ortho-TOLUIDINE

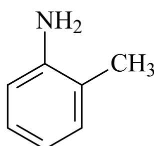
1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Nomenclature*

Chem. Abstr. Serv. Reg. No.: 95–53–4
CAS Name: 2-Methylbenzenamine
Synonyms: 1-Amino-2-methylbenzene; 2-amino-1-methylbenzene; 2-aminotoluene; *ortho*-aminotoluene; 2-methyl-1-aminobenzene; 2-methylaniline; *ortho*-methylaniline; *ortho*-methylbenzenamine; 2-methylphenylamine; 2-tolylamine; *ortho*-tolylamine

1.1.2 *Structural formula, molecular formula, and relative molecular mass*



C_7H_9N Rel. mol. mass: 107.15

1.1.3 *Chemical and physical properties of the pure substance*

Description: Light yellow liquid becoming reddish brown on exposure to air and light (O'Neil, 2006)
Boiling-point: 200–201 °C (O'Neil, 2006)
Melting-point: −14.41 °C (Lide, 2008)
Solubility: Slightly soluble in water; soluble in diethyl ether, ethanol and dilute acids (O'Neil, 2006)
Octanol/water partition coefficient: log P, 1.29–1.32 (Verschueren, 2001)

1.1.4 *Technical products and impurities*

No information was available to the Working Group.

1.1.5 *Analysis*

ortho-Toluidine is the most widely studied of the aromatic amines reviewed in this Volume. Waste-water, air and urine samples have been analysed. The use of LC/MS and LC with electrochemical detection provides detection limits down to the nanomol level. *ortho*-Toluidine is one of the amines detected as a reduction product of azo dyes used as toy colourants. Table 1.1 presents selected methods of detection and quantification of *ortho*-toluidine in various matrices.

Table 1.1. Selected methods of analysis of *ortho*-toluidine in various matrices

Sample matrix	Sample preparation	Assay method	Detection limit	Reference
Toys	Sodium dithionite reductive cleavage of azo dye and analysis of resultant amines	HPLC/UV	0.2 µg/g	Garrigós *et al.* (2002)
Textiles	Extract fabric with citrate buffer; decolorize extract with hydrosulfite; extract with *tert*-butylmethyl ether; concentrate and dilute with methanol	LC-MS/MS	37.9 µg/mL	Sutthivaiyakit *et al.* (2005)
Water	Dissolve in methanol (0.001 mol/L); dilute; add to deionized water	HPLC/ECD	1.94 nmol/L	Mazzo *et al.* (2006)

ECD, electrochemical detection; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; UV, ultraviolet

1.2 Production and use

1.2.1 *Production*

The synthesis of toluidines begins with a mixed-acid (nitric/sulfuric acid) mononitration of toluene, which produces all three isomers (*ortho*, *meta*, *para*), usually in the ratio 15:1:9, respectively. Since the isomeric toluidines themselves cannot be effectively separated by distillation, separation of the isomers is achieved at the nitrotoluene stage. The literature describes a multitude of methods for reducing nitrotoluenes to toluidines. These range from dissolving metal reductions to catalytic

hydrogenation. Currently, high-volume manufacture of toluidines utilizes the same type of continuous vapour-phase hydrogenation process used for aniline manufacture. The hydrogenation catalysts include various supported metals, Raney nickel, copper, molybdenum, tungsten, vanadium, and noble metals (Bowers, 2000).

Commercial production was first reported in the USA in 1922 for *ortho*-toluidine and in 1956 for *ortho*-toluidine hydrochloride (IARC, 1982, 2000). In the late 1970s, production volumes were estimated to be 1.1 to 11 million pounds/year, but this increased to 14.5 to 28.2 million pounds/year by the early 1990s (IARC, 2000). *ortho*-Toluidine hydrochloride has not been commercially produced in the USA since 1975 (HSDB, 2009).

Information was collected in 1994 in Europe for the IUCLID database for substances with a production or import volume greater than 1000 tonnes/year (High Production Volume Chemicals (HPVCs)). *ortho*-Toluidine was included on the list of HPVCs with a range of 100 000 to 500 000 tonnes (Allanou *et al.*, 1999; European Commission, 2000).

The USEPA (2003, 2007) Inventory Update Rule regulation requires manufacturers and importers of certain chemical substances listed in the TSCA Chemical Substance Inventory to report manufacturing information (aggregate production volumes) for chemicals manufactured (including imported) in amounts of 10 000 pounds or greater (in 1986) or 25 000 pounds or greater (in 2003) at a single site. Table 1.2 presents the aggregate production volumes that were reported for *ortho*-toluidine.

Available information indicates that *ortho*-toluidine was produced and/or supplied in the following countries: Canada, France, Germany, Hong Kong Special Administrative Region, India, Italy, Japan, Mexico, the Netherlands, the People’s Republic of China, South Africa, Switzerland, the United Kingdom, and the USA (Chemical Sources International, 2010).

Table 1.2. *ortho*-Toluidine production volumes

Year	Volume (in millions of pounds)
1986	>10–50
1990	>10–50
1994	>10–50
1998	>50–100
2002	>10–50
2006	10–<50

USEPA (2003, 2007)

1.2.2 *Use*

ortho-Toluidine, the isomer produced in greatest volume, has found many commercial applications. By far the single largest use for *ortho*-toluidine is in the preparation of 6-ethyl-*ortho*-toluidine, an intermediate in the manufacture of two very large-volume herbicides, metolachlor and acetochlor. Another important use for *ortho*-toluidine is in rubber chemicals, where it is used in the manufacture of a rubber antioxidant, and of di-*ortho*-tolylguanidine, a nonstaining rubber accelerator. Acetoacet-*ortho*-toluidine, 3-hydroxy-2-naphthoyl-*ortho*-toluidine, 2-toluidine-5-sulfonic acid, and *ortho*-aminoazotoluene are four of the more important dye and pigment intermediates manufactured from *ortho*-toluidine. In addition, *ortho*-toluidine is used to manufacture epoxy resin hardeners such as methylene-bis-2-methylcyclohexylamine, fungicide intermediates such as 2-amino-4-methylbenzothiazole, and *ortho*-fluorobenzoyl chloride. Certain pharmaceutical intermediates are also prepared starting with *ortho*-toluidine (Bowers, 2000).

Other minor uses of *ortho*-toluidine and its hydrochloride salt are as intermediates in organic synthesis and as ingredients in a clinical laboratory reagent for glucose analysis (HSDB, 2009).

1.3 Occurrence and exposure

1.3.1 *Natural occurrence*

ortho-Toluidine is not known to exist as a natural substance.

1.3.2 *Occupational exposure*

Occupational exposure to *ortho*-toluidine can occur during its production, or during the production of dyes, pigments and rubber chemicals manufactured from this compound. Laboratory and medical personnel may be exposed when using *ortho*-toluidine for staining tissues.

(a) Production of ortho-toluidine

In the former USSR (Khlebnikova *et al.*, 1970), air samples at a plant manufacturing *ortho*-toluidine from *ortho*-nitrotoluene (215 samples) generally exceeded the maximum permissible concentrations of 3 mg/m^3 (IARC 1982) by 2–7 times. The highest airborne exposure concentrations were observed during distillation and extraction processes (25–28.6 mg/m^3). Concurrently, in 80–90% of the air samples *ortho*-nitrotoluene levels exceeded the maximum permissible concentration of 1 mg/m^3: values up to approximately 5 mg/m^3 were reported. Dermal deposition of *ortho*-toluidine at 0.01–0.03 mg/dm^2 of skin was measured by collecting 1% acetic acid washes from the wrists, chest and back of individuals at the end of work-shifts (n = 168). After post-shift showers,

dermal levels of *ortho*-toluidine had decreased by a factor of 10. Patches of cloth placed on the workers' overalls collected about 0.10 mg/dm^2 ($n = 46$).

(b) Use of ortho-toluidine in dye production

Measurements in the 1940s in a dye-production plant in the United States indicated that the concentration of *ortho*-toluidine was below 0.5 ppm (2.19 mg/m^3) in the workroom air (breathing zone and area samples) and ranged from < 0.3 ppm to 1.7 ppm in the urine of workers engaged in the production of thioindigo. In addition to inhalatory exposure, exposure from ingestion and skin contact may have occurred (Ott & Langner, 1983).

Exposure to *ortho*-toluidine was reported to occur in an Italian plant producing fuchsin (magenta) and safranine T-based dyes (Rubino *et al.*, 1982), in a German plant producing 4-chloro-*ortho*-toluidine (Stasik, 1988), and in a plant producing azo-dyes in New Jersey, USA (Delzell *et al.*, 1989). No data on exposure levels were provided.

(c) Production and use of rubber antioxidant

ortho-Toluidine, aniline, hydroquinone and toluene were used to synthesize a rubber antioxidant in a chemical plant in the United States. Despite low air concentrations (< 1 ppm; 4.38 mg/m^3), elevated pre-shift urinary *ortho*-toluidine levels of 18 ± 27 µg/L ($n = 46$) and post-shift levels of 104 ± 111 µg/L were detected. The average concentration of *ortho*-toluidine in the pre-shift samples was 17 times higher than that in urine samples of unexposed workers (Ward *et al.*, 1991; Teass *et al.*, 1993).

Sorahan *et al.* (2000) reported worker exposure to *ortho*-toluidine in a plant that produced rubber chemicals in the United Kingdom, but no data on exposure concentrations were provided.

Korinth *et al.* (2006) reported on four workers involved in vulcanising hydraulic rubber articles, who were exposed to *ortho*-toluidine contained in di-*ortho*-tolylguanidine (used as an accelerator for the vulcanization of rubber products) by dermal absorption. The concentrations of *ortho*-toluidine in the air at the workplace ranged between 26.6–93.9 µg/m^3 and *ortho*-toluidine concentrations in urine were between 54.7–242.9 µg/L.

(d) Demolition workers of an SO_2 plant polluted with ortho-toluidine

Labat *et al.* (2006) measured urinary *ortho*-toluidine levels in workers employed in the demolition of a liquid-SO_2 plant polluted with *ortho*-toluidine. The plant had stopped production 20 years earlier. For unexposed workers, urinary concentrations of *ortho*-toluidine ranged between 0.17 and 2.46 µg/g creatinine. Post-shift urinary concentrations for exposed workers ranged between 26.1 and 462 µg/g creatinine. After protective measures were taken, this decreased to 2.4–20.1 µg/g creatinine.

(e) Laboratory workers

Although medical and laboratory personnel represent a significant population of workers potentially exposed to *ortho*-toluidine, air concentrations have been determined to be below 22 µg/l (Environmental Protection Agency, 1984). Kauppinen *et al.* (2003) reported that in 1988 in Finland, five of 26 pathological laboratories used *ortho*-toluidine for staining tissues with a median use of 10 g/year, three of 30 clinical laboratories used it with a median use of 180 g/year, and six of 20 other laboratories with a median use of 10 g/year.

1.3.3 *Environmental occurrence and exposure of the general population*

The general population is known to be exposed ubiquitously to *ortho*-toluidine, although its origin is not known. The levels found are not explained by known exposures.

(a) Air

According to the Toxics Release Inventory (Environmental Protection Agency, 1997), air emissions of *ortho*-toluidine from 23 industrial facilities were approximately 5260 kg in 1995 in the United States.

(b) Water

Surface-water discharges of *ortho*-toluidine from 23 industrial facilities in the United States, as reported in the Toxics Release Inventory, decreased from 242 kg in 1994 to 116 kg in 1995 (Environmental Protection Agency, 1996, 1997).

ortho-Toluidine has been reported to be present in surface-water samples taken from three rivers in Germany, at levels of 0.3–1 µg/L (Neurath *et al.*, 1977). *ortho*-Toluidine has been identified in one secondary effluent from seven industrial and publicly owned treatment works (Ellis *et al.*, 1982) and in wastewaters from synthetic fuel production (Leenheer *et al.*, 1982; Stuermer *et al.*, 1982). It has also been detected in effluents from refineries and chemical production facilities, in process water, river water, groundwater and seawater in the United States (Shackelford & Keith, 1976; Environmental Protection Agency, 1984; NTP, 2005).

(c) Soil

Soil discharges of *ortho*-toluidine from 23 industrial facilities in 1995 in the United States amounted to 5.5 kg, as reported in the Toxics Release Inventory (Environmental Protection Agency, 1997). An estimated 10 000 kg of *ortho*-toluidine were released via underground injection.

(d) Food and beverages

Neurath *et al.* (1977) found unspecified isomers of toluidine in commercially available samples of kale and celery (1.1 mg/kg) and carrots (7.2 mg/kg). *ortho*-Toluidine has been identified in the volatile aroma components of black tea (Vitzthum *et al.*, 1975).

DeBruin *et al.* (1999) measured *ortho*-toluidine at part per billion levels in breast-milk samples from both smokers and nonsmokers (< 0.01 to 0.26 ppb). The concentrations observed did not correlate with smoking status, suggesting that tobacco smoking is not the main source of exposure.

(e) Tobacco smoke

Mainstream smoke from eight US commercial cigarette brands has been found to contain 8.6–144.3 ng *ortho*-toluidine per cigarette (Stabbert *et al.*, 2003). The concentrations of *ortho*-toluidine tend to be much higher in sidestream smoke than in mainstream smoke (IARC, 2004).

Riedel *et al.* (2006) reported urinary excretion of *ortho*-toluidine at 70.1–139.6 ng/24 hours (mean ± SD, 105.2 ± 25.6) for nine nonsmokers and 107.9–258.7 (mean ± SD, 204.2 ± 59.1) for 10 smokers. Higher amounts were reported in an earlier study (El-Bayoumy *et al.*, 1986), with a mean *ortho*-toluidine excretion of 4.1 µg/24 hours in 12 nonsmokers and 6.3 µg/24 hours in 16 smokers.

In a study by Riffelmann and colleagues (1995), urinary concentrations of *ortho*-toluidine in eight smokers averaged 1.7 ± 1.6 µg/L, while it was not detected in eight non-smokers. In another study, current smoking had no influence on background values and on the increase of *ortho*-toluidine adducts to haemoglobin (Gaber *et al.*, 2007).

(f) Hair dyes

In a study from Turkey (Akyüz & Ata, 2008), *ortho*-toluidine was found in 34 of the 54 hair dyes tested, at quantities up to 1547 µg/g.

(g) Prilocaine

Prilocaine, a widely used anaesthetic, is metabolized to *ortho*-toluidine. A component of EMLA cream, it is also used as a pain reliever in neonates (Taddio *et al.*, 1998), during circumcision (Taddio *et al.*, 1997) and during venipuncture in children (Tak & van Bon, 2006).

1.4 Regulations and guidelines

1.4.1 *Europe*

(a) Directive 97/56/EC

According to Directive 97/56/EC on the restrictions on the marketing and use of certain dangerous substances and preparations, the packaging of *ortho*-toluidine and its

preparations must be marked legibly and indelibly as follows: “Restricted to professional users” (European Commission, 1997).

(b) Directive 2002/61/EC

Directive 2002/61/EC restricts the marketing and use of azocolourants (European Commission, 2002). In this Directive, Annex I to Directive 76/769/EEC is amended. Azodyes that, by reductive cleavage of one or more azo groups, may release *ortho*-toluidine in detectable concentrations, i.e. above 30 ppm in the finished articles or in the dyed parts thereof, according to the test method established in accordance with Article 2a of this Directive, may not be used in textile and leather articles that may come into direct and prolonged contact with the human skin or oral cavity.

(c) Directive 2004/37/EC

ortho-Toluidine is regulated by the Directive 2004/37/EC (European Commission, 2004), which applies to activities in which workers are exposed to carcinogens or mutagens of Category 1 and 2. Rules are fixed regarding the employers' obligations of reduction and replacement, prevention and reduction of exposure, unforeseen exposure, foreseeable exposure, access to risk areas, hygiene and individual protection, information for the competent authority, information and training of workers, consultation and participation of workers, health surveillance, record keeping and limit values.

1.4.2 *Germany*

Deviating from the EU classification, *ortho*-toluidine is classified as a Category 1 carcinogen by the German MAK Commission (MAK, 2007). The MAK Commission listed *ortho*-toluidine as a substance for which percutaneous absorption may significantly contribute to systemic exposure. A MAK value was not set. Furthermore, it was classified as a germ-cell mutagen, Class 3A: substances that have been shown to induce genetic damage in germ cells of humans or animals, or that produce mutagenic effects in somatic cells of mammals *in vivo* and have been shown to reach the germ cells in an active form.

1.4.3 *Japan*

The Japan Society for Occupational Health (2007) follows the classification by IARC of *ortho*-toluidine. An occupational exposure limit (OEL) of 1 ppm (4.4 mg/m^3) was reported; skin absorption was noted.

1.4.4 *United Kingdom*

The Health and Safety Commission (HSE, 2007) has set an 8-hour time-weighted average Workplace Exposure Limit (WEL) of 0.2 ppm (0.89 mg/m^3) for *ortho*-toluidine, with a skin notation. HSE also lists *ortho*-toluidine as capable of causing cancer.

1.4.5 *USA*

(a) *ACGIH*

ortho-Toluidine has been assigned an A3 notation, *Confirmed Animal Carcinogen with Unknown Relevance to Humans* (ACGIH, 2001). A TLV-TWA (threshold limit value–time-weighted average) of 2 ppm (8.8 mg/m^3) is recommended. Reported significant percutaneous absorption and its systemic health effects warrant assigning the skin notation (*potential significant contribution to the overall exposure by the cutaneous route*). A methaemoglobin level in blood of 1.5% of total haemoglobin measured during or at the end of the shift is recommended as a Biological Exposure Index (BEI) for biological monitoring of exposure to *ortho*-toluidine.

(b) *NTP*

ortho-Toluidine and *ortho*-toluidine hydrochloride are listed in the NTP *Report on Carcinogens* as *reasonably anticipated to be human carcinogens* (NTP, 2005).

1.4.6 *Other*

(a) *GESTIS*

Table 1.3 presents some international limit values for *ortho*-toluidine (GESTIS, 2007).

Table 1.3. International limit values (2007) for *ortho*-toluidine

Country	Limit value: Eight hours		Limit value: Short-term		Comments
	ppm	mg/m³	ppm	mg/m³	
Austria	0.1	0.5	0.4	2	TRK value (based on technical feasibility)
Belgium	2	8.9			
Canada, Québec	2	8.8			
Denmark	2	9	4	18	
France	2	9			
Hungary				0.5	
Poland		3		9	
Spain	0.2 (skin)	0.89 (skin)			
Switzerland	0.1	0.5			
USA, OSHA	5	22			
United Kingdom	0.2	0.89			

OSHA, Occupational Safety and Health Administration; TRK, technical guiding concentration

2. Studies of Cancer in Humans

2.1 Case reports

Uebelin and Pletscher (1954) studied the occurrence of bladder tumours in workers employed at a factory in Switzerland producing dyestuff intermediates. A total of 97 cases were found in 300 workers exposed to various combinations of 2-naphthylamine, benzidine and other aromatic amines for unspecified periods during 1924–53. A further 650 workers had been exposed only to aniline or other aromatic amines, but not to 2-naphthylamine or benzidine; three of these men developed bladder tumours, but their exact exposures were uncertain. The authors distinguished a subgroup of 35 men who prepared 4-chloro-*ortho*-toluidine from *ortho*-toluidine; no bladder tumours were found among these men. [The Working Group noted that insufficient details were provided concerning person–years at risk or follow-up to evaluate the significance of these observations.]

Gropp (1958) described 98 cases of bladder tumours that occurred from 1903 to 1955 among workers at a German factory. Oettel (1959, 1967) and Oettel *et al.* (1968) also reported these cases and some additional ones. Most of Gropp's cases had had exposure to 2-naphthylamine alone (23 men) or together with other amines, including benzidine, aniline and toluidines (45 men). However, 11 cases were reported as having had exposure only to aniline/toluidine; the population at risk is not known. [The Working Group noted that insufficient details were provided concerning person–years at risk or follow-up to evaluate the significance of these observations.]

Khlebnikova *et al.* (1970) reported bladder tumours in workers engaged in the production of *ortho*-toluidine and/or *para*-toluidine in the USSR during the 1960s. The concentration range of *ortho*-toluidine measured in the air of the working environment was 0.5–28.6 mg/m^3. Two cases of bladder tumours (papillomas) were found when 75 of 81 current toluidine-exposed workers were examined cystoscopically. The 81 workers comprised 35 operators, 18 fitters, and 10 cleaners. A total of 41 workers had worked in *ortho*-toluidine production for more than five years; most of these workers had also been in contact with *para*-toluidine. Six other cases of bladder tumours (including four carcinomas) had been found earlier, upon cystoscopic examination of 16 former workers who had worked with the toluidines for periods ranging from 12 to 17 years. [The Working Group noted that the information contained in the paper with regard to person–years of exposure and to the other substances to which these workers may have had prior or concomitant exposure is insufficient to evaluate the significance of these observations].

Lipkin (1972), in a further report from the USSR, referred to 27 cases of bladder cancer occurring in individuals exposed occupationally to *ortho*- and *para*-toluidine, and to 21 cases in workers exposed to both these chemicals and to 1-naphthylamine. Genin *et al.* (1978) referred to 36 people with bladder cancer who had been occupationally exposed to *ortho*- and *para*-toluidine and to "some other aromatic amino and nitro compounds" in a chemical plant. The cancers were diagnosed in men and women aged

between 25 and 61. [The Working Group noted that no information was given on the sizes of the populations at risk. It is possible, but not explicitly stated, that these cases included some or all of those reported by Khlebnikova *et al.* (1970).]

Conso and Pontal (1982) reported three cases of bladder cancer occurring among 50 workers employed in a factory where *para*-toluenediamine was synthesized from *ortho*-toluidine and *ortho*-aminoazotoluene. [The Working Group noted that although a formal estimate of expected numbers of bladder cancer cases was not provided, three bladder cancers among 50 persons is likely to represent a substantial excess. The relationship of these bladder cancers to *ortho*-toluidine exposure cannot be determined because of co-exposure to *ortho*-aminoazotoluene, which has been classified as possibly carcinogenic to humans (IARC, 1975, 1987).]

2.2 **Cohort studies** (see Table 2.1)

Case and Pearson (1954) and Case *et al.* (1954) reported a historical cohort study of bladder tumours among workers in the British chemical industry engaged in the manufacture of dyestuff intermediates. Groups were defined in terms of exposure to aniline, benzidine, 1- and 2-naphthylamine, auramine and magenta. Many aniline-exposed workers were also exposed to toluidines produced in the same plants. The 21 firms in the survey included the major British producers of aniline, and mortality was observed from 1921 to 1952. There were 812 men classified as having had exposure to aniline but not to the other suspected carcinogens. The death certificate for one of these workers mentioned a bladder tumour; 0.52 such deaths were expected on the basis of national rates [The Working Group noted that exposure of these workers to *ortho*-toluidine is not specified in this paper but can be inferred from knowledge of aniline-production processes].

Rubino *et al.* (1982) reported mortality findings for 906 men first employed in a dyestuff factory in northern Italy between 1922 and 1970 and followed from 1946 to 1976. Mortality was compared with that of the Italian male population. Follow-up was 96% complete and 260 deaths were identified. Thirty-six deaths from urinary bladder cancer were observed (SMR, 29.3; 95% CI, 20.5–40.5). Thirty-one of these deaths occurred among 610 men with exposure to one or more of benzidine, 1-naphthylamine, and 2-naphthylamine (SMR, 35.6; 95% CI, 24.2–50.6). Some of these workers were also exposed to *ortho*-toluidine, among other chemicals, and a significant excess of bladder cancer mortality was observed in the 53 men engaged solely in the manufacture of magenta (IARC, 1993) and safranine T, also involving exposure to one or more of the following chemicals: toluene, *ortho*-nitrotoluene and 4,4′-methylene bis(2-methylaniline), 2,5-diaminotoluene, *ortho*-aminoazotoluene and aniline (5 deaths; SMR, 62.5; 95% CI, 20.3–145.9). No quantitative exposure measurements or data on smoking were available. [The Working Group noted that the clear occupational excess of bladder cancer could not be specifically attributed with certainty to exposure to *ortho*-toluidine or to any one of the other compounds involved.]

Table 2.1. Summary of principal cohort studies of workers exposed to *ortho*-toluidine

Reference	Rubino *et al.* (1982)	Ott & Langner (1983)	Stasik (1988)	Ward *et al.* (1991); Prince *et al.* (2000)	Sorahan *et al.* (2000)
Country	Italy	USA	Germany	USA	UK
Industry	Dyestuff production	Dye production	4-Chloro-*ortho*-toluidine production and processing	Rubber chemicals	Rubber chemicals
Size of the total cohort	906 men	342 men	116 men	1749 (1643 men)	2160 men
Cohort definition	First employed 1922–70	Some employment during 1940–58	Employed before 1970 >1929–82 (mortality) <1967–86 (incidence)	Employed 1946–88	Employed ≥ six months; some employment between 1955–84
Period of follow-up	1946–76	1940–75		1946–94 (mortality) 1973–88 (incidence	1955–96 (mortality) 1971–92 (incidence)
Deaths					
All causes					
N	260	118	19	[190]	1131
SMR (95% CI)	1.5 [1.4–1.7]	1.0 [0.8–1.2]	1.1 (0.7–1.7)	[0.9] [0.8–1.0]	1.0 (1.0–1.1)

Table 2.1 (contd)

Reference	Rubino *et al.* (1982)	Ott & Langner (1983)	Stasik (1988)	Ward *et al.* (1991); Prince *et al.* (2000)	Sorahan *et al.* (2000)
Country	Italy	USA	Germany	USA	UK
All cancers					
N	96	27	5	[49]	305
SMR (95% CI)	2.7 [2.1–3.2]	1.3 [0.8–1.8]	1.5 (0.5–3.4)	[1.0] [0.7–1.3]	1.0 (0.9–1.1)
Bladder cancer mortality					
N	36	0	0	2	17
SMR (95% CI)	29.3 [20.5–40.5]	1.5 urinary cancers expected	0.5 urogenital cancers expected	[2.1] [0.2–7.4]	1.4 (0.8–2.3)
Bladder cancer incidence					
N			8	13	19
SIR (95% CI)			72.7 (31.4–143.3)	3.6 [1.9–6.2]	1.1 (0.6–1.7)
Subgroup exposed to ortho-toluidine					
Size	53	117	Same as full cohort	708	53
Bladder cancer mortality					
N	5	0	0	1	3
SMR (95% CI)	62.5 [20.3–145.9]	[expected number N/A]	0.5 urogenital cancers expected	3.8 [0.1–21.1]	(15.9) [3.3–46.4]

Table 2.1 (contd)

Reference	Rubino *et al.* (1982)	Ott & Langner (1983)	Stasik (1988)	Ward *et al.* (1991); Prince *et al.* (2000)	Sorahan *et al.* (2000)
Country	Italy	USA	Germany	USA	UK
Bladder cancer incidence					
N			8	7	
SIR (95% CI)			72.7 (31.4–143.3)	6.5 [2.6–13.3]	
Other exposures among subgroup exposed to *ortho*-toluidine[a]	4,4′-Methylene bis (2-methylaniline) (2B) Magenta (2B) Safranine T *ortho*-Nitrotoluene (3) 2,5-Diaminotoluene (3) Aniline (3) *ortho*-Aminoazotoluene (2B) Toluene (3)	Multiple exposures including 4-chloro-*ortho*-toluidine (2A) and 4-chloroacetyl-*ortho*-toluidine	*N*-Acetyl-*ortho*-toluidine 6-Chloro-*ortho*-toluidine 4-Chloro-*ortho*-toluidine (2A)	Aniline (3) Hydroquinone (3) Toluene (3) Carbon disulfide Sulfur Benzothiazole 4-Aminobiphenyl (contaminant) (1) 2-Mercaptobenzothiazole (Ward *et al.*, 1996) Nitrobenzene (2B) (Viet *et al.*, 2009)	Aniline (3) 2-Mercaptobenzothiazole Phenyl-β-naphthylamine (3)

[a] Previous IARC overall evaluations of carcinogenicity are given in parentheses.
N, number; SMR, standardized mortality ratio; SIR, standardized incidence ratio

Ott and Langner (1983) studied the mortality of 342 male employees assigned to three aromatic amine-based dye-production areas from 1914 to 1958 at a chemical plant in the United States. All study subjects were currently working as of 1st January 1940 or hired after that date. Expected numbers of deaths for the period 1940–1975 were based on mortality rates for US white males. There were no deaths from bladder cancer, with 1.5 deaths expected from malignant neoplasms of the urinary organs. In one area of the plant, 117 men produced bromindigo and thioindigo with potential exposure to 4-chloro-*ortho*-toluidine and other raw materials and intermediates, including *ortho*-toluidine. No bladder cancer deaths occurred in this subcohort. [The expected figure was not reported.] There was one death from lymphoma [SMR, 0.83] and two deaths from leukaemia [SMR, 2.22]. [The Working Group noted that the interpretation of this study was limited by the small size of the population exposed to 4-chloro-*ortho*-toluidine, and by the ascertainment of deaths only.]

Stasik (1988) reported findings from a mortality study of 335 male workers employed for at least twelve months in the period 1929–1982 in the production and processing of 4-chloro-*ortho*-toluidine at a dyestuffs-manufacturing plant in Frankfurt, Hessen, Germany. Three other monocyclic amines had been used at the plant: *N*-acetyl-*ortho*-toluidine, 6-chloro-*ortho*-toluidine and *ortho*-toluidine. Exposure to 4-chloro-*ortho*-toluidine was reported to be predominant. No deaths from bladder cancer were identified in the period 1929–1982 [expected figure unspecified but estimated on the basis of mortality rates for the Federal Republic of Germany to be about 0.2.] [The Working Group noted that the study had inadequate tracing of deaths and description of methods.] Two incident cases of urothelial carcinoma were subsequently identified in this workforce in workers employed in the 4-chloro-*ortho*-toluidine production plant before improvements in industrial hygiene were made in 1970. As a result of this discovery, a cancer incidence study was established for the 116 subjects employed at this plant before 1970. There was no cancer registry for the region in which the plant was located; expected numbers were therefore based on rates for Saarland, a neighbouring province of the Federal Republic of Germany. A marked excess of bladder cancer cases based on eight cases was reported (SIR, 72.7; 95% CI, 31.4–143.3). No quantitative measure of exposure to 4-chloro-*ortho*-toluidine was available, and exposure to other amines was also present. Median exposure time of the cases before 1970 was 14 years. Three of the eight cases were nonsmokers, one was a former smoker, two were smokers and the smoking habits of the remaining two were unknown. [The Working Group noted that the definition of the subcohort for the cancer incidence survey was made *a posteriori,* the obervational period was unspecified, and case ascertainment was inadequately described. Consequently, some bias in the estimate of excess risk may be present. The excess of bladder cancer could not be attributed with any certainty to *ortho*-toluidine or to any one of the other compounds present.]

Ward *et al.* (1991) reported findings for bladder cancer incidence in workers exposed to *ortho*-toluidine and aniline at a US chemical-manufacturing plant. The study was initiated at the request of union representatives who had noted several bladder cancers

among workers in the department that manufactured chemicals for the rubber industry. Among the major reactants used in these processes were two primary aromatic amines, *ortho*-toluidine and aniline; other reactants and intermediates included 2-mercaptobenzothiazole, hydroquinone, toluene, carbon disulfide, benzothiazole and a proprietary chemical, later identified as nitrobenzene (Ward *et al.*, 1991; Ward *et al.*, 1996; Viet *et al.*, 2009). The study cohort comprised 1749 workers (1643 males, 106 females) employed at the plant in the period 1946–1988. A total of 708 of these workers were considered to have been definitely exposed to *ortho*-toluidine and aniline; 288 workers assigned to maintenance, janitorial, yard work and shipping departments were considered to have been possibly exposed, and the remaining 753 workers were considered to be probably unexposed. Vital status was identified for 1973 through 1988. Bladder-cancer cases were identified from company or union records and confirmed through medical records, or through matching with records at the local cancer registry. Expected numbers of incident bladder cancers were based on local incidence rates. Overall, 13 cases of bladder cancer were observed for the period 1973–1988 (SIR, 3.6; 95% CI, 1.9–6.2), seven of which occurred in the definitely exposed group (SIR, 6.5; 95% CI, 2.6–13.3), four in the possibly exposed group (SIR, 3.7; 95% CI, 1.0–9.4) and the remaining two cases in non-exposed workers (SIR, 1.4; 95% CI 0.2–5.0). Bladder-cancer incidence was particularly elevated in employees who had worked in the exposed department for more than 10 years; six of the seven exposed cases occurred in this subcohort (SIR, 27.2; 95% CI, 10.0–59.2). Data on smoking were available for only 143 study subjects but suggested that confounding from smoking could explain no more than a small fraction of the observed bladder-cancer excess. Other chemical exposures at the plant should be considered. Aniline was present but is not known to induce bladder cancer in humans or animals (IARC, 1987). 4-Aminobiphenyl was identified as a potential low-level contaminant (< 1 ppm) in some bulk samples of process chemicals at the plant in 1990 (Ward & Dankovic, 1991). 4-Aminobiphenyl is an IARC Group-1 carcinogen and is known to be a highly potent human bladder carcinogen (IARC, 1972, 1987). 2-Mercaptobenzothiazole has not been reviewed by IARC, but has shown some evidence of carcinogenicity in rats and equivocal evidence of carcinogenicity in mice (NTP, 1988). An exposure-assessment study conducted at the plant in 1990 (Ward *et al.*, 1996) showed substantially higher urinary concentrations and levels of haemoglobin adducts of *ortho*-toluidine and aniline among exposed workers compared with in-plant controls. Levels of 4-aminobiphenyl adducts were much lower than those of *ortho*-toluidine and aniline, and were similar for exposed and unexposed groups. [The Working Group noted that haemoglobin adducts reflect only recent exposures (Hemminki, 1992), and it is therefore possible that higher levels of 4-aminobiphenyl contamination existed in the past.] A subsequent mortality analysis of the same cohort for the period 1946–1994 (Prince *et al.*, 2000) found only two deaths from bladder cancer in the total cohort (SMR, 2.1; 95% CI, 0.2–7.4). One of these deaths occurred in the definitely exposed group (SMR, 3.8; 95% CI, 0.1–21.1). There were two deaths from lymphohaematopoietic cancers in the definitely exposed group (SMR, 1.2; 95% CI, 0.1–4.2). A further 19 cases

of bladder cancer occurring in this cohort, 18 of which were diagnosed in the later period of 1989–2003, have been identified (Markowitz & Levin, 2004; Markowitz, 2005). Ten of these cases were stated to be members of the definitely exposed subgroup. [The Working Group noted that the authors did not have access to the study master files and were not able to provide expectations for their findings. Nevertheless, this article indicates a continuing excess of occupational bladder cancer at the plant under study.]

Sorahan *et al.* (2000) updated a study of workers exposed to several aromatic amines in a factory manufacturing chemicals for the rubber industry in the United Kingdom (Sorahan & Pope, 1993). All subjects had had at least six months' employment in the factory and some employment in the period 1955–1984. Mortality was examined for the period 1955–1996 and cancer incidence for the period 1971–1992. The updated study included 2160 male production workers, 605 of whom had been exposed to one or more of the four chemicals under investigation (aniline, 2-mercaptobenzothiazole, phenyl-β-naphthylamine, *ortho*-toluidine), including 53 workers who were exposed to *ortho*-toluidine. In the latter subcohort, three bladder cancer deaths were observed (SMR, 15.9; 95% CI, 3.3–46.4). A total of 30 bladder cancers were identified in the overall cohort on the basis of death certificate or cancer registration data. Internal analysis (Poisson regression) revealed a significant association between the risk for bladder cancer and duration of exposure to *ortho*-toluidine (1–4 years, $n = 2$; RR, 6.7; 95% CI, 1.6–28.4; ≥ 5 years, $n = 1$; RR, 7.7; 95% CI, 1.0–56.9).

[The Working Group noted that individual smoking habits were not available for all subjects in any of the published cohort studies. However, the excesses of bladder cancer reported in the four positive studies were much too large to have been due to smoking alone.]

3. Studies of Cancer in Experimental Animals

Studies in experimental animals of carcinogenicity of *ortho*-toluidine and its hydrochloride salt were previously reviewed by IARC (1978, 1982, 1987, 2000). Those found to be adequate and/or reported more fully in later publications are included in this evaluation.

3.1 Oral administration

3.1.1 *Mouse*

Groups of 25 male and 25 female Swiss CD-1 mice, 6–8 weeks of age, were treated with *ortho*-toluidine hydrochloride (purity, 97–99%) in the diet at levels of 16 000 or 32 000 mg/kg diet (ppm) for three months and then, due to toxicity, at levels of 8000 or 16 000 ppm for a further 15 months. Subsequently, the animals were kept without treatment for an additional three months and then killed. The doses were chosen on the

basis of preliminary tests, the higher being the maximum tolerated dose. A simultaneous control group of 25 untreated mice of each sex was used, plus additional controls used for the other compounds tested in the study, and tumour incidences of matched and pooled controls were compared statistically (both separately and together) with those of treated groups. Animals that died during the first six months of the study were discarded without necropsy. In male mice, the incidence of vascular tumours (haemangiomas and haemangiosarcomas combined, observed in abdominal viscera) was 0/14, 5/99 (5%), 5/14 (36%) ($P < 0.025$, Fisher exact test) and 9/11 (82%) ($P < 0.025$, Fisher exact test) in concurrent controls, pooled controls, low-dose and high-dose groups, respectively. In female mice, the incidence of vascular tumours (haemangiomas and haemangiosarcomas combined, observed in abdominal viscera) was 0/15, 9/102 (9%), 5/18 (28%) ($P < 0.05$, Fisher exact test) and 9/21 (43%) ($P < 0.025$, Fisher exact test) in concurrent controls, pooled controls, low-dose and high-dose groups, respectively [the separate incidences for haemangiomas and haemangiosarcomas were not reported] (Weisburger *et al.*, 1978).

Groups of 50 male and 50 female B6C3F$_1$ mice, six weeks of age, were given *ortho*-toluidine hydrochloride (purity > 99%) in the diet at 1000 or 3000 ppm for 102–103 weeks. Concurrent control groups consisted of 20 male and 20 female untreated mice. The mean body weights of both treated males and females were lower than those of the corresponding controls, and were dose-related. Mortality was not significantly related to treatment in either sex. In male mice, the incidence of haemangiomas or haemangiosarcomas (combined, all sites, mainly observed in the abdominal viscera) was increased: 1/19 (5%), 2/50 (4%) and 12/50 (24%) ($P < 0.002$, Cochran–Armitage trend test) in control, low-dose and high-dose groups, respectively. In female mice, the incidence of hepatocellular adenomas or carcinomas (combined) was also increased: 0/20, 4/49 (8%) and 13/50 (26%) ($P < 0.007$, Fisher exact test; $P = 0.001$ trend test) in control, low-dose and high-dose groups, respectively (National Cancer Institute, 1979).

3.1.2 *Rat*

Groups of 25 male Sprague-Dawley CD rats, 6–8 weeks of age, were treated with *ortho*-toluidine hydrochloride (purity 97–99%) in the diet at dose levels of 8000 or 16 000 ppm for three months and then, due to toxicity, at levels of 4000 or 8000 ppm for a further 15 months. Animals were kept without treatment for an additional six months and then killed. The doses were chosen on the basis of preliminary tests, the higher being the maximum tolerated dose. A concurrent control group of 25 untreated male rats was used, plus additional controls used for the other compounds tested in the study, and tumour incidences of matched and pooled controls were compared with those of treated groups. Animals that died during the first six months of the study were discarded without necropsy. The incidence of subcutaneous fibromas and fibrosarcomas (combined) was 0/16, 18/111 (15%), 18/23 (78%) ($P < 0.025$, Fisher exact test) and 21/24 (88%) ($P < 0.025$, Fisher exact test, compared with all controls) in simultaneous controls, pooled controls, low-dose and high-dose groups, respectively. A non-statistically significant

increase in the incidence of transitional-cell carcinomas of the urinary bladder was also observed: 0/16, 5/111 (5%), 3/23 (13%) and 4/24 (17%) in simultaneous controls, pooled controls, low-dose and high-dose groups, respectively (Weisburger *et al.*, 1978).

Groups of 30 male Fischer 344 rats, eight weeks of age, were treated with *ortho*-toluidine hydrochloride [recrystallized product, purity not specified] in the diet at a concentration of 4000 ppm (0.028 mol/kg of diet) for 72 weeks. A control group of 30 untreated male rats was included in the study. The experiment was terminated at 93 weeks. The mean daily dose of *ortho*-toluidine hydrochloride was 0.062 g (0.00043 mol) per rat, and the total dose was 31.3 g (0.22 mol) *ortho*-toluidine hydrochloride per rat. Mean body weights were higher in the treated group than in the control group. Survival of the treated animals was comparable to controls through month 18, then steadily decreased until termination at 22 months. The incidence of fibromas of the skin was 1/27 (4%), 25/30 (83%; $P < 0.001$, Fisher exact test); that of fibromas of the spleen was 0/27, 10/30 (33%; $P < 0.001$, Fisher exact test); that of mammary fibroadenomas 0/27, 11/30 (37%; $P < 0.001$, Fisher exact test); and that of peritoneal sarcomas was 0/27, 9/30 (30%; $P < 0.01$, Fisher exact test) for control and treated groups, respectively (Hecht *et al.*, 1982).

Groups of 50 male and 50 female Fischer 344 rats, six weeks of age, were given *ortho*-toluidine hydrochloride (purity > 99%) in the diet at concentrations of 3000 or 6000 ppm for 101–104 weeks. Matched control groups consisted of 20 male and 20 female untreated rats. Mean body weights of treated male and female rats were lower than those of the corresponding controls, and were dose-related. Mortality was significantly affected by the treatment ($P < 0.001$, Tarone test for positive trend). In males, the incidence of sarcomas, fibrosarcomas, angiosarcomas or osteosarcomas (combined) of multiple organs (mainly subcutis and spleen or bone) was 0/20, 15/50 (30%; $P = 0.003$, Fisher exact test) and 37/49 (76%; $P < 0.001$, Fisher exact test); that of subcutaneous integumentary fibromas was 0/20, 28/50 (56%; $P < 0.001$, Fisher exact test) and 27/49 (55%; $P < 0.001$, Fisher exact test); and that of mesotheliomas of multiple organs or tunica vaginalis was 0/20, 17/50 (34%; $P < 0.001$, Fisher exact test) and 9/49 (18%; $P = 0.036$, Fisher exact test) in controls, low-dose and high-dose groups, respectively. In females, the incidence of transitional cell carcinomas of the urinary bladder was 0/20, 9/45 (20%; $P = 0.028$, Fisher exact test) and 22/47 (47%; $P < 0.001$, Fisher exact test); that of sarcomas, fibrosarcomas, osteosarcomas or angiosarcomas (combined) of multiple organs (mainly subcutis and spleen or bone) was 0/20, 3/50 (6%) and 21/49 (43%; $P = 0.001$, Fisher exact test); and that of mammary adenomas and fibroadenomas (combined) was 7/20 (35%), 20/50 (40%) and 35/49 (71%) ($P = 0.006$), in controls, low-dose and high-dose groups, respectively (National Cancer Institute, 1979).

Three groups of 20 male Fischer 344/N rats, 45 days of age, were given *ortho*-toluidine hydrochloride (purity > 99%) at a concentration of 5000 ppm in the diet for up to 26 weeks. Group 1 was treated with *ortho*-toluidine hydrochloride for 13 weeks and then killed. Group 2 was treated for 13 weeks and then kept untreated for an additional 13 weeks, when animals were killed. Group 3 was treated with *ortho*-toluidine

hydrochloride for 26 weeks and then killed. The average of group mean compound consumption was 301 mg/kg/day for Group 1, 304 mg/kg/day for Group 2 and 285 mg/kg/day for Group 3. Groups of 10 untreated male Fischer 344/N rats served as controls for each of the treated groups. Hyperplasia in the transitional epithelium of the urinary bladder was observed in 10/20 (50%) ($P < 0.01$, Fisher exact test) rats exposed to *ortho*-toluidine hydrochloride for 13 weeks and in 17/20 (85%) ($P < 0.01$, Fisher exact test) rats exposed for 26 weeks. No hyperplasia was observed in the concurrent control animals (0/10). It was also reported that mesotheliomas in the epididymis were observed in 2/20 (10%) male rats exposed to *ortho*-toluidine hydrochloride for 13 weeks and held for an additional 13 weeks. No mesotheliomas were seen in concurrent controls (0/10) (NTP, 1996).

3.2 Subcutaneous injection

3.2.1 *Hamster*

Groups of 15 male and 15 female Syrian golden hamsters, eight weeks of age, were given subcutaneous injections of 1.9 mmol/kg bw (2 mg/kg bw) *ortho*-toluidine (free base) [recrystallized product, purity not specified] in peanut oil once per week for 52 weeks. Control groups of 15 male and 15 female hamsters were given 52 subcutaneous injections of peanut oil. Animals were observed until moribund. The experiment was terminated after 87 weeks. Mean body weights in the treated groups were similar to those of the control groups. Mean survival times were shorter in the treated groups, being 61.3 and 57.8 weeks in male and female treated hamsters, respectively, compared with 75.5 and 68.7 weeks in male and female controls, respectively. The incidence of tumours in the treated groups was not significantly different from that in the control groups [details on the incidence of specific tumours were not reported] (Hecht *et al.*, 1983). [The Working Group noted the small number of animals, low dose and short duration of treatment.]

3.3 Carcinogenicity of metabolites

3.3.1 *Rat*

Groups of 30 male Fischer 344 rats, eight weeks of age, were treated with *ortho*-nitrosotoluene [recrystallized product, purity not specified] in the diet at a concentration of 3380 ppm (0.028 mol/kg of diet) for 72 weeks. A control group of 30 untreated male rats was used. The experiment was terminated at 93 weeks. The mean daily dose of *ortho*-nitrosotoluene was 0.051 g (0.00042 mol) per rat and the total dose was 25.7 g (0.21 mol) *ortho*-nitrosotoluene per rat. Mean body weights were higher in the treated group than in the control group. The incidence of fibromas of the skin was 1/27 (4%), 19/29 (66%) ($P < 0.001$, Fisher exact test); that of fibromas of the spleen was 0/27, 14/29 (48%) ($P < 0.001$, Fisher exact test); that of hepatocellular carcinomas was 0/27, 18/29 (62%) ($P < 0.001$, Fisher exact test); and that of urinary bladder tumours was 0/27, 15/29 (52%)

($P < 0.01$, Fisher exact test) in control and treated groups, respectively (Hecht *et al.*, 1982).

3.3.2 *Hamster*

Groups of 15 male and 15 female Syrian golden hamsters, eight weeks of age, were given subcutaneous injections of 1.9 mmol/kg bw (2 mg/kg bw) *ortho*-nitrosotoluene [recrystallized product, purity not specified] in peanut oil once per week for 52 weeks. Control groups of 15 male and 15 female hamsters were given 52 subcutaneous injections of peanut oil. Animals were observed until moribund. The experiment was terminated after 87 weeks. Mean body weights in the treated groups were similar to those of the control groups. Mean survival times were shorter in the treated groups, being 45.4 and 51.1 weeks in male and female treated hamsters, respectively, compared with 75.5 and 68.7 weeks in male and female controls, respectively. The incidence of tumours in the treated groups was not significantly different from that in the control groups [details on the incidence of specific tumours were not reported] (Hecht *et al.*, 1983). [The Working Group noted the small number of animals, the low dose and the short duration of treatment.]

4. Mechanistic and Other Relevant Data

4.1 Absorption, distribution, metabolism, elimination

4.1.1 *Humans*

ortho-Toluidine occurs in the environment and is a constituent of tobacco smoke (Patrianakos & Hoffmann, 1979). Riedel *et al.* (2006) measured this amine in urine from smokers and nonsmokers using gas-chromatography/mass-spectrometry/negative-ion chemical ionization (GC-MS-NICI) with a deuterated *ortho*-toluidine standard to assess recovery. Excretion of *ortho*-toluidine was significantly higher in 10 smokers (204 ng/24 h, 117 ng/L) than in 10 nonsmokers (104 ng/24 h, 55 ng/L). A large variation was observed among nonsmokers, which was attributed to one subject with *ortho*-toluidine concentrations of 318 ng/L or 731 ng/24 h, the highest of all subjects. After excluding this subject, the difference for *ortho*-toluidine excretion between smokers and nonsmokers remained significant. Urinary arylamine excretion in smokers was associated with the extent of smoking, as assessed by daily cigarette consumption ($P < 0.177$), urinary excretion of nicotine equivalents (nicotine plus its five major metabolites; $P < 0.021$), cotinine in saliva ($P < 0.118$), and carbon monoxide in exhaled breath ($P < 0.373$). All nonsmokers had quantifiable amounts of *ortho*-toluidine in their urine, confirming that other environmental sources of exposure also occur. This study evaluated its results in comparison with those of three other studies on *ortho*-toluidine excretion in smokers and

nonsmokers (el-Bayoumy *et al.*, 1986; Riffelmann *et al.*, 1995; Ward *et al.*, 1996). While only one of those studies reported significant differences between smokers and nonsmokers (Riffelmann *et al.*, 1995), higher absolute values were reported for non-occupationally exposed nonsmokers in all three studies. Possible reasons for this were suggested to be alkaline rather than acid hydrolysis, leading to cleavage of *N*-acetylated metabolites; the method of detection, i.e. electrochemical or electron capture *vs* GC-MS-NICI; and non-occupational exposure to *ortho*-toluidine.

ortho-Toluidine is a major metabolite of prilocaine (*N*-[α-propyl-aminopropionyl]-*ortho*-toluidine), a widely used local anaesthetic. Gaber *et al.* (2007) assessed the impact of prilocaine-treatment on the formation of haemoglobin (Hb) adducts from *ortho*-toluidine by obtaining blood samples from 20 patients undergoing head and neck surgery and six healthy volunteers, before and 24 h after receiving local anaesthesia with prilocaine (Xylonest®, 100 mg). Hb adducts of *ortho*-toluidine and 4-aminobiphenyl were determined by GC/MS. *ortho*-Toluidine-Hb adducts were significantly increased at 24 h after prilocaine treatment by 22 ± 13 ng/g Hb ($P < 0.0001$), which corresponds to about 0.034 ± 0.021% of the prilocaine dose. This corresponds to a 6–360-fold increase of *ortho*-toluidine adduct levels in patients. Because of an extremely high background level, the increase was only 1.6-fold in one patient (40.9 ng/g before *vs* 64.4 ng/g Hb at 24 h after prilocaine injection). Self-reported smoking status and 4-aminobiphenyl-Hb adducts were used to control for smoking-related effects. Current smoking had no influence on background values or on the increase of *ortho*-toluidine adducts by prilocaine. The latter did not alter 4-aminobiphenyl-Hb adduct levels, which were significantly higher in eight smokers, 0.15 ± 0.1 ng/g Hb, than in 16 nonsmokers, 0.04 ± 0.04 ng/g Hb ($P < 0.01$). Data for *ortho*-toluidine are similar to those given in a previous report (Ward *et al.*, 1996), which found similar values of Hb adducts in smoking and nonsmoking unexposed workers; among exposed workers, smokers and non-smokers showed similar results. Since smokers have significantly higher CYP1A2 activity compared with nonsmokers (Sesardic *et al.*, 1988), the absence of any effect of smoking status on the increase of *ortho*-toluidine adducts after exposure to prilocaine does not support a role for this enzyme in activation of *ortho*-toluidine. The results are also consistent with those of studies in rats demonstrating no significant increase in *ortho*-toluidine-Hb adducts after induction of CYP1A2 by β-naphthoflavone (DeBord *et al.*, 1992). Human CYP2A6 and 2E1 have also been shown to play a role in the activation of another monocyclic amine, 2,6-dimethylaniline (Gan *et al.*, 2001).

4.1.2 *Experimental systems*

The metabolism of prilocaine in the mouse has been investigated (Akerman *et al.*, 1966). In female Sprague-Dawley rats, the tissue distribution of ^{14}C-prilocaine, dosed intramuscularly at 10 mg/kg bw, was in the following decreasing order: lung, kidney, spleen, brain, heart, liver and blood. Lung distribution was maximal after 10 min and remained at the highest level of ^{14}C-prilocaine during the entire 120 min of the study. The

rapid in-vivo decomposition of ^{14}C-prilocaine, when given intraperitoneally at 10 mg/kg bw to DSS mice, was unaffected by the CYP inhibitor SKF 525A (25 mg/kg bw, i.p.). However, SKF 525A greatly reduced the metabolism of lidocaine (a substituted xylidene, *ortho*-diethylamino-2,6-dimethylacetanilide). In experiments with rat and mouse liver slices *in vitro*, *ortho*-toluidine was tentatively identified as a metabolite of lidocaine.

The ability of *ortho*-toluidine to affect xenobiotic biotransformation in male Sprague-Dawley rats was investigated (Leslie *et al.*, 1988). After intraperitoneal injections of 10 mg/kg bw *ortho*-toluidine, daily for 7 consecutive days, there was an increase in hepatic CYP content and activities of ethoxyresorufin-*O*-deethylase, ethoxycoumarin-*O*-deethylase, and aldrin epoxidase. At 100 mg/kg bw, *ortho*-toluidine increased metabolic activity at several hydroxylation sites of androstenedione and caused a small decrease in testosterone synthesis. Administration of *ortho*-toluidine at either 10 or 100 mg/kg bw, was not associated with a change in aniline *para*-hydroxylase, epoxide hydrolase, or aminopyrine *N*-demethylase activities. Similar results were observed with male Wistar rats given intraperitoneal injections of 75 mg/kg bw *ortho*-toluidine for three consecutive days (Gnojkowski *et al.*, 1984). The hepatic activities of microsomal aryl hydrocarbon hydroxylase (predominantly catalysed by CYP1A iso-enzyme activity) and NADPH-cytochrome *c* reductase and the content of cytochrome *b5* were enhanced. No effect was observed on epoxide hydrolase, aminopyrine demethylase or glutathione *S*-transferase activities or CYP content. Another study used caffeine metabolism to investigate whether the three isomers of toluidine induce CYP1A2 activity (Jodynis-Liebert & Matuszewska, 1999). Male Wistar rats were fasted overnight and given an oral dose of 1, 10, or 60 mg/kg bw of each isomer. After 24 hours, rats were orally dosed with 10 mg/kg bw of caffeine and killed three hours later. All toluidines were inducers of CYP1A2. However, *ortho*-toluidine was the most effective with large increases observed at 1 mg/kg bw, and its effect was dose-dependent. This study is quite different from the two studies discussed above with regard to route of administration and total time of exposure to *ortho*-toluidine.

In-vivo biotransformation of *ortho*-toluidine has been assessed in male F344 rats (Son *et al.*, 1980). Following a 50- or 400- mg/kg bw subcuteneous dose of *ortho*-[methyl-^{14}C]toluidine, > 75% of the radioactivity was recovered in urine and < 3.5% in faeces after 48 hours. Major routes of metabolism were *N*-acetylation and hydroxylation at the 4-position. Minor pathways included hydroxylation at the 6-position, oxidation of the methyl group, and oxidation of the amino group. Sulfate conjugates predominated over glucuronides. Studies should be designed to determine specific P450s, *N*-acetyl-transferases, or other enzymes involved in the metabolism of *ortho*-toluidine.

4.2 Genetic and related effects

The genetic toxicology of *ortho*-toluidine has been extensively studied in two international collaborative trials for evaluation of short-term tests for carcinogens (Ashby, 1981; 1985). A review (Danford, 1991) summarized the conclusions of these trials. The genetic toxicology of *ortho*-toluidine has also been reviewed more briefly, in the context

of carcinogenesis, by Sellers and Markowitz (1992). There seems to be substantial variation in results between different laboratories and minor variations in protocols (see Table 4.1 for details and references; this Table is reproduced from Monograph Volume 77; IARC, 2000).

Most of the data from bacterial or bacteriophage assay-systems show negative or at most weakly positive results. *ortho*-Toluidine gave positive results for induction of bacteriophage lambda, but only when tested in the presence of exogenous metabolic activation (Thomson, 1981). It failed to induce SOS activity in *Salmonella typhimurium* TA1535/PSK1002 (Nakamura *et al.*, 1987). At very high concentrations (20 mg per plate), it was differentially toxic towards *Escherichia coli* strains differing in capacity for recombinational repair, in the absence of S9 mix (Rosenkranz & Poirier, 1979). However, this result was not reproduced in two further studies carried out in other laboratories, with lower concentrations (Green, 1981; Tweats, 1981). *ortho*-Toluidine gave positive results for forward mutation in recombination-deficient strains of *Bacillus subtilis* (Kada, 1981).

A large series of studies have been reported using *S. typhimurium* strains TA100, TA102, TA1535, TA1537, TA1538, TA98 and TA97. Almost all of the results were negative, although there are sporadic reports of positive responses, only in the presence of S9 mix, with strains TA100, TA98, TA1535 and TA1538 (Table 4.2).

Miller *et al.* (1986) tested the mutagenic potential of some chemical components of dental materials including *ortho*-toluidine, and found that this compound was not mutagenic in the Ames *Salmonella*/microsome mutagenicity spot test and the plate-incorporation test with tester strains TA97, TA98, TA100, and TA104, with or without S9 mix. Analogues of *ortho*-toluidine, such as *para*-toluidine and *N,N*-dimethyl-*para*-toluidine were also not mutagenic.

Gupta *et al.* (1987) tested the mutagenicity of *ortho*-toluidine and its potential metabolites in the *Salmonella*/mammalian microsome mutagenicity assay. The compound was not mutagenic in the presence or absence of rat liver S9 in *Salmonella* strains TA98 and TA100. However, its *N*-oxidized metabolites, *N*-hydroxy-*ortho*-toluidine and *ortho*-nitrotoluene showed a marked mutagenic response in TA100, but only in the presence of S9 mix at dose levels of 0.5–2.0 μM per plate; a linear dose–response curve was observed. Other potential metabolites such as *N*-acetyl-*ortho*-toluidine, *N*-acetyl-*N*-hydroxy-*ortho*-toluidine, *N*-acetoxy-*N*-acetyl-*ortho*-toluidine, 2-OH-6-methyl-acetanilide, *ortho*-azoxytoluene, and *ortho*-azotoluene were found to be inactive in TA100 and TA98, with or without activation.

In *Saccharomyces cerevisiae*, *ortho*-toluidine induced “petite” mutants in strain D5 (Ferguson, 1985) but it was negative for mitotic crossing-over and gene conversion in strain D7 (Metha and von Borstel, 1985; Brooks *et al.*, 1985). The *S. cerevisiae* deletion (DEL) assay can detect a wide variety of nonmutagenic carcinogens, including carcinogens that are not detectable with the Ames test (Schiestl *et al.* 1989). Carls and Schiestl (1994) employed the *S. cerevisiae* DEL assay to test the genotoxicity of *ortho*-toluidine, and found that it increased the frequency of DEL recombination

Table 4.1. Genetic and related effects of *ortho*-toluidine (reproduced from *IARC Monographs* Volume 77)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Prophage induction, SOS repair, DNA strand breaks or cross-links	NT	+	2500	Thomson (1981)
Prophage induction, SOS repair, DNA strand breaks or cross-links (*Salmonella typhimurium* TA1535/pSK1002)	–	–	1670	Nakamura *et al*. (1987)
Escherichia coli pol A/W3110-P3478, differential toxicity (liquid suspension tests)	–	–	250 µg/plate	Rosenkranz *et al.* (1981)
Escherichia coli rec strains, differential toxicity	+	NT	20 µL/disc	Rosenkranz & Poirier (1979)
Escherichia coli rec strains, differential toxicity	?	–	2500	Green (1981)
Escherichia coli rec strains, differential toxicity	–	–	1000	Tweats (1981)
Bacillus subtilis rec strains, forward mutation	+	+	20 µL/disc	Kada (1981)
Salmonella typhimurium, TM677, forward mutation	NT	–	500 µg/plate	Skopek *et al.* (1981)
Salmonella typhimurium, forward mutation	–	–	500 µg/plate	Liber (1985)
Salmonella typhimurium TA100, TA1535, TA1537, TA98, reverse mutation	–	–	5000 µg/plate	McCann *et al.* (1975)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1000 µg/plate	Simmon (1979)
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	1000 µg/plate	Tanaka (1980)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	10 µL/plate	Baker & Bonin (1981)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA92, TA98, reverse mutation	–	–	2000 µg/plate	Brooks & Dean (1981)
Salmonella typhimurium TA100, TA98, TA1537, reverse mutation	–	–	5000 µg/plate	MacDonald (1981)
Salmonella typhimurium TA100, TA1537, TA98, reverse mutation	–	–	1000 µg/plate	Nagao & Takahashi (1981)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	10 000 µg/plate	Richold & Jones (1981)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	2000 µg/plate	Rowland & Severs (1981)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, G46, C3076, reverse mutation	–	–	1000 µg/mL agar	Thompson *et al.* (1983)
Salmonella typhimurium TA100, TA98, TA97, TA102, reverse mutation	–	–	10 000 µg/plate	Baker & Bonin (1985)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1000 µg/plate	Falck *et al.* (1985)
Salmonella typhimurium TA100, TA102, TA98, TA97, reverse mutation	–	–	2000 µg/plate	Matsushima *et al.* (1985)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	5000 µg/plate	Rexroat & Probst (1985)
Salmonella typhimurium TA100, reverse mutation	–	+[c]	2000 µg/plate	Zeiger & Haworth (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium BA13 (L-arabinose resistance), forward mutation	NT	+	480 µg/plate	Dorado & Pueyo (1988)
Salmonella typhimurium TA1535, TA1538, reverse mutation	–	–	250 µg/plate	Rosenkranz & Poirier (1979)
Salmonella typhimurium TA1535, TA1537, TA98, reverse mutation	–	–	300 µg/mL	Gatehouse (1981)
Salmonella typhimurium TA1535, reverse mutation	–	–	1000 µg/plate	Zeiger & Haworth (1985)
Salmonella typhimurium TA1538, reverse mutation	–	–	50 µg/plate	Ferretti *et al.* (1977)
Salmonella typhimurium TA1538, reverse mutation	–	–	50 µg/plate	Garner & Nutman (1977)
Salmonella typhimurium TA1538, reverse mutation	–	+[d]	10 µg/mL	Gatehouse (1981)
Salmonella typhimurium TA98, reverse mutation	–	NT	100 µg/plate	Nagao *et al.* (1977)
Salmonella typhimurium TA98, reverse mutation	NT	–	25 µg/plate	Nagao *et al.* (1978)
Salmonella typhimurium TA98, reverse mutation	NT	+	1.3 µg/plate	Kawalek *et al.* (1983)
Salmonella typhimurium TA98, TA97, reverse mutation	–	–	500 µg/plate	Zeiger & Haworth (1985)
Escherichia coli WP2 *uvrA*, reverse mutation	–	–	1000 µg/plate	Gatehouse (1981)
	–	–	1000 µg/mL agar	Thompson *et al.* (1983)
Escherichia coli WP2 *uvrA*, reverse mutation	–	–	1000 µg/plate	Falck *et al.* (1985)
Aspergillus nidulans, forward mutation	–	NT	504	Carere *et al.* (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Aspergillus nidulans, genetic crossing-over	–	NT	2520	Carere *et al.* (1985)
Saccharomyces cerevisae, DNA repair-deficient strains, differential toxicity	+	+	300	Sharp & Parry (1981a)
Saccharomyces cerevisiae, gene conversion	–	–	333 µg/plate	Jagannath *et al.* (1981)
Saccharomyces cerevisiae, strain XV185-14C, gene conversion	–	+	2222	Mehta & von Borstel (1981)
Saccharomyces cerevisiae, gene conversion	+	NT	50	Sharp & Parry (1981b)
Saccharomyces cerevisiae, gene conversion	NT	–	2 µL/mL	Zimmermann & Scheel (1981)
Saccharomyces cerevisiae, gene conversion, reverse mutation	–	–	500	Arni *et al.* (1985)
Saccharomyces cerevisiae, gene conversion	–	–	2000	Brooks *et al.* (1985)
Saccharomyces cerevisiae, gene conversion, forward/reverse mutation	–	–	1000	Inge-Vechtomov *et al.* (1985)
Saccharomyces cerevisiae, gene conversion, reverse mutation	–	–	500	Parry & Eckardt (1985a)
Saccharomyces cerevisiae, deletion assay	+	+	1000	Carls & Schiestl (1994)
Saccharomyces cerevisiae, interchromosomal recombination	–	–	5000	Carls & Schiestl (1994)
Saccharomyces cerevisiae, forward ‘petite’ mutation	+	NT	2500	Ferguson (1985)
Saccharomyces cerevisiae, strain XV185-14C, reverse mutation	–	–	2222	Mehta & von Borstel (1981)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Saccharomyces cerevisiae, reverse mutation	–	–	21.2	Harrington & Nestmann (1985)
Saccharomyces cerevisiae, gene conversion	–	+	1512	Mehta & von Borstel (1985)
Saccharomyces cerevisiae, aneuploidy	+	+	50	Parry & Sharp (1981)
Saccharomyces cerevisiae, aneuploidy	+	+	NR	Parry & Eckardt (1985b)
Saccharomyces cerevisiae, aneuploidy	–	NT	1.5 µL/mL	Zimmermann *et al.* (1985)
Drosophila melanogaster, genetic crossing-over, somatic mutation or recombination	+		0.94 mM in feed[e]	Würgler *et al.* (1985)
Drosophila melanogaster, somatic mutation (and recombination)	(+)		10 700	Fujikawa *et al.* (1985)
Drosophila melanogaster, somatic mutation (and recombination)	+		1 mM in feed	Vogel (1985)
Drosophila melanogaster, somatic mutation (and recombination)	+		1 mM in feed	Batiste-Alentorn *et al.* (1991)
Drosophila melanogaster, somatic mutation	–		2 mM in feed	Batiste-Alentorn *et al.* (1994)
Drosophila melanogaster, somatic mutation (and recombination), wing-spot test	+		5 mM in feed	Batiste-Alentorn *et al.* (1995)
DNA strand breaks, cross-links or related damage, rat hepatocytes *in vitro*	+	NT	319	Bradley (1985)
DNA strand breaks, cross-links or related damage, Chinese hamster ovary cells *in vitro*	+	+	4280	Douglas *et al.* (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks, cross-links or related damage, Chinese hamster ovary cells *in vitro*	–	(+)	2140	Lakhanisky & Hendrickx (1985)
DNA strand breaks (Comet assay), MCL-5 cells	+	NT	454	Martin *et al.* (1999)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	500 nmol/mL	Thompson *et al.* (1983)
Unscheduled DNA synthesis, Sprague-Dawley rat primary hepatocytes	–	NT	10.7	Kornbrust & Barfknecht (1984)
Unscheduled DNA synthesis, rat primary hepatocytes	+	NT	107	Glauert *et al.* (1985)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	53.5	Probst & Hill (1985)
Unscheduled DNA synthesis, rat primary hepatocytes	–	NT	10	Williams *et al.* (1985)
Unscheduled DNA synthesis, Sprague-Dawley rat primary hepatocytes	–	NT	10.7	Barfknecht *et al.* (1987)
Unscheduled DNA synthesis, golden Syrian hamster primary hepatocytes *in vitro*	–	NT	10.7	Kornbrust & Barfknecht (1984)
Unscheduled DNA synthesis, golden Syrian hamster primary hepatocytes *in vitro*	–	NT	10.7	Barfknecht *et al.* (1987)
Gene mutation, *Hprt* locus, ouabain resistance, Chinese hamster ovary cells *in vitro*	–	–	500	Zdzienicka & Simons (1985)
Gene mutation, Chinese hamster lung V79 cells *Hprt* locus *in vitro*	–	–	2000	Fox & Delow (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Gene mutation, Chinese hamster lung V79 cells, *Hprt* locus *in vitro*	(+)	–	500	Kuroda *et al.* (1985)
Gene mutation, Chinese hamster lung V79 cells *Hprt* locus *in vitro*	–	–	10 µL/mL	Lee & Webber (1985)
Gene mutation, Chinese hamster lung V79 cells, oubain resistance *in vitro*	–	–	535	Kuroki & Munakata (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	–	1100[f]	Amacher & Turner (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus and *Hprt* locus *in vitro*	–	–	1.3 µL/mL	Knaap & Langebroek (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	–	10 µL/mL	Lee & Webber (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	+	NT	0.3 µL/mL	Myhr *et al.* (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	–	–	500[g]	Oberly *et al.* (1985)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	(+)	–	1004	Styles *et al.* (1985)
Gene mutation, mouse lymphoma L5178Y cells, ouabain or thioguanine resistance *in vitro*	–	+	200	Garner & Campbell (1985)
Gene mutation, mouse lymphoma L5178Y cells, ouabain or trifluorothymidine resistance, *in vitro*	–	–	1004	Styles *et al.* (1985)
Gene mutation, Balb/c 3T3 cells, ouabain resistance *in vitro*	NT	(+)	250	Matthews *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	+	–	300	Perry & Thomson (1981)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	–	–	1070	Douglas *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	+	+	50	Gulati *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	+	–	500	Lane *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	–	–	2140	Natarajan *et al.* (1985)
Sister chromatid exchange, Chinese hamster cells *in vitro*	+	+	268	van Went (1985)
Sister chromatid exchange, RL_4 rat liver cells *in vitro*	+	NT	21.8	Priston & Dean (1985)
Micronucleus formation, Chinese hamster ovary cells *in vitro*	–	–	1070	Douglas *et al.* (1985)
Micronucleus formation, Syrian hamster embryo cells *in vitro*	+	NT	NR	Fritzenschaf *et al.* (1993)
Chromosomal aberrations, Chinese hamster CH1-L liver fibroblasts *in vitro*	+	NT	12	Danford (1985)
Chromosomal aberrations, Chinese hamster cells *in vitro*	+	+	250	Gulati *et al.* (1985)
Chromosomal aberrations, Chinese hamster cells *in vitro*	–	+	1000	Ishidate & Sofuni (1985)
Chromosomal aberrations, Chinese hamster cells *in vitro*	–	–	2140	Natarajan *et al.* (1985)
Chromosomal aberrations, Chinese hamster cells *in vitro*	–	(+)	300	Palitti *et al.* (1985)
Chromosomal aberrations, RL_4 rat liver cells *in vitro*	+	NT	700	Priston & Dean (1985)
Aneuploidy, Chinese hamster CH1-L liver fibroblasts *in vitro*	+	NT	60	Danford (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cell transformation, C3H/10T1/2 mouse cells	–	(+)	600	Lawrence & McGregor (1985)
Cell transformation, BALB/c3T3 mouse cells	–	+[h]	150	Matthews *et al.* (1985)
Cell transformation, C3H/10T1/2 mouse cells	+	NT	500	Nesnow *et al.* (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay	+	NT	1	Barrett & Lamb (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay	+	NT	100	Sanner & Rivedal (1985)
Cell transformation, Syrian hamster embryo cells, clonal assay	+	NT	750	Kerckaert *et al.* (1998)
Cell transformation, baby hamster kidney BHK-21 cells	+	+	NG	Daniel & Dehnel (1981)
Cell transformation, baby hamster kidney BHK-21 cells	NT	+	250	Styles (1981)
Cell transformation, Chinese hamster ovary cells	–	–	0.5 µL/mL	Zdzienicka *et al.* (1985)
Cell transformation, RLV/Fischer rat embryo cells	(+)	NT	10	Suk & Humphreys (1985)
Cell transformation, SA7/Syrian hamster embryo cells	(+)	NT	965	Hatch & Anderson (1985)
Inhibition of intercellular communication, Chinese hamster V79 cells rodent *in vitro*	+	NT	5	Elmore *et al.* (1985)
Inhibition of intercellular communication, Chinese hamster V79 cells rodent *in vitro*	+	NT	5	Scott *et al.* (1985)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro*[i]	–	NT	535	Umeda *et al.* (1985)
Gene mutation, human TK6 cells *in vitro*	+	+	150	Crespi *et al.* (1985)
Gene mutation, human AHH-1 cells *in vitro*	NT	+	300	Crespi *et al.* (1985)
Sister chromatid exchange, human lymphocytes *in vitro*	?	–	100	Obe *et al.* (1985)
Sister chromatid exchange, human lymphocytes *in vitro*	+	NT	1000	Lindahl-Kiessling *et al.* (1989)
Micronucleus formation, human lymphocytes *in vitro*	+	–	2000	Vian *et al.* (1993)
Unscheduled DNA synthesis, HeLa S3 cells *in vitro*	–	+	0.05 µL/mL	Barrett (1985)
Body fluids from Sprague-Dawley rats (urine), microbial mutagenicity (*S. typhimurium* TA98)	–	+[c]	300 mg/kg bw, po × 1	Tanaka (1980)
Body fluids from WAG/Rij rats (plasma), sister chromatid exchange, Chinese hamster ovary cells *in vitro*	+		400 mg/kg bw, ip × 1	Darroudi & Natarajan (1985)
DNA strand breaks, cross-links or related damage, animal cells *in vivo*	+		100	Cesarone *et al.* (1982)
Sister chromatid exchange, $B6C3F_1$ mouse bone marrow cells *in vivo*	(+)		200	Neal & Probst (1983)
Sister chromatid exchange, animal cells *in vivo*	+		600	McFee *et al.* (1989)

Table 4.1 (contd)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Micronucleus formation, B6C3F$_1$ mice *in vivo*	–		0.338 mL/kg ip × 2	Salamone *et al.* (1981)
Micronucleus formation, CD-1 mice *in vivo*	–		0.16 mL/kg ip × 2	Tsuchimoto & Matter (1981)
Chromosomal aberrations, B6C3F$_1$ mice bone marrow cells *in vivo*	–		300 ip × 1	McFee *et al*. (1989)
Micronucleus formation, B6C3F$_1$ mice bone marrow cells *in vivo*	–		300 ip × 1	McFee *et al*. (1989)
Micronucleus test, *Pleurodeles waltl in vivo*	+		20 µg/mL	Fernandez *et al.* (1989)
Binding (covalent) to RNA or protein, Crl:CD rat liver *in vivo*	+		500 po × 1	Brock *et al*. (1990)
Sperm morphology, (CBA × BALB/c)F$_1$ mice *in vivo*	?		0.25 ip × 5	Topham (1981)
Sperm morphology, (CBA × BALB/c)F$_1$ mice *in vivo*	–		0.4 ip × 5	Topham (1980)

[a] +, positive; (+), weakly positive; –, negative; NT, not tested; ?, inconclusive; NR, not reported
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro test, µg/mL; in-vivo test, mg/kg bw/day; ip, intraperitoneal; po, oral
[c] Active only with 30% hamster liver S9; not with rat liver S9
[d] S9 from phenobarbital-treated rats
[e] Acute feeding
[f] Cytotoxic dose; higher toxicity observed with S9
[g] Toxicity higher in the presence of S9
[h] Activation by co-cultivation with X-irradiated primary rat hepatocytes
[i] Growth of V79 (T2-14) 6-thioguanine-resistant cells

Table 4.2. Genetic and related effects of metabolites of *ortho*-toluidine (reproduced from *IARC Monographs* Volume 77)

Test system	Results[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
***N*-Hydroxy-*ortho*-toluidine**				
Salmonella typhimurium TA100, reverse mutation	–	+	0.16 µg/plate	Gupta *et al*. (1987)
Salmonella typhimurium TA98, reverse mutation	–	–	0.62 µg/plate	Gupta *et al*. (1987)
***N*-Acetyl-*ortho*-toluidine**				
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	3.75 µg/plate	Gupta *et al*. (1987)
***N*-Acetyl-*N*-hydroxy-*ortho*-toluidine**				
Salmonella typhimurium TA100, reverse mutation	–	–	4.1 µg/plate	Gupta *et al*. (1987)
Salmonella typhimurium TA98, reverse mutation	–	+	2.1 µg/plate	Gupta *et al*. (1987)
***N*-Acetoxy-*N*-acetyl-*ortho*-toluidine**				
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	5.2 µg/plate	Gupta *et al*. (1987)

[a] +, positive; –, negative
[b] LED, lowest effective dose; HID, highest ineffective dose

2.6-fold in the absence of S9 and more than five-fold in the presence of S9. In the absence of S9 an increase in DEL recombination-frequency was first seen at a concentration of 3 mg/ml, whereas in the presence of S9 an increase appeared at 1 mg/ml. The frequency of intrachromosomal recombination was not increased in this test.

In *S. cerevisiae*, treatment with *ortho*-toluidine resulted in differential toxicity in repair-proficient and -deficient strains (Sharp and Parry, 1981a). However, inconsistent data were seen in all other assays with this species of yeast. Of eight assays for gene conversion carried out in different laboratories, one positive result was reported only when exogenous metabolic activation was present (Mehta and von Borstel, 1981) and another only when it was absent (Sharp and Parry, 1981b). Although *ortho*-toluidine caused a recombinogenic event leading to deletion (in either the presence or absence of exogenous metabolic activation), it failed to cause intra-chromosomal recombination in the same yeast strain (Carls and Schiestl, 1994). It was a mitochondrial "petite" mutagen (Ferguson, 1985), but failed to give a positive response for forward mutation in a nuclear gene (Inge-Vechtomov *et al.*, 1985). It gave a positive result in one of seven assays for reverse mutation. However, it caused aneuploidy in two of three assays (Parry and Sharp, 1981; Parry and Eckardt, 1985b). Assays for forward mutation or genetic crossing-over in *Aspergillus nidulans* gave completely negative results, as did a forward mutation assay in *Schizosaccharomyces pombe*.

Uniformly negative results were found for reverse mutation in *E. coli* strains WP2 or WP2 *uvrA*. Where positive responses have been seen in microbial assays, they have generally required variations to the standard test procedures, including the use of the fluctuation protocol, or incorporating the addition of norharman or lithocholic acid. High concentrations of S9 mix, or special types of S9 mix may also be important.

In the *Escherichia coli* K-12 *uvrB/recA* DNA repair host-mediated assay, *ortho*-toluidine was shown to be not mutagenic, with or without metabolic activation (Hellmér and Bolcsfoldi, 1992).

ortho-Toluidine caused DNA strand breakage in various animal cell lines *in vitro*, in the absence of exogenous metabolic activation. The alkaline single-cell gel electrophoresis (comet) assay revealed DNA breakage after *ortho*-toluidine treatment in a metabolically competent human mammary cell line, MCL-5, as well as in primary cultures of cells isolated from human breast milk. The response was substantially increased when the cells were incubated in the presence of the DNA-repair inhibitors hydroxyurea and cytosine arabinoside. Only one of eight studies showed that treatment with *ortho*-toluidine could lead to unscheduled DNA synthesis (Glauert *et al.*, 1985). A single study suggested a weak positive effect in gene mutation at the *Hprt* locus in V79 Chinese hamster cells, although two other similar studies gave negative results. *ortho*-Toluidine failed to cause mutation to ouabain resistance in V79 Chinese hamster cells (Zdzienicka and Simons, 1985). Two of six studies suggested a positive response at the *Tk* locus but not usually at other loci in mouse lymphoma L5178Y cells. However, there are isolated reports of *ortho*-toluidine increasing gene mutations at loci other than *Tk* in

L5178Y cells or in other animal cells *in vitro*, but only in the presence of exogenous metabolic activation.

There have been occasional reports of *ortho*-toluidine causing chromosomal aberrations (Danford, 1985; Gulati *et al.*, 1985; Ishidate and Sofuni, 1985; Palitti *et al.*, 1985; Priston and Dean, 1985) or micronuclei (Fritzenschaf *et al.*, 1993) in various cultured cell lines. Manifestation of these effects required incubation times longer than three hours. In some of these studies, S9 mix was required, while in others it reduced the effect. Most studies of effects on sister chromatid exchange, in either animal or human cells, have revealed positive results, even in the absence of exogenous metabolic activation. *ortho*-Toluidine caused aneuploidy in mammalian cells *in vitro* (Danford, 1985), and increased cell transformation in all but one of 11 studies. The latter effects did not generally appear to require exogenous metabolic activation, although it should be noted that the cell types have some endogenous metabolic capability. In two of three studies, *ortho*-toluidine inhibited intracellular communication (Elmore *et al.*, 1985; Scott *et al.*, 1985).

Several in-vivo studies have been conducted. *ortho*-Toluidine gave a positive result in a host-mediated assay for bacterial mutagenesis. It increased somatic mutation (Fujikawa *et al.*, 1985; Vogel, 1985) but not genetic crossing-over in *Drosophila melanogaster*, and enhanced sister chromatid exchange in rodent models (McFee *et al.*, 1989). Only one of four studies in mice and one study in a newt model (Fernandez *et al.*, 1989), suggested that it enhanced micronucleus frequency. Studies on sperm morphology have given equivocal data (Topham, 1980; 1981).

Although *ortho*-toluidine shows negative results in most genotoxicity tests, there are several reports that show positive results. The bacterial mutagenicity tests, predominantly negative, were in general agreement with other studies with monocyclic aromatic amines. Only 37 of 87 of the short-term tests used in a large collaborative study showed a positive result (Ashby *et al.*, 1985). *ortho*-Toluidine was negative in the Ames mutagenicity test (Brennan and Schiestl, 1999) but it gave positive results in assays detecting unscheduled DNA synthesis or strand breaks (Danford, 1991).

The *ortho*-toluidine metabolite 4-amino-3-methylphenol increased the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in calf-thymus DNA in the presence of Cu(II). This metabolite auto-oxidizes to form the aminomethylphenoxyl radical, which reacts with O_2 to form the superoxide ($O_2^{\cdot-}$) radical and H_2O_2. The reactive species generated by H_2O_2 in the presence of Cu (I) contributes to the formation of DNA damage. Metal-mediated DNA damage by *ortho*-toluidine metabolites through H_2O_2 may play a role in the carcinogenicity of *ortho*-toluidine (Ohkuma *et al.*, 1999).

DNA-adduct formation was measured in in-vitro and in-vivo experiments with *ortho*-toluidine and other amines. Calf-thymus DNA was modified *in vitro* by reaction with activated *ortho*-toluidine. Female Wistar rats ($n = 2$) were given a single dose of the arylamine by oral gavage and were killed after 24 hours. Hepatic DNA and DNA modified *in vitro* were hydrolysed enzymatically to individual 2′-deoxyribonucleosides. Adducts were determined by use of HPLC/MS/MS by comparison with synthesized

standards. *ortho*-Toluidine formed adducts to 2′-deoxyguanosine and 2′-deoxyadenosine after in-vitro reaction with DNA. It also formed hydrolysable haemoglobin adducts in treated rats (Jones and Sabbioni 2003).

Bolognesi *et al.* (1980) investigated the DNA damage induced by administration to mice of 2,4-dinitroaniline, *ortho*-toluidine, and *para*-toluidine. DNA damage was measured by use of the alkaline filter-elution technique. Target organs for the ultimate carcinogens in mice appear to be the liver and kidney. The DNA damage was evident four hours after administration of a single dose of *ortho*-toluidine and *para*-toluidine. The value obtained after treatment with 2.4-dinitroaniline fell within the range of controls.

Brock *et al.* (1990) studied the hepatic macromolecular binding and tissue distribution of *ortho*-toluidine and *para*-toluidine in rats. The degree of binding to hepatic macromolecules appeared to be at a maximum for both compounds at 24–48 hours following dosing. At 24 hours, the level of DNA binding of *ortho*-toluidine was about 1.2-fold lower than that of *para*-toluidine. The binding to RNA and protein was also lower for *ortho*-toluidine than for *para*-toluidine, although the difference was not as large as that observed for DNA binding. There were subtle differences in tissue distribution for each isomer.

The formation of *ortho*-toluidine-Hb adducts was first detected by Birner and Neumann (1988) in a study with rats. The HbI (haemoglobin-binding index) was in the same range as that observed for aniline and *ortho*-chlorotoluidine.

Haemoglobin (Hb) and albumin (Alb) adducts of *ortho*-toluidine were quantified in blood samples collected from rats after a single injection (Cheever *et al.*, 1992). Mild alkaline hydrolysis of Hb adducted with [^{14}C]-labelled *ortho*-toluidine followed by extraction with ethyl acetate resulted in recovery of 63% of the bound radioactivity. A single radio-labelled peak identified as *ortho*-toluidine by GC-MS was found after HPLC analysis. In subsequent experiments Hb and Alb adduct levels were determined by HPLC analysis of this cleavage product by means of fluorescence detection. The detection limit for *ortho*-toluidine was 450 pg/injection or 5 pmol/mg Hb. Mean adduct levels for Hb increased rapidly over the first four hours, with the highest level (ng/mg Hb ± SD) 3.7 ± 0.5 detected 24 hours after administration of *ortho*-toluidine at a dose of 50 mg/kg bw. The adduct levels for pooled Alb samples increased from 0.7 ng/mg Alb at two hours to 2.5 ng/mg Alb at four hours, but were not detectable at 24 hours after dosing. Hb adducts showed a linear relationship with *ortho*-toluidine doses of 10, 20, 40, 50, and 100 mg/kg bw. The Hb-adduct half-life was 11 days after a single 100 mg/kg bw dose. Hb-adduct levels were still quantifiable (1.3 ± 0.2 ng/mg Hb) by HPLC/fluorescence at 28 days after a 100-mg/kg bw dose of *ortho*-toluidine (Cheever *et al.*, 1992).

The binding characteristics of *ortho*-toluidine to rat haemoglobin (Hb) and albumin (Alb) were studied by DeBord *et al.* (1992). Sprague-Dawley rats were given [^{14}C]-labelled *ortho*-toluidine intraperitoneally at 10, 20, 40, 50, or 100 mg/kg bw, and were killed at 2, 4, 8, 18, 24, 48, or 72 hours, or 7, 14, 28 days. Haemoglobin and albumin were isolated from blood, and *ortho*-toluidine was determined by liquid scintillation counting. For albumin, a maximum binding occurred at 50 mg/kg bw at the 4-h time point (15.6 ng

ortho-toluidine/mg Alb); the maximum binding to Hb was observed at 24 h at the 100-mg/kg bw dose (23.0 ± 5.1 ng *ortho*-toluidine/mg Hb). *ortho*-Toluidine-Alb binding was not linear, but *ortho*-toluidine-Hb appeared to increase linearly in a dose-dependent manner. The biological half-lives of *ortho*-toluidine bound to Alb or Hb were observed to be 2.6 and 12.3 days, respectively, after rats were given a single dose of [^{14}C]-labelled *ortho*-toluidine. An approximately two-fold increase in radioactivity bound to Hb was observed after i.p. administration of 100 mg/kg bw [^{14}C]-labelled *ortho*-toluidine compared with oral intubation.

Suzuki *et al.* (2005) evaluated the liver and peripheral blood micronucleus assays with nine chemicals including *ortho*-toluidine in young male Fischer F344 or SD rats. *ortho*-Toluidine significantly increased the numbers of micronucleated hepatocytes (MNHEPs), micronucleated reticulocytes (MNRETs) and the number of reticulocytes (RETs) in the peripheral blood of the test animals. Results of this study were in agreement with the report that *ortho*-toluidine induced hepatocellular carcinoma and hemangiosarcoma in mice, and cancer in multiple organs in rats (IARC, 2000). However, Nakai *et al.* (1994) reported that *ortho*-toluidine was negative in a mouse bone-marrow micronucleus assay.

McFee *et al.* (1989) tested the in-vivo genotoxicity of *ortho*-toluidine in B6C3F1 mice, and found that bone-marrow cells from mice given intraperitoneal injections to up to the maximum tolerated dose of *ortho*-toluidine hydrochloride did not show an increased frequency of chromosomal aberrations or micronuclei, but the frequency of sister chromatid exchange was increased in two successive tests.

ortho-Toluidine induced an increase of intra-chromosomal recombination in *S. cerevisiae* strains RS112 and EG133 of greater than three- and six-fold at doses of 5 and 6 mg/ml, respectively, although statistical significance ($P < 0.05$) was reached only at the highest dose. The frequency of recombination was reduced by the presence of the antioxidant *N*-acetyl-cysteine. The cytotoxicity of *ortho*-toluidine was also reduced in the presence of this free-radical scavenger. Superoxide dismutase-deficient strains of *S. cerevisiae,* however, were hypersensitive to the cytotoxicity induced by *ortho*-toluidine (Brennan and Schiestl, 1999). These results indicate that the genotoxicity of *ortho*-toluidine was at least in part due to the formation of free radicals.

In *Drosophila melanogaster*, both positive and negative responses were obtained in the somatic cell w/w^{co} test with repair-proficient strains (Vogel, 1985; Würgler and Vogel, 1986). Inconclusive results were also obtained for the reversion at w/w^{z} in the excision-repair deficient *mei*-9 strain (Fujikawa *et al.*, 1985; Batiste-Alentorn *et al.*, 1991). Mutagenicity of several aromatic amines was tested by means of the w/w^{+} somatic assay of *D. melanogaster* with the wild-type strain Leiden Standard (LS) and an insecticide-resistant stock Hikone-R (HK-R). *ortho*-Toluidine was found to be positive in this test (Rodriguez-Arnaiz & Aranda, 1994). Batiste-Alentorn *et al.* (1995) further conducted the *D. melanogaster* wing-spot somatic mutation and recombination assay on 10 selected carcinogens including *ortho*-toluidine. Third-instar larvae, 72 hours old and trans-heterozygous for two recessive wing-cell markers, i.e. *multiple wing hairs (mwh*) and

flare[3] (*flr*[3]), were given three concentrations of each carcinogen in the feed during the rest of their development until pupation, and genotoxic effects were measured as significant increases in the appearance of visible mutant-hair clones on the adult wing-blade. It was found that six of the carcinogens tested, including *ortho*-toluidine, produced significant increases in wing-spot frequency.

Ward *et al.* (1996) monitored aromatic amine exposures in workers at a chemical plant with a known bladder-cancer excess. Data were obtained for a total of 73 workers, including 46 of 64 exposed workers who were employed in the rubber-chemicals department and had the potential for exposure to aniline and *ortho*-toluidine, and 27 of 52 unexposed workers employed in other departments where aniline and *ortho*-toluidine were not used or produced. They found that post-shift urinary *ortho*-toluidine concentrations averaged (± standard deviation) 2.8 μg/L (± 1.4 μg/L) in unexposed subjects and 98.7 μg/L (± 119.4 μg/L) in exposed subjects ($P = 0.0001$). Average aniline-Hb adducts and *ortho*-toluidine-Hb adducts were also significantly higher ($P = 0.0001$) among exposed workers than among unexposed control subjects (3163 pg/g ± 1302 pg/g for unexposed *vs* 17 441 ± 8867 pg/g for exposed in the case of aniline-Hb adducts; 3515 ± 6036 pg/g for unexposed *vs* 40, 830 ± 32 518 pg/g for exposed in the case of *ortho*-toluidine-Hb adducts). Average levels of adducts to *4*-aminobiphenyl, a potential contaminant of process chemicals, were not significantly different ($P = 0.48$). The adduct data suggest that among these workers, *ortho*-toluidine exposure substantially exceeded aniline exposure and that *4*-aminobiphenyl exposure, if it occurred at all, was not widespread. The authors concluded that occupational exposure to *ortho*-toluidine was the most likely causal agent of the bladder-cancer excess observed among workers in the rubber-chemicals department of the plant under study (Ward *et al.*, 1996).

Marques *et al.* (1996) investigated the ability of *N*-(acyloxy)arylamine derived from 2-, 3- and 4-methylaniline (*ortho*-toluidine is 2-methylaniline), 2,3- and 2,4-dimethyl-aniline to bind to DNA by reacting with deoxyguanosine (dG), and dG nucleotides. The predominant products from reactions with dG and the nucleotides were characterized as *N*-(deoxyguanosine-8-yl)-arylamines. Analyses of the [^{1}H]- and [^{13}C]-NMR spectra suggested that the adducts containing a methyl substituent *ortho* to the arylamine nitrogen (i.e. toluidine) had a higher percentage of *syn* conformers. With other aromatic amines, the occurrence of *syn* conformers has been associated with higher propensity for base mispairing and higher tumorigenic responses (Cho *et al.*, 1994; Eckel and Krugh 1994). It was observed that some aromatic amines containing methyl substituents in the *ortho* position tend to be more mutagenic and tumorigenic than analogues with no substituents in the *ortho* position (El-Bayoumy *et al.*, 1981; Nussbaum *et al.*, 1983). Theoretical simulation studies indicated substantial percentages of low-energy *syn* conformers, increasing with the substitution pattern in the order *para* < *meta* < *ortho* < *ortho-para* < *ortho-meta*. The results demonstrate that although single-ring arylamines are considered weak carcinogens, their electrophilic *N*-acetoxy derivatives, which are plausible metabolic intermediates, react with DNA to yield covalent adducts structurally identical to those derivatives from carcinogenic polyarylamines such as 2-aminofluorene and

4-aminobiphenyl. Furthermore, the conformational perturbation induced in DNA by the formation of monoarylamine-DNA adducts, especially those with *ortho* substituents, may contribute to the biological activities of these compounds (Marques *et al.*, 1996). [The Working Group noted as a *caveat* that these conformational studies were done with the nucleosides but not with oligonucleotides.]

Marques *et al.* (1997) further studied the effects of the substitution site on the oxidation potentials of these alkylanilines, the mutagenicities of the corresponding *N*-hydroxyalkylanilines, and the conformations of the alkylaniline-DNA adducts. It was found that the adducts from *ortho*-substituted alkylanilines may be intrinsically more mutagenic than their *meta*- and *para*-substituted analogues. There were higher percentages of low-energy *syn* conformers in the adducts that contained alkyl groups *ortho* to the arylamine nitrogen as opposed to adducts not bearing *ortho* substituents. It was suggested that the conformational properties of the DNA adducts, in particular their ability to adopt the sy*n* conformation, may be determinant factors for the genotoxic responses elicited by certain alkylanilines (i.e. *ortho*-toluidine [2-methylaniline] and 2,6-dimethylaniline). Beland *et al.* (1997) confirmed, on the basis of spectroscopic and theoretical data, that DNA adducts of these single-ring amines containing alkyl groups *ortho* to the amine function (e.g. *ortho*-toluidine) had a greater percentage of *syn* conformers around the glycosyl bond than those not bearing such groups.

4.3 Mechanistic considerations

The classification of *ortho*-toluidine as carcinogenic to humans has been controversial for some time. Recently, the classification has been upgraded to Category 1 (carcinogenic to humans) of the MAK List in Germany (DFG, 2007). Epidemiological observations were impaired by the fact that workers, for instance in the rubber industry, were usually exposed to other aromatic amines as well, mostly aniline or 4-aminobiphenyl. Haemoglobin adducts have been used to monitor rubber-industry workers from the rubber-chemical department. Their average blood levels of *ortho*-toluidine-Hb adducts were significantly higher than those in non-exposed controls, whereas 4-aminobiphenyl-Hb adduct levels were not higher than in controls (Ward *et al.*, 1996). Interestingly, the adduct levels were increased even under conditions where airborne exposures were below the OSHA time-weighted average permissible exposure limits. There has been discussion about the possibility that workers with bladder tumours could have been exposed to 4-aminobiphenyl in the 1950s and early 1960s, when diphenylamine was produced that often contained 4-aminobiphenyl as a contaminant (Freudenthal & Anderson, 1997). It remains remarkable that even today haemoglobin adducts of *ortho*-toluidine are increased at certain workplaces under improved hygiene conditions. This may partly be due to the fact that these amines are readily absorbed through the skin, particularly if the skin barrier is damaged. Frequent use of skin-barrier creams increases the absorption and internal exposure (Korinth *et al.*, 2007). Drugs may also contribute to the exposure; prilocaine, a

local anaesthetic, is hydrolysed *in vivo* and leads to a massive increase of the amount of *ortho*-toluidine-Hb adducts (Gaber *et al.*, 2007).

In line with the hypothesis that alkylation in the *ortho*-position relative to the amino group enhances the activity, *ortho*-toluidine is more potent than aniline and *para*-toluidine. It produces predominantly sarcoma in rats. The acute toxic effects of *ortho*-toluidine are comparable to those of other monocyclic aromatic amines, i.e. methaemoglobin formation, haemosiderosis, fibrosis, while both liver and kidneys are affected. In contrast to the carcinogenic potency, however, *ortho*-toluidine is less toxic than aniline, and as toxic as *para*-toluidine. The haemoglobin binding-indices for these three compounds – 4, 22 and 2, respectively – reflect this well (Neumann, 2005). It is concluded that both genotoxic and acute toxic effects are necessary to explain the experimental tumour formation induced by *ortho*-toluidine.

5. Summary of Data Reported

5.1 Exposure data

The aromatic amine *ortho*-toluidine is used in the production of dyes, pigments and rubber chemicals, and in laboratories to stain tissues. The main route of occupational exposure is by dermal contact. *ortho*-Toluidine is detected ubiquitously in the general population, but its origin is not known. It is not known to exist as a natural substance. Detectable levels of *ortho*-toluidine have been found in surface water, in effluents and soil near industrial facilities, in breast milk and in other food items. *ortho*-Toluidine is also present in tobacco smoke, although this does not appear to be a main source of exposure. *ortho*-Toluidine was detected in patients following treatment with the anaesthetic prilocaine, a metabolic precursor of *ortho*-toluidine. Analytical techniques have been developed to detect *ortho*-toluidine in very small quantities in toys.

5.2 Human carcinogenicity data

The five principal relevant cohort studies of chemical production workers available for evaluation were carried out in Germany, Italy, the United Kingdom, and the United States (two studies). Four of these studies reported highly elevated bladder-cancer risks in *ortho*-toluidine-exposed workers; the earlier study from the USA had limited power to detect any excess risk. Smoking differences could be excluded with confidence as the sole explanation of the elevated risks. In the Italian study, confounding by concomitant exposure to various recognized occupational bladder carcinogens was considered to be possible. This is not the case for the positive German, United Kingdom and US studies, because any known bladder carcinogens were present only at trace levels. In addition,

other exposures were very different from study to study. The epidemiological data provide strong evidence that *ortho*-toluidine causes bladder cancer.

5.3 Animal carcinogenicity data

ortho-Toluidine was tested for carcinogenicity as its hydrochloride salt in two experiments in mice and in four experiments in rats, and as the free base in one limited experiment in hamsters. When administered in the diet to mice, *ortho*-toluidine hydrochloride increased the incidences of hepatocellular carcinomas or adenomas and hemangiosarcomas at multiple sites. When administered in the diet to rats, *ortho*-toluidine hydrochloride increased the incidences of sarcomas of multiple organs, subcutaneous fibromas and mesotheliomas, transitional cell carcinomas of the urinary bladder, and mammary gland fibroadenomas and adenomas. When *ortho*-toluidine was administered as the free base by subcutaneous injection to hamsters, the number of tumours produced was not significantly different from that in controls. *ortho*-Nitrosotoluene, a metabolite of *ortho*-toluidine, was tested in one study in rats and one study in hamsters. When administered in the diet to rats, *ortho*-nitrosotoluene increased the incidence of fibromas of the skin and spleen, hepatocellular carcinomas and urinary bladder tumours. When administered by subcutaneous administration to hamsters, *ortho*-nitrosotoluene did not produce an increase in tumours.

5.4 Other relevant data

In contrast to most other aromatic amines, *ortho*-toluidine is metabolised in rats and humans by CYPs other than 1A2. Candidates for the responsible CYPs are CYP2A6 and 2E1. The major routes of metabolism (assessed in the rat) were *N*-acetylation and hydroxylation at the 4-position. The oxidation of the amino group was a minor pathway. Adducts to haemoglobin, albumin and DNA are formed in rodents after *ortho*-toluidine administration *in vivo*. An additional pathway to DNA-damage induction by *ortho*-toluidine is inferred by the observation that the *ortho*-toluidine metabolite 4-amino-3-methylphenol increased the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in calf thymus DNA in the presence of Cu(II). The levels of *ortho*-toluidine-Hb adducts were tenfold higher among exposed workers than among unexposed control subjects. Prilocaine, a widely used local anaesthetic, is metabolized to *ortho*-toluidine, leading to *ortho*-toluidine-Hb adducts after prilocaine treatment. In line with what is generally observed with monocyclic aromatic amines, *ortho*-toluidine was inactive in most bacterial genotoxicity tests, although there are a few reports showing positive results, most of them either at very high doses or after introduction of variations in the standard test procedures. *ortho*-Toluidine produced increases in intra-chromosomal recombination in *S. cerevisiae*. In cultured mammalian cells, *ortho*-toluidine showed predominantly negative results with some exceptions: in liver and peripheral blood of rats *ortho*-toluidine significantly increased the number of micronucleated hepatocytes and micronucleated reticulocytes.

DNA damage measured by the alkaline filter-elution technique was induced by administration of *ortho*-toluidine to mice. In line with the hypothesis that alkylation in the *ortho*-position to the amino group enhances carcinogenicity, *ortho*-toluidine is a more potent animal carcinogen than are aniline and *p*-toluidine. Both genotoxicity and acute toxic effects, necessary to explain the experimental tumour formation by *ortho*-toluidine, have clearly been shown.

6. Evaluation

6.1 Cancer in humans

There is *sufficient evidence* in humans for the carcinogenicity of *ortho*-toluidine. *ortho*-Toluidine causes cancer of the urinary bladder.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of *ortho*-toluidine.

6.3 Overall evaluation

ortho-Toluidine is *carcinogenic to humans (Group 1).*

The Working Group was aware of the existence of numerous dyes and colourants that contain *ortho*-toluidine as a structural element, but a full evaluation of this group of dyes was beyond the scope of this Monograph. The local anaesthetic prilocaine, which is metabolized to *ortho*-toluidine, has been shown to cause methaemoglobinaemia and haemoglobin-adduct formation in treated patients.

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