeks. Administration of 2-nitrofluorene did not result in the formation of skin papillomas, even at the highest dose, whereas the positive control induced the formation of many papillomas (Möller *et al.*, 1993a).

3.2 Rat

3.2.1 Oral administration

Six male and three female Minnesota rats [age unspecified] were fed a diet containing 2.37 mmol [500 mg]/kg of 2-nitrofluorene [purity unspecified] for 23 weeks, and were then placed on a basal diet until they developed tumours or became moribund. Three males and three females fed a basal diet served as controls. At necropsy (300 days), animals were analysed grossly and histologically for tumours. Two females (67%) in the treated group had one adenocarcinoma of the mammary gland and one squamous cell carcinoma in the ear duct. No tumours were observed in the controls (Morris *et al.*, 1950). [The Working Group noted that the small number of animals hampered an evaluation of the study.]

Nine male and nine female Holtzman rats [age unspecified] were fed a diet containing 1.62 mmol [342 mg]/kg of 2-nitrofluorene [purity unspecified] for 8 months, after which they were placed on a basal diet for 2 additional months and

Table 3.1 Studies of the carcinogenicity of 2-nitrofluorene in mice and rats

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, SENCAR (F) 13 or 19 wks Möller *et al.* (1993a)	Topical application Study 1: 0 (control), 10 µg DMBA (positive control), 50 or 150 µg 2-NF in acetone followed by TPA for 13 wks (2 µg) and an additional 7 wks (5 µg) Study 2: 0 (control), 5 µg DMBA (positive control), 500 or 1500 µg 2-NF in acetone followed by TPA for 19 wks 20 or 25 mice/group	The positive control had the expected incidence of skin tumours. No skin papilloma was observed in any 2-NF -treated group.	NS	The absence of initiation may have been due to a lack of metabolism to a putative metabolite.
Rat, Minnesota albino (F) 310 d Morris *et al.* (1950)	Oral administration (diet) 0 (control) or 500 mg/kg of diet (total estimated dose, 756 mg) for 23 wks then normal diet until moribund death or tumour development Control: 3 M, 3 F; 2-NF: 6 M, 3 F	Mammary gland (adenocarcinoma): F–0/3, 2/3 (67%) Ear duct (squamous cell carcinoma): M–0/3, 1/6 (17%)	NS	The number of animals was too small to evaluate the study effectively.
Rat, Holtzman (M, F) 10 mo Miller *et al.* (1955)	Oral administration 0 (control) or 1.62 mmol/kg of diet for 8 mo then normal diet for 2 mo Control: 18 M, 18 F; 2-NF, 9 M, 9 F	Mammary gland (all tumours): F–1/18 (6%), 4/9 (44%)* Liver (all tumours): M–0/18, 1/9 (11%) Small intestine (all tumours): M–0/18, 1/9 (11%)	*$P < 0.05$	
Rat, Holtzman (M) 12 mo Miller *et al.* (1955)	Oral administration 0 (control) or 1.62 mmol/kg of diet for 12 mo Control, 10; treated, 20	Forestomach (squamous cell carcinoma): 0/10, 17/18 (94%)* Liver (all tumours): 0/10, 13/18 (72%)* Ear duct (all tumours): 0/10, 4/18 (22%) Small intestine (epithelial): 0/10, 2/18 (11%) Mammary gland (all tumours): 0/10, 1/18 (11%)	*$P < 0.05$	Some tumour types not specified

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Wistar (M) 24 mo Cui *et al.* (1995)	Oral administration 0, 0.24, 0.95 or 2.37 mmol/kg of diet for 11 mo; surviving animals held for an additional 13 mo before necropsy, histological analysis and DNA adduct determination 18–20/group	Liver (hepatocellular carcinoma): 0/20, 2/18 (11%), 15/19 (79%)*, 20/20 (100%)** Forestomach (squamous cell carcinoma): 0/20, 10/18 (55%), 16/19 (84%), 10/20 (50%) Cortical kidney (renal cell carcinoma): 0/20, 1/18 (5%), 15/19 (79%)*, 11/20 (55%)	*$P < 0.01$ **$P < 0.001$	Purity, > 98%; high- and mid-dose animals died before end of experiment (high-dose animals within 10–13 months); body weight was reduced in the high-dose group. DNA adducts correlated with tumour locations.
Rat, Minnesota Albino (M, F) 310 d Morris *et al.* (1950)	Topical application 0 (control) or 3 drops in acetone, 3 ×/wk for 6 mo then 6 drops, 3 ×/wk (total dose, 69 mg) Control: 3 M, 3 F; treated: 7 M, 7 F	Mammary gland (carcinoma): M–0/3, 1/7 (14%) Adrenal gland (carcinoma): M–0/3, 3/7 (43%) Lung (lymphosarcoma): 0/3, 2/7 (28%) Skin (subcutaneous fibroma): M–0/3, 1/7 (14%) Salivary gland (anaplastic carcinoma): M–0/3, 1/7 (14%)	NS	The number of animals was too small to evaluate the study effectively.

d, day; DMBA, 7,12-dimethylbenz[*a*]anthracene; F, female; M, male; mo, month; 2-NF, 2-nitrofluorene; NS, not significant; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; wk, week

evaluated grossly and histologically for tumours. A group of 18 males and 18 females were fed the basal diet alone and served as controls. Four females in the treated group developed mammary gland tumours (44%) and one fibroadenoma was observed in a control female (1 out of 18). Most 2-nitrofluorene-treated rats developed multiple papillomas or squamous cell carcinomas in the forestomach (5 out of 7 males and 2 out of 2 females examined) (Miller *et al.*, 1955).

To confirm these findings, groups of 10 and 20 male Holtzman rats [age unspecified] were fed a basal diet containing 0 and 1.62 mmol/kg of 2-nitrofluorene [purity unspecified] for 12 months. In the treated group, 17 out of 18 (94%; $P < 0.05$) survivors had squamous cell carcinomas of the forestomach, 13 out of 18 (72%) had liver tumours ($P < 0.05$) [type not specified], 4 out of 18 (22%) had tumours of the ear duct, 2 out of 18 (11%) had tumours in the epithelium of the small intestine and 1 out of 18 (5%) had a tumour of the mammary gland. No tumours were observed in the control group (Miller *et al.*, 1955).

Groups of 18–20 male Wistar rats [age unspecified] were fed 0, 0.24, 0.95 or 2.37 mmol/kg of 2-nitrofluorene (purity, > 98%) in the diet for 11 months, and were then placed on a basal diet for an additional 13 months before gross or histological evaluation. The incidence of tumours was: hepatocellular carcinoma – 2 out of 18 (11%) low-dose, 15 out of 19 (79%; $P < 0.01$) mid-dose and 20 out of 20 (100%; $P < 0.01$) high-dose rats; forestomach squamous cell carcinoma – 10 out of 18 (5%) low-dose, 16 out of 19 (84%) mid-dose and 11 out of 20 (55%) high-dose rats; and cortical kidney [renal cell] carcinoma – 1 out of 18 (5%) low-dose, 15 out of 19 (79%; $P < 0.01$) mid-dose and 10 out of 20 (50%; $P < 0.05$) high-dose rats. No tumours were observed in the control animals (Cui *et al.*, 1995). [The Working Group noted that the high-dose animals died within 10–13 months.]

3.2.2 Skin application

Seven male and three female Minnesota rats [age unspecified] received a single topical application of 69 mg of 2-nitrofluorene [purity unspecified] and were then maintained on a basal diet and analysed grossly and histologically for tumours at approximately 300 days (approximate average life-span of the group). An untreated group of three males and three females served as controls. No tumours were observed in females or in the controls. In the males, 1 out of 7 (14%) had mammary gland carcinoma, 3 out of 7 (43%) had adrenal gland carcinoma, 2 out of 7 (28%) had lung lymphosarcoma, 1 out of 7 (14%) had subcutaneous fibroma and 1 out of 7 (14%) had anaplastic carcinoma of the salivary glands. The incidence of these tumours was not statistically significant compared with controls (Morris *et al.*, 1950). [The Working Group noted that the small number of animals hampered an evaluation of the study.]

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, excretion

The metabolism of 2-nitrofluorene *in vivo* has been reviewed extensively (Möller, 1988, 1994).

4.1.1 Humans

No data were available to the Working Group.

4.1.2 Experimental systems

(a) In-vivo studies

In Sprague-Dawley rats treated with a single oral dose of 1 mg/kg body weight (bw) of 2-nitro[9-^{14}C]fluorene, 2-nitrofluorene was excreted rapidly in the urine and faeces; 60%

was excreted in the urine and 30% in the faeces (Möller *et al.*, 1985, 1987a). The major free products (unconjugated) were 5-hydroxy (OH)- and 7-OH-2-acetylaminofluorene (5- and 7-OH-2-AAF), but also *N*-, 1, 3-, 8- and 9-OH-2-AAF were identified as metabolites (Möller *et al.*, 1985, 1987a). No difference in the excretion profiles of 5- and 7-OH-2-AAF was observed in rats pretreated intraperitoneally with β-naphthoflavone, an inducer of cytochrome P450 (CYP) 1A1/2; however, the relative proportion of excreted hydroxylated 2-nitrofluorenes increased substantially after treatment with β-naphthoflavone (Möller *et al.*, 1987a).

Similar experiments were performed in conventional and germ-free AGUS rats (single oral dose of 5 mg of [^{14}C]2-nitrofluorene per rat) (Möller *et al.*, 1988). The metabolism of 2-nitrofluorene was similar in AGUS and Sprague-Dawley rats (Möller *et al.*, 1987a, 1988). However, the excretion profiles of metabolites in germ-free animals differed considerably from those in conventional animals. Whereas 7- and 5-OH-2-AAF were the major metabolites of 2-nitrofluorene in conventional animals (3- and 1-OH-2-AAF were excreted as minor metabolites), five different hydroxylated 2-nitrofluorenes were observed in the excreta from germ-free animals (Möller *et al.*, 1988). Hydroxylated 2-nitrofluorenes were also observed in another study of germ-free Wistar rats that received 1 mmol/kg bw of 2-nitrofluorene, whereas very little OH-2-AAF was detected in the urine (Scheepers *et al.*, 1994a).

The metabolism of 2-nitrofluorene was studied in isolated perfused rat lung and liver (Möller *et al.*, 1987b). The compound was metabolized in the lung to hydroxylated 2-nitrofluorenes, mainly 9-hydroxy-2-nitrofluorene. After intratracheal administration, 2-nitrofluorene was rapidly excreted into the perfusate, indicating that other organs might be exposed to the unmetabolized compound. In the liver, hydroxylated 2-nitrofluorenes were further conjugated to glucuronides and were excreted in the bile. Mutagenicity was only observed in the bile after treatment with β-glucuronidase, suggesting that biliary 2-nitrofluorene glucuronides could be hydrolysed by the β-glucuronidase expressed in the colon (Möller *et al.*, 1987b). Törnquist *et al.* (1990) compared the absorption of 2-nitrofluorene in isolated, perfused and ventilated lungs of treated rats with that in control animals and animals pretreated with β-naphthoflavone. The clearance of intratracheally instilled 2-nitrofluorene increased approximately fourfold after treatment with β-naphthoflavone.

The pharmacokinetics of 2-nitrofluorene was studied in Wistar rats with cannulated bile ducts after intravenous administration of a single oral dose of 6 μmol/kg bw of ^{3}H-labelled 2-nitrofluorene (Mulder *et al.*, 1990). 2-Nitrofluorene was removed from the blood in a biphasic manner: the initial removal was very rapid, with a half-time of 2.5 minutes, which was followed by a much slower phase (half-time > 2.5 hours). Excretion *via* the bile was also biphasic, with half-time values of 9 minutes and 1 hour, respectively. After 2 hours, approximately 40% of the 2-nitrofluorene dose had been excreted in the bile. In contrast, the excretion of 2-nitrofluorene and its metabolites in urine was low (20% within 24 hours), possibly due to reabsorption of the parent compound or its metabolites from the gut and subsequent enterohepatic recirculation (Mulder *et al.*, 1990). The glucuronide conjugate of 9-hydroxy-2-nirofluorene was identified as the major metabolite.

The time-dependent metabolism of 2-nitrofluorene was studied after a single intraperitoneal injection of 1 mmol/kg bw of 2-nitrofluorene in Sprague-Dawley rats. In contrast to previous studies of the metabolism of 2-nitrofluorene following its oral administration, OH-AAFs (*N*-, 1-, 3-, 5-, 7- or 9-OH-2-AAF), 2-AAF and 2-aminofluorene were not detected in the urine by high-performance liquid chromatography or nuclear magnetic resonance analysis. After hydrolysis with β-glucuronidase/arylsulfatase,

five hydroxylated 2-nitrofluorenes were isolated and structurally identified as *trans*-6,9-dihydroxy-2-nitrofluorene, and 6- , 7-, 8- and 9-hydroxy-2-nitrofluorene. Furthermore, two conjugated metabolites were identified as 6- and 7-[(hydroxysulfonyl)oxy]-2-nitrofluorene (Castañeda-Acosta *et al.*, 1997).

The acylated derivative, 2-formylaminofluorene (2-FAF), was identified in rats and albino rabbits treated with 2-nitrofluorene (Tatsumi & Amano, 1987). 2-FAF was formed by the *N*-formylation of 2-aminofluorene catalysed by liver formamidase in the presence of *N*-formyl-L-kynurenine. The metabolism of 2-nitrofluorene, 2-aminofluorene, 2-FAF and 2-AAF was compared in rats and dogs (Ueda *et al.*, 2001a). 2-AAF and its hydroxylated derivatives, 5- and 7-OH-2-AAF, were the major metabolites identified in the urine and faeces of rats, but FAF and its hydroxylated derivatives, 5- and 7-hydroxy-2-FAF, were mainly excreted in dogs.

The metabolism of 2-nitrofluorene *in vivo* was also studied in goldfish after exposure to 5 mg/L for 2 days (Ueda *et al.*, 2001b); the metabolites identified were 7-OH-2-AAF, 7-hydroxy-2-aminofluorene, 2-FAF, 2-AAF and 2-aminofluorene.

(b) In-vitro studies

The metabolism of 2-nitrofluorene in human lung tissue was studied after its incubation with surgical resectate containing adenocarcinoma and normal surrounding tissue (Gøtze *et al.*, 1994). 9-Hydroxy-2-nitrofluorene was detected as the main metabolite.

After the incubation of 2-nitrofluorene with rat pulmonary microsomes, 9-hydroxy-2-nitrofluorene was the predominant metabolite formed (Törnquist *et al.*, 1990). In microsomes isolated from rats pretreated with β-naphthoflavone, the formation of 9- and X-hydroxy-2-nitrofluorene increased significantly. In lung microsomes, the formation of X-hydroxy-2-nitrofluorene was inhibited *in vitro* by the addition of α-naphthoflavone, an inhibitor of CYP1A1/2. Anti-P4502B1-immunoglobulin G inhibited the formation of 9-hydroxy-2-nitrofluorene, whereas that of X-hydroxy-2-nitrofluorene was unaffected (Törnquist *et al.*, 1990), indicating that CYP2B1 catalyses the hydroxylation of 2-nitrofluorene at the 9-position.

The metabolism of 2-nitrofluorene was studied *in vitro* in isolated rat lung cells (Gøtze *et al.*, 1994). The main metabolites in rat lung Clara cells and rat alveolar type II cells were 9- and X-hydroxy-2-nitrofluorene. The formation of 9-hydroxy-2-nitrofluorene was efficiently catalysed by CYP2B1 in Clara cells and increased with the age-dependent up-regulation of CYP2B1 in the lung (Törnquist *et al.*, 1988). Alveolar type II cells showed a preferentially formed X-hydroxy-2-nitrofluorene and metabolism increased after pretreatment of the rats with β-naphthoflavone.

The reduction of 2-nitrofluorene and 9-hydroxy-2-nitrofluorene in the rat mammary gland was investigated *in vitro* (Ritter *et al.*, 2000). Cytosolic fractions catalysed the nicotinamide adenine dinucleotide- and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent formation of 2-aminofluorene and 9-hydroxy-2-aminofluorene, respectively. Nitroreduction was primarily catalysed by a xanthine oxidase and partially by a diaphorase (Ritter *et al.*, 2000). Similar results were obtained in rat skin, in which the reduction of 2-nitrofluorene was mainly catalysed by xanthine oxidase (Ueda *et al.*, 2003). Skin cytosols from various mammals including rabbits, hamsters, guinea-pigs, mice and rats all exhibited significant nitroreductase activity towards 2-nitrofluorene (Ueda *et al.*, 2005). The species differences reflected differences of relative aldehyde oxidase and xanthine oxidase activities.

In liver preparations of sea bream (*Pagrus major*), 2-nitrofluorene was effectively reduced to 2-aminofluorene by CYP enzymes or aldehyde oxidase (Ueda *et al.*, 2002). 2-Aminofluorene,

2-AAF and 2-FAF were oxidized to their 7- or 5-hydroxy derivatives by CYPs.

4.2 Genetic and related effects

The genetic and related effects of 2-NF and some of its metabolites have been reviewed (Beije & Möller, 1988a; Purohit & Basu, 2000;. IPCS, 2003).

4.2.1 Humans

No data were available to the Working Group.

4.2.2 Experimental systems

(a) Formation of DNA and protein adducts

(i) In-vivo studies

See Fig 4.1

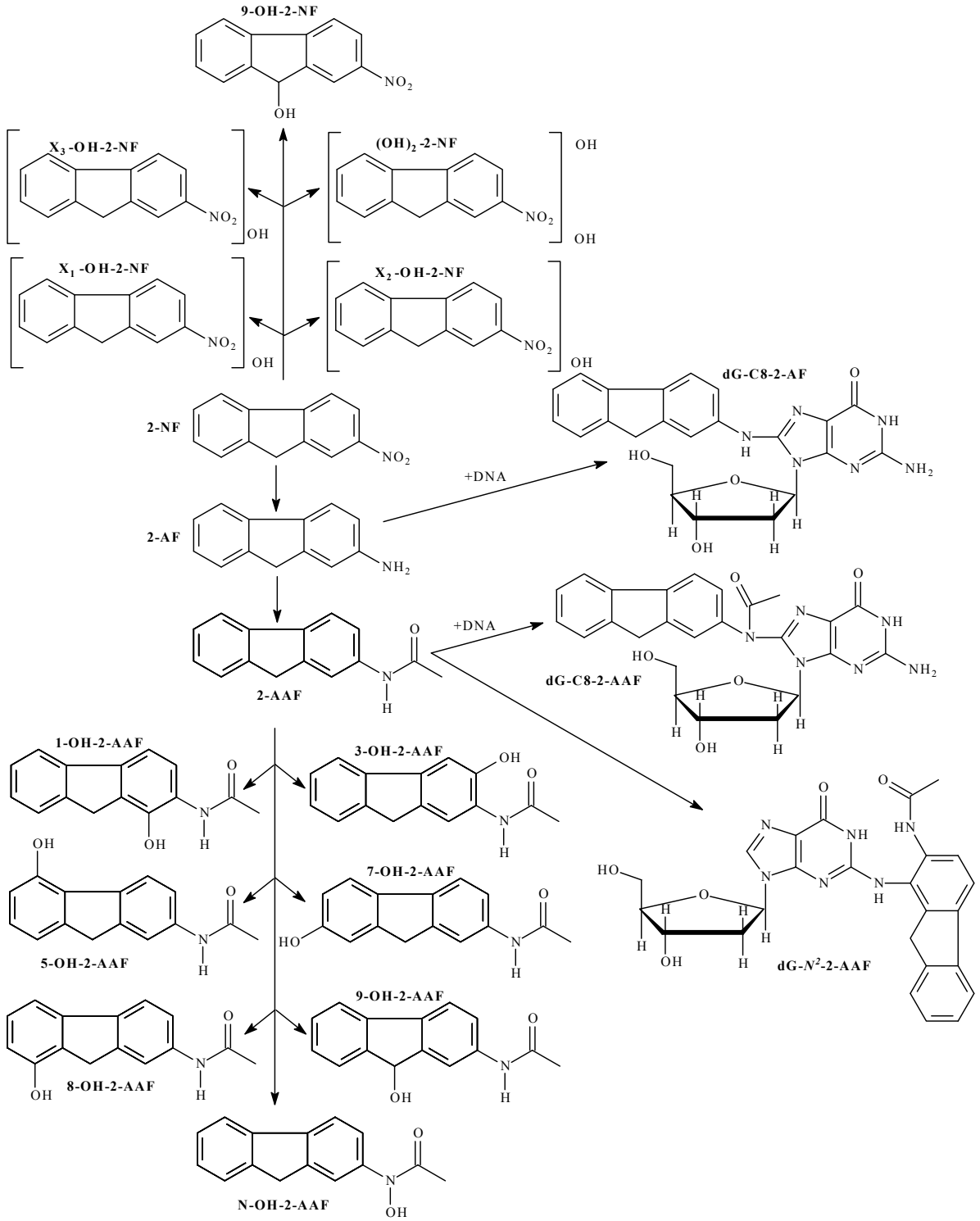
[^{3}H]-Labelled 2-nitrofluorene (a single oral dose of 6 µmol/kg bw) was used to demonstrate DNA binding in the liver, kidney, forestomach and gut mucosa of Wistar rats (Wierckx *et al.*, 1990). The formation of DNA adducts by 2-nitrofluorene (measured using the ^{32}P-postlabelling method) was predominantly studied in rats (Möller *et al.*, 1993b; Möller & Zeisig, 1993; Cui *et al.*, 1995, 1999). Four major DNA adducts were detectable (adducts A–D). DNA adduct D was chromatographically indistinguishable from *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-2-AF) and DNA adduct C co-migrated with C3-(deoxyguanosin-N^2-yl)-2-acetylaminofluorene (dG-N^2-2-AAF), whereas adducts A and B could not be identified.

In Wistar rats treated with 2-nitrofluorene, the formation of DNA adducts was dose- and time-dependent, with higher levels in tumour target tissues (liver, kidney and forestomach) compared with non-tumour target organs (heart, spleen and glandular stomach) (Cui *et al.*, 1995, 1999). Animals were treated with 0.24–2.4 mmol/kg of 2-nitrofluorene in the diet and were killed after 1, 2, 6 and 10 days or 11 months after continuous feeding. The four 2-nitrofluorene-induced DNA adducts showed different kinetics of formation and persistence. A and B were the major DNA adducts detected after 11 months of feeding. All of the adducts persisted after the cessation of treatment, and adducts A and C (i.e., dG-N^2-2-AAF) were only repaired to a minor extent (Cui *et al.*, 1999). Another study in which Wistar rats were treated with 200 mg/kg bw of 2-nitrofluorene for 48 hours showed that oral administration was more potent in the formation of DNA adducts than intraperitoneal administration (Möller *et al.*, 1993b). Nuclear magnetic resonance spectroscopy and restrained molecular dynamics showed that the presence of dG-N^2-2-AAF in DNA increased the thermal and thermodynamic stability of duplex DNA (Zaliznyak *et al.*, 2006), which has important implications for the recognition of this adduct by the DNA-repair machinery and may explain its persistence in rat tissue DNA *in vivo*.

The role of the intestinal microflora on the formation of 2-nitrofluorene-induced DNA adducts was investigated in several studies. Conventional and germ-free AGUS rats were treated with a single oral dose of 35 mg/kg bw of 2-nitrofluorene for 48 hours, and DNA adduct formation was analysed in the liver, kidney, lung and heart by ^{32}P-postlabelling. The presence of intestinal microflora enhanced the formation of DNA adducts in all tissues examined, and dG-C8-2-AF was the major adduct detected (Möller *et al.*, 1994). Similar results were obtained in rats and mice with human microflora (Scheepers *et al.*, 1994a; Hirayama *et al.*, 2000).

Wistar rats received an intraperitoneal injection of 100 mg/kg bw of 2-nitrofluorene, or 5-, 7- or 9-hydroxy-2-nitrofluorene and were killed 72 hours later (Cui *et al.*, 1996). 2-Nitrofluorene and 9-hydroxy-2-nitrofluorene had similar potency for the formation of DNA adducts, while 5- and 7-hydroxy-2-nitrofluorene generated approximately fourfold less DNA adducts. The ^{32}P-thin-layer chromatography autoradiograms revealed

Fig. 4.1 Metabolism of and formation of DNA adducts by 2-nitrofluorene



2-AAF, 2-acetylaminofluorene; 2-AF, 2-aminofluorene; 2-NF, 2-nitrofluorene; 9-OH-2-NF, 9-hydroxy-2-NF; dG-C8-2-AAF, *N*-(deoxyguanosin-8-yl)-2-AAF; dG-C8-2-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; dG-N^2-2-AAF, C3-(deoxyguanosin-N^2-yl)-2-AAF; X-OH-2-AAF, X-hydroxy-2-AAF (X = 1, 3, 5, 7, 8, or 9); X-OH-2-NF, X-hydroxy-2-NF (X = 1,2,3)

that 2-nitrofluorene and 9-hydroxy-2-nitrofluorene showed three adduct spots with similar positions on the chromatogram. 7-Hydroxy-2-nitrofluoene showed four weak DNA adduct spots while 5-hydroxy-2-nitrofluorene did not induce a distinct adduct spot pattern.

^{3}H-Labelled 2-nitrofluorene (a single oral dose of 6 μmol/kg bw) was used to demonstrate protein binding *in vivo* in Wistar rats (Wierckx *et al.*, 1990). After oral administration of 1 mmol/kg bw of 2-nitrofluorene, the number of haemoglobin adducts in the blood was low compared with administration of 2-aminofluorene (5.1 ± 0.6 versus 0.04 ± 0.01 μmol/g haemoglobin) and no haemoglobin adducts were detected in the blood in the absence of intestinal microflora (Scheepers *et al.*, 1994a, b).

(ii) In-vitro studies

DNA binding by ^{3}H-labelled 2-nitrofluorene was catalysed by rat hepatic microsomes and was CYP-dependent (Wierckx *et al.*, 1990). 2-Nitrofluorene binding was also observed in rat hepatocytes and a higher degree of binding was found in those pretreated with Aroclor 1254, an inducer of various CYP isoenzymes, indicating that DNA-reactive intermediates of 2-nitrofluorene are formed by CYP enzymes (Wierckx *et al.*, 1990).

(b) Mutagenesis of 2-nitrofluorene

In-vitro studies

Urinary mutagenicity in 2-nitrofluorene-treated Sprague-Dawley rats was observed in *Salmonella typhimurium* strain TA98 (in the presence or absence of an exogenous metabolic activation system) and was associated with a range of OH-2-AAFs in addition to several hydroxy-2-nitrofluorenes (Möller *et al.*, 1987a, 1989). The mutagenicity (measured in TA98 in the presence or absence of an exogenous metabolic activation system), in particular the direct-acting mutagenicity, detected in excreta from 2-nitrofluorene-treated germ-free rats was considerably higher than that in the excreta from conventional 2-nitrofluorene-treated animals (Möller *et al.*, 1988). 9-Hydroxy-2-nitrofluorene was mainly liable for the total urinary mutagenicity, whereas 2-nitrofluorene was only responsible for a small part (~2%). Thus, the 2-nitrofluorene metabolite that mainly contributes to its direct-acting mutagenicity is formed in germ-free animals or by metabolic routes in which microflora are not involved (Möller *et al.*, 1987a, 1988).

When evaluated previously (IARC, 1989), more than 200 independent reports were already available on the mutagenicity of 2-nitrofluorene in *S. typhimurium*, because the compound was often used as positive reference compound. These studies gave generally positive results in the standard *S. typhimurium* tester strains TA97, TA98, TA100, TA1538 or TA1978, and these results have been confirmed in more recent studies (Jurado *et al.*, 1994; Nohmi *et al.*, 1995; Cui *et al.*, 1996; Hughes *et al.*, 1997; Suzuki *et al.*, 1997; Yamada *et al.*, 1997). When TA98 was compared with the corresponding nitroreductase-deficient strain, TA98NR, the mutagenicity of 2-nitrofluorene decreased, while stronger mutagenicity (~12-fold) was observed in TA98 with high nitroreductase activity (TA98NR+), compared with the standard tester strain (Hagiwara *et al.*, 1993). In strain TA98AT+, which overexpresses *O*-acetyltransferase, 2-nitrofluorene showed a ~20-fold increase in mutagenicity compared with TA98. Thus, nitroreduction followed by *O*-acetylation appears to be the mutagenic activation pathway of 2-nitrofluorene in *S. typhimurium* (Hagiwara *et al.*, 1993). The importance of nitroreduction followed by *O*-acetylation in 2-nitrofluorene-induced mutagenicity has also been observed in other studies (Watanabe *et al.*, 1989, 1990). It has also been suggested that part of the mutagenicity of 2-nitrofluorene in *Salmonella* is related to oxidative stress (i.e., formation of 8-hydroxydeoxyguanosine), because *Salmonella* strains that are deficient in 8-oxo-guanine DNA glycosylase were shown to

exhibit enhanced mutagenicity when incubated with 2-nitrofluorene (Suzuki *et al.*, 1997).

The bacterial mutagenicity of 2-nitrofluorene, and 5-, 7- and 9-hydroxy-2-nitrofluorene was compared in strain TA98 (in the absence of an exogenous metabolic activation system) (Cui *et al.*, 1996). 5- and 9-Hydroxy-2-nitrofluorene were less mutagenic (two- to four-fold) compared with 2-nitrofluorene (absence of an exogenous metabolic activation system), while 7-hydroxy-2-nitrofluorene was twice as mutagenic as 2-nitrofluorene. 2-Nitrofluorene, and 5- and 7-hydroxy-2-nitrofluorene were stronger mutagens in the absence of an exogenous metabolic activation system, while 9-hydroxy-2-nitrofluorene had a reverse pattern and displayed stronger mutagenicity in the presence of metabolic activation.

Additional studies also confirmed the mutagenicity of 2-nitrofluorene in *Escherichia coli* (Kranendonk *et al.*, 1996; Hoffmann *et al.*, 2001).

2-Nitrofluorene induced mutations in mouse lymphoma L5178Y $Tk^{+/-}$ cells (Amacher *et al.*, 1979; Oberly *et al.*, 1984, 1996). In contrast, no mutagenicity by 2-nitrofluorene was found at the thymidine kinase locus in human B-lymphoblastoid cells that constitutively express CYP1A1 (designated h1A1v2) (Durant *et al.*, 1996). 2-Nitrofluorene gave positive results in the hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) forward mutation assay in Chinese hamster ovary cells (Oberly *et al.*, 1990), but did not induce mutations at the *Hprt* locus in Chinese hamster V79 cells with (V79NH) or without (V79-MZ) endogenous acetyltransferase activity that stably express human *CYP1A2* (Kappers *et al.*, 2000).

Chromosomal aberrations were observed in Chinese hamster lung cells treated with 2-nitrofluorene, which was more clastogenic in the presence than in the absence of an exogenous metabolic activation system (Matsuoka *et al.*, 1991). 2-Nitrofluorene induced micronuclei in Chinese hamster V79 cells, rat epithelial intestinal IEC-17 and IEC-18 cells, mouse BALB/c 3T3 cells and embryonic human liver HuFoe-15 cells (Glatt *et al.*, 1990; Gu *et al.*, 1992). 2-Nitrofluorene showed higher clastogenicity in Chinese hamster lung cells that express bacterial *O*-acetyltransferase or human *N,O*-acetyltransferase (1 or 2) (Watanabe *et al.*, 1994).

(c) Other genetic effects of 2-nitrofluorene

(i) In-vivo studies

Studies reviewed in the previous *Monograph* (IARC, 1989) are summarized below. Oral administration of 2-nitrofluorene (125–500 mg/kg bw) to Chinese hamsters induced sister chromatid exchange in bone-marrow cells, while intraperitoneal injection of 50–200 mg/kg bw had no effect. 2-Nitrofluorene did not induce micronuclei in the bone marrow of treated mice, or unscheduled DNA synthesis in the liver of rats treated orally with up to 50 mg/kg bw. In the mouse host-mediated recombination assay, 2-nitrofluorene (at 125–1600 mg/kg bw) induced mutation in *S. typhimurium*, but not recombination in *Saccharomyces cerevisiae* D3. Treatment of *E. coli* with 2-nitrofluorene induced binding of cellular DNA to the bacterial envelope, while conflicting results were reported regarding its ability to induce prophages in *E. coli*. 2-Nitrofluorene preferentially inhibited the growth of DNA repair-deficient *E. coli* and *Bacillus subtilis* (rec assay) (IARC, 1989).

In more recent studies, oral treatment of Fischer 344 rats with 2-nitrofluorene (125 mg/kg bw) caused significant micronucleus formation in the liver, but not in the bone marrow (Parton & Garriott, 1997), while micronuclei were not detected in the peripheral reticulocytes isolated from ddY mice treated intraperitoneally (80 mg/kg bw) (Murakami *et al.*, 1996).

The expression profile induced by 2-nitrofluorene in the liver was examined using Affimetrix GeneChip technology after the treatment of Wistar rats with 44 mg/kg per day

for 1, 3, 7 and 14 days, a dose known to induce liver tumours in a 2-year rat bioassay (Ellinger-Ziegelbauer *et al.*, 2005). Cellular pathways affected were related to oxidative stress/DNA-damage response, and the activation of proliferative and survival signalling. Interestingly, 2-nitrofluorene deregulated genes that comprised cellular responses to various forms of oxidative stress, including the target genes of the reactive oxygen species-response transcription factor Nrf-2, NADPH:quinone oxidoreductase, haeme oxygenase 1, glutathione-synthase (light chain)) and other genes known to be induced under oxidative stress conditions (e.g., epoxide hydrolase 1) (Ellinger-Ziegelbauer *et al.*, 2005), indicating that oxidative stress might be a tumour promoter in 2-nitrofluorene-induced carcinogenesis.

(ii) In-vitro studies

2-Nitrofluorene induced DNA damage in *S. typhimurium* (SOS *umu* test) (Oda *et al.*, 1992, 1993, 1996) and *E. coli* (SOS chromotest) (IARC, 1989; Mersch-Sundermann *et al.*, 1991, 1992), and inhibited DNA synthesis in HeLa cells. Conflicting results were obtained in the unscheduled DNA synthesis assay: one study reported that 2-nitrofluorene gave positive results in mouse and rat hepatocytes, whereas another showed a negative response in rat hepatocytes. 2-Nitrofluorene induced sister chromatid exchange in cultured Chinese hamster ovary and V79 cells in the presence of an exogenous metabolic activation system. It induced morphological transformation in Syrian hamster embryo cells co-cultured with hamster hepatocytes (IARC, 1989).

In human lymphoblastoid MCL-5 cells that express high levels of native *CYP1A1* and that carry *CYP1A2*, *CYP2A6*, *CYP3A4* and *CYP2E1* as cDNA in plasmids, 2-nitrofluorene showed a positive response in the alkaline single-cell gel electrophoresis (comet) assay, in the presence or absence of the DNA-repair inhibitors hydroxyurea or cytosine arabinoside (Martin *et al.*, 1999). In contrast, it did not cause any marked DNA damage (measured by the alkaline elution technique) in isolated rabbit lung cells (Becher *et al.*, 1993).

4.3 Other relevant data

2-Nitrofluorene showed estrogenic activity (measured in an estrogen reporter assay using estrogen-responsive yeast and human breast cancer MCF-7 cells) after incubation with liver microsomes from of 3-methylcholanthrene-treated rats in the presence of NADPH, whereas no estrogenic activity was observed in liver microsomes from untreated or phenobarbital-treated rats (Fujimoto *et al.*, 2003). 7-Hydroxy-2-nitrofluorene was the main metabolite identified in liver microsomes isolated from 3-methylcholanthrene-treated rats, whereas little of the metabolite was formed in those of untreated or phenobarbital-treated rats. Rat recombinant *CYP1A1* catalysed the formation of 7-hydroxy-2-nitrofluorene which appears to be responsible for the estrogenic activity of 2-nitrofluorene.

4.4 Mechanistic considerations

2-Nitrofluorene can enter an oxidative or a reductive pathway (Möller *et al.*, 1987a, b, 1988). 2-Aminofluorene and 2-AAF, which are both carcinogenic in rodents (Heflich & Neft, 1994), are the main reductive metabolites that are formed after oral administration of 2-nitrofluorene and are mediated by the intestinal microflora; 2-nitrofluorene is mainly excreted as OH-2-AAFs in urine and faeces (Möller *et al.*, 1988). A minor metabolic pathway results in the formation of hydroxylated nitrofluorenes after oral administration of 2-nitrofluorene to conventional rats; hydroxylated nitrofluorenes are major metabolites in germ-free rats after oral administration and in perfusates from isolated

rat lung and liver (Möller *et al.*, 1987b, 1988). While OH-2-AAFs show low mutagenic activity, hydroxylated nitrofluorenes, although not detected individually, appear to be the mutagenic metabolites of 2-nitrofluorene (Beije & Möller, 1988b; Möller *et al.*, 1988).

2-Nitrofluorene induces the formation of DNA adducts *in vivo*, one of which has been characterized as dG-C8-2-AF (Wierckx *et al.*, 1990; Möller *et al.*, 1993b; Möller & Zeisig, 1993). Together with other known 2-aminofluorene- and 2-AAF-related DNA adducts (i.e., dG-C8-2-AF and dG-N^2-2-AAF), these lesions are suspected to be pre-mutagenic (Heflich & Neft, 1994). The formation of these DNA adducts suggests that nitroreduction, the first step of which is its conversion to 2-aminofluorene, is a major route for the metabolic activation of 2-nitrofluorene, and appears to be catalysed mainly by the cytosolic nitroreductase xanthine oxidase and partially by a diaphorase (Ritter *et al.*, 2000). After oral administration of 2-nitrofluorene to rats, dG-C8-AF was the major adduct detected in liver while dG-C8-AAF was found as a minor adduct (Möller & Zeisig, 1993).

2-Nitrofluorene is both an initiator and a promoter of preneoplastic liver lesions in rats (Möller *et al.*, 1989), and resulted in tumour formation in different organs when administered to rats orally or by topical application (Miller *et al.*, 1955; Cui *et al.*, 1995). In addition to some common target organs, such as the liver and kidney, the forestomach appears to be a prime target organ for 2-nitrofluorene when compared with 2-aminofluorene and 2-AAF (Miller *et al.*, 1955). This difference could possibly be related to genotoxic hydroxylated derivatives of 2-nitrofluorene, such as 7- and 9-hydroxy-2-nitrofluorene, which are not formed after exposure to 2-aminofluorene or 2-AAF (Cui *et al.*, 1995). Tumour development and DNA adduct formation were dose- and time-dependent, and the levels of DNA adducts within the first days during a 2-year bioassay correlated with the localization of tumours, indicating that the formation of DNA adducts is an important factor for tumour development (Cui *et al.*, 1995).

5. Summary of Data Reported

5.1 Exposure data

Diesel exhaust was identified as the primary source of exposure for 2-nitrofluorene. No evidence was found that it has been produced in commercial quantities or used for purposes other than laboratory applications. 2-Nitrofluorene was detected in both the gas phase and particulate phase of air samples collected at urban locations in the low picagram per cubic metre range, but was not detected in air sampled at rural and remote locations. Analyses of the blood and urine indicated that workers in some occupations and the general population are exposed.

5.2 Human carcinogenicity data

No data were available to the Working Group.

5.3 Animal carcinogenicity data

The carcinogenesis of 2-nitrofluorene was evaluated in one initiation–promotion study by topical application in mice, and after oral administration in the diet in three studies and topical application in one study in rats. It caused squamous cell carcinomas of the forestomach and hepatocellular carcinoma in two separate feeding studies and cortical kidney (renal cell) carcinoma in one study in rats. Two studies of dietary exposure and topical application in rats were not included in the evaluation due to the small numbers of animals tested and insufficient methodological details. The results of the initiation–promotion study were negative.

5.4 Mechanistic and other relevant data

No data on the absorption, distribution, metabolism and excretion or genetic and related effects of 2-nitrofluorene in humans were available to the Working Group. When given to rats, the compound was rapidly excreted as a mixture of hydroxylated metabolites of aminofluorene and acetylaminofluorene produced by nitroreduction and *O*-acetylation. These metabolites were mutagenic in bacteria. 2-Nitrofluorene induced mutations in mouse lymphoma cells and had cytogenetic effects in experimental animals. The nitroreduction of 2-nitrofluorene, which is catalysed by cytosolic nitroreductases, is the major activation pathway that leads to the formation of DNA-binding metabolites and DNA adducts. The major adduct detected in the liver, i.e., the target organ for carcinogenesis, after oral administration was deoxyguanosin-8-yl-2-aminofluorene, although 2-acetylaminofluorene-related DNA adducts may also contribute to the mutagenic potency of 2-nitrofluorene. When administered to experimental animals orally or by topical application, 2-nitrofluorene caused tumour formation in different organs, including the liver, kidney and forestomach, in a time- and dose-dependent manner, which was linked with the increased formation of DNA adducts. The initial levels of DNA adducts correlated well with the location of tumours.

Collectively, these data provide *strong evidence* in animals and *weak evidence* in humans that the formation of DNA adducts plays an important role in the tumour development induced by 2-nitrofluorene.

6. Evaluation

6.1 Cancer in humans

There is *inadequate evidence* in humans for the carcinogenicity of 2-nitrofluorene.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of 2-nitrofluorene.

6.3 Overall evaluation

2-Nitrofluorene is *possibly carcinogenic to humans (Group 2B).*

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