EVIDENCE ON THE CARCINOGENICITY OF

5-CHLORO-*O*-TOLUIDINE
AND ITS STRONG ACID SALTS

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PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code 25249.5 *et seq.*) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The Act specifies that "a chemical is known to the state to cause cancer or reproductive toxicity...if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity." The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment of the California Environmental Protection Agency. The "state's qualified experts" regarding findings of carcinogenicity are identified as the members of the Carcinogen Identification Committee of the OEHHA Science Advisory Board (22 CCR 12301).

5-Chloro-*o*-toluidine was discussed as a high priority candidate for Committee review during a public committee meeting held in Sacramento, California, on July 22, 1996 and at a public workshop held November 15, 1996. Public input was solicited on the priority of this chemical in two public comment periods, each of 60 days duration. Once the chemical was selected for Committee review, a public request for pertinent information was made.

This draft document *Evidence on the Carcinogenicity of 5-Chloro-o-Toluidine and Its Strong Acid Salts* was developed to provide the Committee with relevant information for use in its deliberations, and reviews the available scientific evidence on the carcinogenic potential of 5-chloro-*o*-toluidine and its strong acid salts. The meeting where the Committee is to discuss this evidence has been tentatively scheduled for September 25, 1997. Written public comment on the document should be submitted to OEHHA by August 20, 1997, if it is to be considered by the Committee in advance of the meeting. During the September meeting, the public will be given the opportunity to present verbal comments to the Committee.

1 EXECUTIVE SUMMARY

5-Chloro-*o*-toluidine (CAS number 95-79-4) and its hydrochloride salt are used in dye-stuff manufacture, as chemical intermediates in the synthesis of other dyes, and as dyes for cotton, silk, nylon and cellulose. Strong acid salts of 5-chloro-*o*-toluidine, including the hydrochloride, can be expected to exist in a dissociated state in solution and *in vivo*, and thus are considered toxicologically equivalent to the free or protonated amine forms. 5-Chloro-*o*-toluidine has been shown to induce tumors at multiple sites in both sexes of the mouse when administered in the diet. A high incidence of hemangiosarcomas, a relatively uncommon tumor of the vasculature, was observed in both male and female mice. Significantly increased incidences of hepatocellular carcinomas and hepatocellular carcinomas and adenomas (combined) were also observed in male and female mice. There was no evidence of carcinogenicity in female rats fed 5-chloro-*o*-toluidine in the diet. Findings in the male rat were equivocal; however, it is likely that the doses of 5-chloro-*o*-toluidine administered to male rats were below the maximally tolerated dose range.
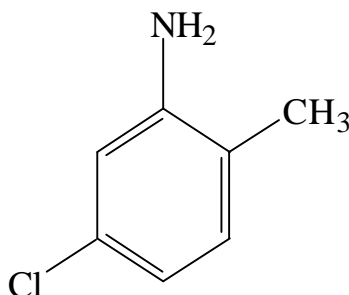
Based on the information reviewed in the preparation of this document, there is evidence for the carcinogenicity of *5-chloro-o-toluidine and its strong acid salts* at multiple sites in both sexes of the mouse. Chemical structural analogies with known carcinogens contribute to the weight of evidence.

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2   INTRODUCTION

2.1   Identity of 5-chloro-*o*-toluidine

5-Chloro-*o*-toluidine : $ClC_6H_3(CH_3)NH_2$
Molecular Weight = 141.6   CAS Registry No. 95-79-4

Synonyms: 5-chloro-2-methylbenzenamine;   5-chloro-2-methylaniline;
            p-chloro-*o*-aminotoluene; 4-chloro-2-aminotoluene; C.I. Azoic
            Diazo Component 32.

With respect to nomenclature, the term *toluidine* refers to methylaniline
compounds.   For methylanilines, the spatial relationships of the methyl,
chlorine, and amino groups are expressed relative to the primary amine.
Other chemical names are also correct; for example, methylaniline is also
aminotoluene (see Synonyms above). Caution should be taken when reading
the chemical name of a toluidine, because the numbering system will depend
on the particular nomenclature system that is used.

5-Chloro-*o*-toluidine is a monocyclic aromatic amine and is a grayish-white
solid that is soluble in hot alcohol (HSDB, 1997). The melting point is 26°C
and the boiling point is 239°C (Lide, 1996); the melting point of the
hydrochloride is 265-267°C (Henschler, 1994). The octanol-water partition
coefficient (K_{ow}) of 5-chloro-*o*-toluidine has not been measured. Estimates of
log K_{ow} based on properties of individual chemical groups range from 2.20 to
2.58 (HSDB, 1997; Arnold *et al.*, 1990). 5-Chloro-*o*-toluidine, like other
primary amines, undergoes oxidation when exposed to air. The hydrochloride

salt is not oxidized in air (Matthews *et al.*, 1993). The hydrochloride and other strong acid salts can be expected to exist in a dissociated state in solution and *in vivo,* and thus are considered toxicologically equivalent to the free or protonated amine forms.

2.2   Occurrence and use

5-Chloro-*o*-toluidine is one of several structurally related aromatic amines used in the dye industry. More specifically, 5-chloro-*o*-toluidine and its hydrochloride salt are used in dye-stuff manufacture, as chemical intermediates in the synthesis of other dyes, and as dyes for cotton, silk, nylon and cellulose (NCI, 1979a). Both 5-chloro-*o*-toluidine and its hydrochloride salt are known as C.I. Azoic Diazo Component 32, which is a component of azoic dyes (NCI, 1979a). Azoic dyes are formed on textile fibers, especially cellulose, by the reaction of selected diazo and coupling components.

The U.S. production level for 5-chloro-*o*-toluidine reported in the U.S. Environmental Protection Agency's (EPA) 1990 Toxic Substances Control Act (TSCA) Inventory Update was 57,378 pounds (Sherlock, 1995). Readily absorbed by the skin and by inhalation, exposure is thought to be limited primarily to workers in the chemical and dye manufacturing and textile industries (NCI, 1979a; Parmeggiani, 1983). Releases to the environment may occur during production and use; 5-chloro-*o*-toluidine has been detected in tap water drawn from the Rhine in the Netherlands (HSDB, 1997).

3   DATA ON 5-CHLORO-O-TOLUIDINE CARCINOGENICITY

3.1   Epidemiological studies of carcinogenicity in humans

No data on long-term effects of human exposure to 5-chloro-*o*-toluidine were found in literature searches performed by OEHHA. One early occupational study incorrectly identified 5-chloro-*o*-toluidine as the chemical to which workers were exposed (Currie, 1933). The chemical structure and the melting and boiling points reported by the author indicate that the chemical was 4-chloro-*o*-toluidine.

Epidemiological studies on the structurally related primary arylamine 4-chloro-*o*-toluidine suggest that this structural isomer increased the risk of bladder cancer in those occupationally exposed (Stasik, 1988 and 1991; Popp *et al.,* 1992). The International Agency for Research on Cancer (IARC, 1990) found that there was limited human evidence for the carcinogenicity of 4-chloro-*o*-toluidine on the basis of findings of bladder and other cancers in the epidemiological studies reviewed.

3.2   Carcinogenicity studies in animals

The National Cancer Institute conducted carcinogenicity studies in which rats and mice of both sexes received 5-chloro-*o*-toluidine in the diet (NCI, 1979a). Statistically significant increases in hemangiosarcomas and liver tumors were observed in male and female mice fed 5-chloro-*o*-toluidine. In rats, no statistically significant increases in treatment-related tumors were observed; however, a statistically significant dose-related trend was observed for adrenal pheochromocytomas in male rats. The NCI concluded that 5-chloro-*o*-toluidine "was carcinogenic to $B6C3F_1$ mice, inducing hemangiosarcomas and hepatocellular carcinomas in both males and females" (NCI, 1979a). "There was no conclusive evidence of the carcinogenicity of the compound in Fischer 344 rats" (NCI, 1979a). The NCI carcinogenicity studies for 5-chloro-*o*-toluidine and results are described in greater detail below.

The findings of carcinogenicity for 5-chloro-*o*-toluidine are similar to those for the 4-chloro analog. In dietary studies of 4-chloro-*o*-toluidine, hemangiosarcomas or hemangiomas were observed in male and female CD-1 albino and $B6C3F_1$ mice (Weisburger *et al*., 1978; NCI, 1979b); and adrenal pheochromocytomas were observed to increase with dose in male Fischer rats ($p = 0.014$ Cochran Armitage trend test) (NCI, 1979b). In addition, chromophobe adenomas of the pituitary gland were observed to occur in both male and female rats receiving 4-chloro-*o*-toluidine (NCI, 1979b), an observation not made for the 5-chloro analog.

Rat dietary exposure: NCI, 1979a

Groups of 50 male and 50 female Fischer 344 rats received 2500 ppm or 5000 ppm 5-chloro-*o*-toluidine in the diet for 78 weeks, followed by a 26 week observation period. Control groups for each sex, containing 20 animals, received plain diet. The design of this study, one of the early series

conducted by NCI, is not consistent with the standard protocol used by the National Toxicology Program. The control groups had 20 rather than 50 animals, and the test animals were housed in the same room as animals exposed to several other potentially carcinogenic test materials. Thus studies run under current protocol would have greater power than the NCI studies.

The test material was technical-grade 5-chloro-*o*-toluidine, purity unspecified; however, analysis of the test material by gas chromatography yielded a single peak (NCI, 1979a).

In male rats a significant positive association between administered dose and the incidence of adrenal pheochromocytomas was observed (0/20 controls; 2/49 low-dose group; 7/48 high-dose group). By trend test the result was statistically significant (Cochran-Armitage trend test, $p = 0.019$), however, a pairwise comparison between high dose and control group was only marginally significant by the Fisher exact test ($p = 0.076$). While suggestive of a treatment-related effect, the NCI found the result not sufficient to conclude that the increase in adrenal pheochromocytomas in the male rat is attributable to the carcinogenic action of 5-chloro-*o*-toluidine. No other significant positive association was observed for tumors at any site in male or female rats. Body weight gain was delayed in the female animals, suggesting that the doses administered were in the maximally tolerated range for females. Males experienced no delay in body weight gain, nor did they exhibit other signs of toxicity, suggesting that the doses administered were below the maximally tolerated dose range for male rats.

Mouse dietary exposure: NCI, 1979a

Groups of 50 male and 50 female B6C3F$_1$ mice received 2000 ppm or 4000 ppm 5-chloro-*o*-toluidine in the diet for 78 weeks followed by a 13 week observation period. Control groups for each sex, containing 20 animals, received plain diet. The design of this study, one of the early series conducted by NCI, is not consistent with the standard protocol used by the National Toxicology Program. The control groups had 20 rather than 50 animals, and the test animals were housed in the same room as animals exposed to several other potentially carcinogenic test materials. Thus studies run under current protocol would have greater power than the NCI studies.

The test material was technical-grade 5-chloro-*o*-toluidine, purity unspecified; however, analysis of the test material by gas chromatography yielded a single peak (NCI, 1979a).

In both males and females, body weight gain was delayed by the same amount in both dose groups, and survival was reduced in a dose-dependent manner, suggesting that the administered doses were in the maximally tolerated range. Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumors. A significant positive association between administered dose and the incidence of hemangiosarcomas was observed in both male and female mice (Cochran-Armitage trend test; $p < 0.001$). The increased incidence of hemangiosarcomas in high-dose animals of both sexes was statistically significant as compared to controls (Fisher exact test; $p < 0.001$). Hemangiosarcomas were found most frequently in the periepididymal adipose or adjacent pelvic tissues in males and in the periuterine adipose tissue in females; less frequently they were found in the spleen, perirenal adipose tissue, or muscle. In addition, metastases of primary hemangiosarcomas were observed in treated mice of both sexes. The incidences of hepatocellular carcinomas and hepatocellular adenomas and carcinomas combined were significantly increased in both males (high-dose only) and females (low- and high-dose groups). The dose-related trends for hepatocellular carcinoma and for hepatocellular adenomas and carcinomas combined were also significant and positive in both sexes. Tumor incidences are reported in detail in Table 1.

Table 1: **Tumors in B6C3F$_1$ mice receiving 5-chloro-*o*-toluidine (2000 ppm or 4000 ppm in the diet) for 78 weeks followed by 13 weeks observation.**

		Dose, ppm[a]		
Tumor Site and Type		0	2000	4000
Males				
All Sites	Hemangiosarcoma	1/20[c]	11/50	37/48[f]
Liver	Hepatocellular carcinoma	4/20[d]	19/50	25/47[g]
	Hepatocellular adenomas and carcinomas (combined)	4/20[e]	20/50	27/47[h]
Females				
All Sites	Hemangiosarcoma	0/20[c]	6/50	22/43[f]
Liver	Hepatocellular carcinoma	0/20[c]	19/50[f]	26/43[f]
	Hepatocellular adenomas and carcinomas (combined)	0/20[c]	21/50[f]	31/43[f]

[a]   Concentration of 5-chloro-*o*-toluidine in feed.

[b]   Number of lesion-bearing animals/total examined.

[c, d, e]   Dose-related trend was significant by the Cochran-Armitage trend test ([c] $p < 0.001$; [d] $p = 0.007$; [e] $p = 0.003$).

[e, f, h]   Incidence relative to control group was significant by the Fisher Exact test ([f] $p < 0.001$; [g] $p = 0.011$; [h] $p = 0.005$).

3.3   Other relevant data

In addition to the reported animal bioassays, additional evidence relating to the possible carcinogenicity of 5-chloro-*o*-toluidine is available.   The evidence includes studies on genetic toxicity, cell transformation and macromolecular binding, predicted pharmacokinetics, and structure-activity comparisons.

3.3.1 Genetic toxicology

Although chemical structure features predict that 5-chloro-*o*-toluidine would be genotoxic (Ashby and Tennant, 1988), this has not been demonstrated experimentally.

Bacterial systems

One report of positive mutagenic activity by 5-chloro-*o*-toluidine in *S. typhimurium* has been identified in the published literature, although the results were cited only as unpublished data (Seiler, 1977). Haworth *et al*. (1983) did not find 5-chloro-*o*-toluidine mutagenic in *S. typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, using a preincubation suspension procedure in either the presence or absence of a metabolically active liver extract from Aroclor treated rats or hamsters (liver S9). Similarly, Ono *et al*. (1992) reported negative findings for TA 1535 with and without metabolic activation. In reporting their results, Haworth *et al*. stated that negative results obtained under preincubation suspension conditions should be confirmed with a plate incorporation procedure, which permits testing at higher doses. Such plate incorporation studies with 5-chloro-*o*-toluidine have not been published. However, increased mutagenicity in *S. typhimurium* was observed with a closely related toluidine analog, 4-chloro-*o*-toluidine, when metabolic activation took place under plate incorporation conditions as compared to a preincubation procedure (Zimmer *et al*., 1980).

5-Chloro-*o*-toluidine was not observed to induce prophage lambda in *E. coli* in the presence or absence of rat liver S9 (DeMarini and Brooks, 1992). Inspection of the data reveals that for 5-chloro-*o*-toluidine, a dose-dependent decrease in induced PFUs/plate occurred. Such a decrease may indicate that unobserved toxicity was occurring at the lower doses.

In vitro mammalian systems

McGregor *et al.* (1988) reported that *in vitro* 5-chloro-*o*-toluidine was not mutagenic in a mouse lymphoma cell forward mutation assay; however, the chemical was toxic in four of the six assays conducted using doses ranging from 250-325 ug/ml, and among these four assays, lethality was present in two. Yoshimi *et al.* (1988) reported that 5-chloro-*o*-toluidine did not meet the criterion for a positive response in an *in vitro* unscheduled rat hepatocyte DNA synthesis (UDS) assay. 5-Chloro-*o*-toluidine, at 1.6-16 ug/ml and in the absence or presence of liver S9, did not induce sister chromatid exchange or chromosome aberrations in an *in vitro* Chinese hamster ovary cell assay (Galloway *et al.,*1987). The 4-chloro- analogue was genotoxic in these assays when tested at higher doses (Appendix A).

In vivo mammalian systems

Seiler (1977) tested several compounds, including 5-chloro-*o*-toluidine, for their abilities to inhibit testicular DNA synthesis in the mouse. 5-Chloro-*o*-toluidine (200 mg/kg bw, ingestion) caused a 52% decrease in the *in vivo* thymidine incorporation into mouse testicular DNA. Seiler (1977) found that *in vivo* inhibition of testicular DNA synthesis was strongly correlated with carcinogenic or mutagenic activity.

Structurally related toluidines, in particular 4-chloro-*o*-toluidine, have been tested in several short-term tests for genotoxicity. The results of these investigations are summarized in Appendix A.

3.3.2 Other short term tests

Cell proliferation

Miyagawa *et al.* (1995) tested several Ames-negative (i.e., nonmutagenic in the *S. typhimurium* assay) mouse hepatocarcinogens, including 5-chloro-*o*-toluidine, for the ability to induce replicative DNA synthesis (RDS) in an *in vivo-in vitro* isolated mouse hepatocyte system. A single exposure of mice by gavage to 50 or 100 mg/kg 5-chloro-*o*-toluidine resulted in increased RDS by the isolated hepatocytes (Miyagawa *et al.*, 1995). The highest dose tested

was one half of the LD_{50}. The induction of RDS has been shown to be positively correlated with carcinogenic activity in rodent bioassays for Ames-negative chemicals (Uno *et al.*, 1994).

Cell transformation

Matthews *et al.* (1993) reported that 5-chloro-*o*-toluidine transformed mouse BALB/c-3T3 cells. As discussed in more detail in Appendix A, the chemically related toluidines *o*-toluidine, 3-chloro-*p*-toluidine, and 4-chloro-*o*-toluidine also tested positive in the standardized protocol. Similar to the findings in other cell systems, the toluidines were cytotoxic to mouse BALB/c-3T3 cells under the conditions of this assay (Matthews *et al.*, 1993).

Protein binding

5-Chloro-*o*-toluidine binds covalently to Wistar rat and B6C3F$_1$ mouse hemoglobin *in vivo*, presumably through an arylnitroso metabolite (Birner and Neumann, 1988; Sabbioni and Sepai, 1995). The metabolic pathways leading to formation of the hemoglobin binding species (*i.e.,* an arylnitroso compound) and the DNA binding species (*i.e.,* a nitrenium ion) share an arylhydroxylamine as a common precursor (Damani, 1982; Sabbioni and Sepai, 1995). Thus the presence of hemoglobin binding is indicative of the potential for DNA binding via formation of a putative DNA reactive species.

3.3.3 Pharmacokinetics and Metabolism

No published studies on 5-chloro-*o*-toluidine pharmacokinetics or metabolism were found. Information in the published literature however suggests primary arylamines share common metabolic features, in particular reaction at the primary amino nitrogen.

Primary arylamines undergo enzyme catalyzed oxidation reactions which result in the formation of arylhydroxylamines. The reaction takes place at the amino nitrogen under conditions where the unprotonated form predominates (Damani, 1982; Nelson, 1985). Cytochrome P450-dependent monooxygenases (P450) participate in the metabolism of primary arylamines, but other enzymes (*e.g.*, peroxidases) may also play a role (Nelson, 1985). Ring hydroxylation may occur, but it is diminished if substituents occur on the

ortho (-*o*-) and para (-*p*-) positions (Damani, 1982). The arylhydroxylamine metabolite common to primary arylamine metabolism may be converted to a resonance stabilized nitrenium ion, and this DNA reactive electrophile is suggested to be responsible for arylamine carcinogenicity (Damani, 1982; Nelson, 1985; Sabbioni and Sepai, 1995). Arylhydroxylamines may be further bioactivated by O-sulfation or O-glucuronidation, although activation through sulfation may be predominant (Hanna and Banks, 1985). The glucuronide conjugates can undergo hydrolysis in an acidic environment (*e.g.*, the bladder) to regenerate the free arylhydroxylamine (Damani, 1982). Arylhydroxylamines may also undergo further oxidation to an arylnitroso compound, which is considered responsible for some non-cancer toxicities and protein binding (Nelson, 1985; Sabbioni and Sepai, 1995).

Metabolism of aromatic amines by acetylation of the primary amino group also occurs. Acetylation frequently represents a detoxification pathway, however, acetylation products may also undergo subsequent activation to putative DNA reactive species (i.e., nitrenium ions) (Nelson, 1985). N-acetylation is polymorphic in many animals including humans and the extent of such polymorphism is dependent on the substrate and the ethnic and geographic characteristics of the population (Weber and Hein, 1985).

Other metabolites of primary arylamines include acetanilides, anthranilic acides, and azo condensation products arising from the reaction of nitroso and arylhydroxylamine species (Son *et al.*, 1980; Boeren *et al.*, 1992; Knowles and Gupta, 1970).

Studies on the metabolism of chlorotoluidines other than the 5-chloro-*o*-toluidine are briefly summarized in Appendix B.

3.3.4 *Structure-Activity comparisons*

Considerable literature exists that documents the experimental carcinogenicity and mutagenicity of aromatic amines. Comparison of the carcinogenic and mutagenic activities of several monocyclic primary aromatic amines, in particular *o*-toluidine and 4-chloro-*o*-toluidine, suggests that these compounds may share common modes of action with 5-chloro-*o*-toluidine. Relevant

studies of the carcinogenicity, genotoxicity, macromolecular binding activity and metabolism of selected toluidines are briefly summarized below.

Carcinogenicity

Carcinogenicity bioassays on *o*-toluidine, 4-, and 5-chloro-*o*-toluidine have been carried out by NCI in mice and rats (NCI, 1979a,b,c; Weisburger, 1983). Whereas 4- and 5-chloro-*o*-toluidine were clearly carcinogenic in mice only, *o*-toluidine was carcinogenic in both species. A commonality in the types of tumors induced in mice was observed between the three compounds. *o*-Toluidine induced liver tumors in female mice and tumors of the blood vessels in male mice (NCI, 1979c), 4-chloro-*o*-toluidine induced tumors of the blood vessels in male and female mice (NCI, 1979b), and 5-chloro-*o*-toluidine induced tumors of the blood vessels and the liver in both sexes of the mouse (NCI, 1979a). These data suggest that the presence and position of the chlorine atom affects the carcinogenic outcome and indicate that the three toluidines share similar carcinogenic properties.

In general, the level of carcinogenicity concern of a monocyclic aromatic amine is determined by considering several factors, including the presence or absence of heteroatoms in the ring, the number and position of amino groups, and the nature, number and position of additional substituents. The structure-activity prediction program Oncologic, which is based on a set of rules generated by USEPA experts from data on known carcinogens, indicates a "High-Moderate" concern for carcinogenicity to 5-chloro-*o*-toluidine. Assignment of this level of concern is based on the following considerations, 1) the methyl group ortho to the amino group indicates a high-moderate level of concern, and 2) the additional chloro group generally raises the level of concern. A "High-Moderate" level of concern is the highest level found for agents, such as 5-chloro-*o*-toluidine, which do not actually appear in the program's source database of known carcinogens.

Genotoxicity and other short-term studies

Using a plate incorporation protocol, mutagenicity towards *S. typhimurium* was observed for halogenated toluidines in the presence of liver S9 in three studies. Specifically, 4-chloro-*o*-toluidine and 2-chloro-*p*-toluidine were mutagenic in strain TA100 (in the presence of rat or mouse S9) (Zimmer *et*

al., 1980), and 4-chloro-*o*-toluidine was mutagenic in strain TA1535, but not in *E. Coli* WP$_2$ (Rashid *et al*., 1984), and in strains TA98 and TA100 (Ggglemann *et al*., 1996). 4-Chloro-*o*-toluidine-associated toxicity was observed at high doses in the latter study (Ggglemann *et al*., 1996). Using a preincubation suspension system, Zimmer *et al.* (1980) and Haworth *et al*. (1985) reported that 4-chloro-*o*-toluidine was negative in the *S. typhimurium* mutagenicity assay.

Neither 3-chloro-*p*-, 4-, nor 5-chloro-*o*-toluidine induced the synthesis of DNA repair enzymes in *S. typhimurium* strain TA1535, in either the presence or absence of metabolic activation (Ono *et al*., 1992). *In vitro* exposure of rat hepatocyte cultures to 2-chloro-*p*-toluidine, but not to 2-chloro-*m*-toluidine or 5-chloro-*o*-toluidine resulted in UDS (Yoshimi *et al*., 1988). Prophage λ was not induced in *E.Coli* by either 3-chloro-*p*-toluidine or 5-chloro-*o*-toluidine (with or without liver S9); however, both compounds were toxic at the highest dose and an inverse dose response relationship was observed for 5-chloro-*o*-toluidine (DeMarini and Brooks, 1992).

5-Chloro-*o*-toluidine, 4-chloro-*o*-toluidine, 3-chloro-*p*-toluidine, and *o*-toluidine each tested positive in a standardized BALB/c-3T3 cell transformation protocol (Matthews *et al.,* 1993). 4-Chloro-*o*-toluidine, but not the non-halogenated toluidines, caused DNA damage in hamster lung fibroblasts (Zimmer *et al*., 1980). 4-Chloro-*o*-toluidine induced sister chromatid exchange and chromosomal aberrations in hamster ovary cells, but 5-chloro-*o*-toluidine did not when tested at considerably lower doses (Galloway *et al*., 1987).

The combined results from genotoxicity and other short-term studies with the chlorinated toluidines indicate that these compounds have produced inconsistent and variable responses in these assays. Possible explanations for these inconsistencies include the different sensitivities of preincubation suspension and plate incorporation protocols, and the expression of chemical toxicity in several of the studies.

Macromolecular binding

Birner and Neumann (1988) reported nearly identical *in vivo* rat hemoglobin binding indices (HBI) for 5-chloro-*o*-toluidine and 4-chloro-*o*-toluidine. The

rat HBIs for *o*-toluidine and 6-chloro-*o*-toluidine were each lower by roughly a factor of 5. In mice the HBIs for *o*-toluidine, 4-chloro-*o*-toluidine, and 5-chloro-*o*-toluidine were similar (6-chloro-*o*-toluidine was not tested in mice). Although the binding of chlorotoluidines to hemoglobin does not itself reflect DNA reactivity, both macromolecular binding end-points appear to require a common precursor metabolite, an arylhydroxylamine (Damani, 1982; Sabbioni and Sepai, 1995).

Metabolism

Metabolism studies of three toluidine compounds in the rat suggest that the same metabolic pathways are involved in the metabolism of these compounds, and that similar critical metabolites are formed, such as the arylhydroxylamines, which are precursors of DNA reactive electrophilic nitrenium ions (Damani, 1982, Sabbioni and Sepai, 1995). Exposure of rats to *o*-toluidine (Son *et al.*, 1980), 2-chloro-*p*-toluidine (Boeren *et al.*, 1992), or 4-chloro-*o*-toluidine (Knowles and Gupta, 1970; Hill *et al.*, 1979) resulted in a complex metabolite profile that included arylhydroxylamines. The arylhydroxylamines were found either uncombined or as part of a condensation product. Other metabolites included methyl group hydroxylation and oxidation products, condensation products, glucuronide and sulfate conjugates, and in the case of the non-halogenated *o*-toluidine, ring hydroxylation products. More detailed descriptions of these studies are found in Appendix B.

3.3.5 Pathology

The liver tumors observed in male and female mice were diagnosed as hepatocellular adenomas and carcinomas (NCI, 1979a). It is generally considered that these tumor phenotypes are related in origin, and that the adenomas may progress to carcinomas. They are therefore usually aggregated for carcinogen identification and risk assessment purposes. The vascular tumors observed in male and female mice were diagnosed as hemangiosarcomas (NCI, 1979a). Occurring primarily in the periepidydimal fat or adjacent pelvic tissues in males and in the periuterine pelvic fat in females, hemangiosarcomas were also found in the spleen, perirenal tissue and skeletal muscle. These tumors were highly malignant; many were locally

invasive and metastases to the lung, heart and spleen were frequently observed (NCI, 1979a).

3.4   Mechanism

The types of tumors observed to occur in mice exposed to 5-chloro-*o*-toluidine are consistent with the animal cancer bioassay findings of other aromatic amines. The most common tumors induced in mice by aromatic amines are liver tumors, followed by tumors of the blood vessels (i.e., hemangiomas, hemangiosarcomas) (Weisburger, 1983). More specifically, the induction by 5-chloro-*o*-toluidine of liver and blood vessel tumors in both sexes of the mouse is consistent with the carcinogenic action of the closely related isomer 4-chloro-*o*-toluidine, which increased the incidence of blood vessel tumors in male and female mice (NCI, 1979b), and with the non-chlorinated analogue *o*-toluidine, which increased the incidence of liver tumors in female mice and blood vessel tumors in male mice (NCI, 1979c). Although no epidemiologic data were available on 5-chloro-*o*-toluidine-exposed individuals, human cancers are associated with exposure to other aromatic amines, with bladder tumors being the most frequent tumor type observed (Weisburger, 1983). Thus, the positive carcinogenicity findings in male and female mice are interpreted as an indication of the general, rather than species- or tissue-specific carcinogenic activity of 5-chloro-*o*-toluidine.

There are only a handful of published studies on 5-chloro-*o*-toluidine that address issues of mechanism, and none on metabolism, thus the mechanism by which 5-chloro-*o*-toluidine induces tumors in mice remains unknown. By analogy with other carcinogenic aromatic amines, it may be hypothesized that 5-chloro-*o*-toluidine undergoes bioactivation to a DNA-reactive metabolite, and that the liver and blood vessel tumors occur as a consequence of genotoxicity. At the present time, however, there is little evidence to unequivocally support or to challenge this hypothesis. The available relevant data are summarized below.

Although chemical structure features predict that 5-chloro-*o*-toluidine is genotoxic (Ashby and Tennant, 1988), the mutagenicity and clastogenicity studies conducted in bacteria and cultured mammalian cells and published to date have been negative (Haworth *et al.*, 1983; Ono *et al.*, 1992; DeMarini

and Brooks, 1992; McGregor *et al*., 1988; Galloway *et al*., 1987). These negative responses may be due, in part, to the observed toxicity of the compound in these *in vitro* systems (Haworth *et al*., 1983; DeMarini and Brooks, 1992; McGregor *et al*., 1988) and, in the case of the *S. typhimurium* reverse mutation studies, to the apparent insensitivity of suspension preincubation protocols, as compared with plate incorporation protocols (Haworth *et al*., 1983; Zimmer *et al*., 1980). In three independently conducted *S. typhimurium* reverse mutations assays utilizing a plate incorporation procedure 4-chloro-*o*-toluidine tested positive (Zimmer *et al*., 1980; Rashid *et al*., 1984; Gggelmann *et al*., 1996) and negative in assays utilizing suspension procedures (Haworth *et al*., 1983; Zimmer *et al*., 1980). To date, no mutagenicity studies in *S. typhimurium* of 5-chloro-*o*-toluidine utilizing plate incorporation procedures have been published.

5-Chloro-*o*-toluidine has been shown to interfere with DNA metabolism, inhibiting thymidine incorporation into mouse testicular DNA *in vivo* (Seiler, 1977) and increasing replicative hepatocyte DNA synthesis *in vitro* (Miyagawa *et al*., 1995), and to transform mouse cells in culture (Matthews *et al*., 1993).

No data on the ability of 5-chloro-*o*-toluidine to bind to DNA have been reported, however, *in vivo* studies with mice and rats have demonstrated covalent binding of 5-chloro-*o*-toluidine (or a reactive metabolite) to hemoglobin (Birner and Neumann, 1988; Sabbioni and Sepai, 1995). The capacity of 5-chloro-*o*-toluidine - like 4-chloro-*o*-toluidine - to bind to hemoglobin (Birner and Neumann, 1988), suggests the presence of an arylhydroxylamine, which can also be metabolized to a DNA reactive metabolite (Damani, 1982; Sabbioni and Sepai, 1995). Although the metabolism of 5-chloro-*o*-toluidine has not been investigated, studies of three related toluidine compounds, including 4-chloro-*o*-toluidine, have demonstrated the formation of an arylhydroxylamine (Hill *et al*., 1979; Son *et al*., 1980; Boeren *et al*., 1992).

Thus, given that 1) the hemoglobin binding index of 5-chloro-*o*-toluidine is nearly identical to that of the 4-chloro isomer (Birner and Neumann, 1988), 2) 4-chloro-*o*-toluidine (or a reactive metabolite) has been shown to bind to DNA, as well as RNA and protein (Hill *et al*., 1979), and 3) structure activity analyses predict a high degree of similarity between the metabolism and the

bioactivation products of 5- and 4-chloro-*o*-toluidine, it is plausible to hypothesize that 5-chloro-*o*-toluidine is metabolized to a DNA reactive species, and induces tumors as a consequence of the ensuing genotoxicity. It is important to emphasize that other mechanisms of action may also be operative, however, the limited data available in the published literature on the mechanism of action of 5-chloro-*o*-toluidine precludes further speculation on alternative mechanisms.

4   SUMMARY AND CONCLUSIONS

4.1   Summary of evidence

Carcinogenicity has been observed in both sexes of the mouse in diet studies conducted by the NCI (1979a). The incidence of liver tumors (*i.e.*, hepatocellular carcinomas; hepatocellular adenomas and carcinomas, combined) was statistically significantly increased above control levels in both males and females. In addition, hemangiosarcomas, a relatively uncommon tumor type, were observed in high incidence in both males and females. The non-mutagenic responses of bacteria to 5-chloro-*o*-toluidine in short-term tests may be an artifact of the experimental protocol. Similar negative responses were overcome with a related isomer and known animal carcinogen, 4-chloro-*o*-toluidine, when an alternate protocol was used. 5-Chloro-*o*-toluidine interferes with DNA metabolism and transforms mouse cells in culture. Additional supporting evidence is provided by hemoglobin binding studies in mice and rats, and by structure activity comparisons with 4-chloro-*o*-toluidine and its hydrochloride, which exhibit carcinogenic activity in laboratory experiments similar to that seen with 5-chloro-*o*-toluidine.

4.2   Conclusion

Based on the information reviewed in the preparation of this document, there is evidence for the carcinogenicity of *5-chloro-o-toluidine and its strong acid salts* at multiple sites in both sexes of the mouse. Chemical structural analogies with known carcinogens contribute to the weight of evidence.

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APPENDIX A

Genetic Toxicology of 4-chloro-*o*-toluidine and related toluidines

Bacterial systems

Zimmer *et al.* (1980) tested 4-chloro-*o*-toluidine, 2-chloro-*p*-toluidine, *o*-, *m*-, and *p*-toluidine in *S. typhimurium* in the presence of rat or mouse liver S9 during plate incorporation. The non-chlorinated toluidines were negative. The chlorinated toluidines were positive in strain TA100 with activation by rat liver S9 and were negative in strains TA98 and TA 1537. The 4-chloro-*o*-toluidine was 6-fold more potent that the 2-chloro-*p*-toluidine and exhibited a different dose-response curve. For the 4-chloro-*o*-toluidine, the number of revertants per plate was decreased when the suspension preincubation system was used, and the mouse liver S9 extract was not an efficient activator. Rashid *et al*. (1984) tested 4-chloro-*o*-toluidine in *S. typhimurium* using plate incorporation and rat liver S9. Positive results at 1 - 325 ug/plate were obtained with strain TA1535, and not with strains TA 98, 100, 1537, or 1538. A mutagenic response was not observed for 4-chloro-*o*-toluidine in several strains of *E. coli* WP$_2$, with or without metabolic activation. Gggelmann *et al*. (1996) showed that 4-chloro-*o*-toluidine was positive in *S. typhimurium* in the presence of rat liver S9 (plate incorporation protocol). The increased revertants/plate occurred with strains TA100 (100-600 ug/plate) and TA98 (375-2250 ug/plate). Higher doses led to toxicity. Mutagenicity was not observed, with or without metabolic activation, in strains TA1535 (200-1500 ug/plate) and TA1537 (200-1500 ug/plate).

Ono *et al*. (1992) studied the induced synthesis of DNA repair enzymes in *S. typhimurium* (TA1535) in the presence or absence of metabolic activation, carried out in a suspension assay system. Exposure of the cells to 3-chloro-*p*-, 4-, or 5-chloro-*o*-toluidine, did not lead to the induced synthesis of DNA repair enzymes. Unscheduled DNA synthesis was measured *in vitro* in rat hepatocyte culture after exposure to 2-chloro-*p*-toluidine, 2-chloro-*m*-toluidine, and 5-chloro-*o*-toluidine (Yoshimi *et al*., 1988). Only 2-chloro-*p*-toluidine tested positive under the conditions of the assay. To study the mutagenesis of toxicants that do not respond in the *S. typhimurium* (Ames)

test, DeMarini and Brooks (1992) measured the induction of prophage λ in *E..Coli* by a series of compounds including 3-chloro-*p*-toluidine and 5-chloro-*o*-toluidine. Induced virus was measured by plaque-forming units (PFUs), and a three-fold increase over background was used as the criterion for a positive result. Each toluidine tested negative in the presence or absence of rat liver S9, added in a preincubation suspension system. Each compound was also toxic at the highest dose (8.8 nM for 3-chloro-*p*-toluidine and 18 nM for 5-chloro-*o*-toluidine). Inspection of the data reveals that for 5-chloro-*o*-toluidine, a dose dependent decrease in induced PFUs/plate occurred. Such a decrease may indicate that unobserved toxicity was occurring at the lower doses.

DNA repair was effected by 4-chloro-*o*-toluidine in *S. typhimurium* (TA 1538 and 1978) and *E. coli* (strain WP$_2$) in the absence of a rat S9 (Rashid *et al*., 1980). The induction of DNA repair enzymes did not occur (with or without S9) in *S. typhimurium* (TA1535) in a cell suspension system in the presence of 3- or 4-chloro-*o*-toluidine (Ono *et al*., 1992).

These data indicate that the toluidines induce variable responses in the various bacterial tests for mutagenicity. Inspection of the assay procedures, however, suggests that the mutagenic activities of the toluidines may remain undetected because of toxicity. For example, the use of a suspension system has been shown to exaggerate the toxicity of some chlorotoluidines whereas the plate incorporation system allowed the expression of mutagenicity (Gggleman *et al*., 1996).

In vitro mammalian systems

Matthews *et al.* (1993) reported *o*-toluidine, 3-chloro-*p*-toluidine, 4-, and 5-chloro-*o*-toluidine tested positive in a standardized BALB/c-3T3 cell transformation protocol. A rank-order, based on statistical significance (t-statistic) and correction for the sensitivity of each individual experiment for transformation in the presence of benzo[*a*]pyrene, was calculated for the toluidines. Compared to the 114 tested chemicals, the four toluidines were ranked by the authors as active transformers. Each toluidine was also toxic to the BALB/c-3T3 cells. Except for the 4-chloro-*o*-toluidine which was present as the hydrochloride, the tested toluidines were observed to oxidize when exposed to air.

4-Chloro-*o*-toluidine caused DNA damage in Chinese hamster lung fibroblasts (V79), whereas the non-halogenated toluidines did not (Zimmer *et al.*, 1980) (use of S9 not mentioned). Data presented in a review of NTP-sponsored genotoxicity studies (Galloway *et al.*, 1987) indicated that 4-chloro-*o*-toluidine induced sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells. For sister chromatid exchanges, 50-200 ug/ml were effective doses in the presence or absence of metabolic activation. For chromosomal aberrations the effective doses were 125-175 ug/ml (no activation) and 300-400 ug/ml (with activation). The authors reported that 5-chloro-*o*-toluidine at doses of 1.6 - 16 ug/ml did not exhibit clastogenic activity in the same test systems. In another study, 4-chloro-*o*-toluidine was not observed to be clastogenic in human lymphocytes (chromosomal aberrations, sister chromatid exchange) or V79 Chinese hamster cells (spindle disturbances) (Ggglemann *et al.*,1996).

Binding studies

The covalent binding of 4-chloro-*o*-toluidine to tissue DNA, RNA, and protein was measured in Osborne-Mendel rats who were exposed by intraperitoneal (i.p.) injection (Hill *et al.*, 1979). Twenty-four hours after exposure, the liver tissue exhibited the highest levels of 4-chloro-*o*-toluidine-DNA binding (10 ng/mg tissue). Levels of 4-chloro-*o*-toluidine covalently bound to liver protein and RNA were 28 and 33 ng/mg tissue, respectively. The DNA chemical binding index was estimated as 720- and 42- pg 4-chloro-o-toluidine bound per mg DNA/mg administered 4-chloro-*o*-toluidine (i.p.) per kg body weight for liver and kidney, respectively. Stasik (1991), in a review article, also reported the covalent binding of 4-chloro-*o*-toluidine to DNA.

Four chlorotoluidines and the nonchlorinated *p*-toluidine were tested for their ability to bind, *in vivo*, to rat (female Wistar) and mouse (female B6C3F$_1$) hemoglobin (Hb) (Birner and Neumann, 1988). Binding was quantified as the hemoglobin binding index (HBI) and was calculated as mmol compound per mol Hb per applied dose and took into account the recovery of the chemicals during the preparative procedures. In rat, the HBIs were: *o*-toluidine, 4.0 ± 0.65; 4-chloro-*o*-toluidine, 28 ± 3; 5-chloro-*o*-toluidine, 28 ± 4; and 6-chloro-*o*-toluidine, 0.8 ± 0.2. In mice the HBIs were: *o*-toluidine, 2.1 ± 0.4; 4-

chloro-*o*-toluidine, 2.5 ± 0.4; and 5-chloro-*o*-toluidine, 1.0 ± 0.3. Clearly the binding to Hb was more efficient in the female rats than in the female mice. As discussed previously, multiple chlorotoluidine metabolites are likely to bind to biological macromolecules and hemoglobin binding may reflect the action of nonmutagenic metabolites (Sabbioni and Sepai, 1995). The metabolites responsible for binding to hemoglobin and to nucleic acids, however, share a common precursor, an arylhydroxylamine, thus the presence of hemoglobin binding is indicative of the potential for DNA binding via formation of a putative DNA reactive species.

APPENDIX B

Metabolism studies on *o*-toluidine, 2-chloro-*p*-toluidine, and 4-chloro-*o*-toluidine

Male rats (Fischer 344) were exposed subcutaneously for 48-hours to [^{14}C (CH$_3$)]-*o*-toluidine (50 or 400 mg/kg body weight) (Son *et al.*, 1980). Among the urinary metabolites were acetanilides, anthranilic acids, and phenols that were conjugated to sulfate or glucuronide, and an azo condensation product which was postulated to arise from the reaction of a nitroso metabolite and the hydroxylamine. Boeren *et al.* (1992) reported that 2-chloro-*p*-toluidine was metabolized by rat liver microsomes to a series of oxygenated products that included condensation products presumably derived from the reaction between nitroso and hydroxylamine products. The addition of cytochrome P450 inhibitors or the exclusion of required cytochrome P450 cofactors only partially decreased product formation, suggesting that non-P450 enzymes are also involved in the metabolism of 2-chloro-*p*-toluidine. Ring hydroxylated products were not observed.

Knowles and Gupta (1970) conducted a 72 hour urinary analysis of [^{14}C (CH$_3$)] 4-chloro-*o*-toluidine metabolites, following intraperitoneal administration to Sprague-Dawley rats. 4-Chloro-*o*-toluidine, 5-chloroanthranilic acid (5-chloro-2-carboxy-aniline), and 4-chloro-2-methylacetanilide were detected in the ethylacetate soluble fraction as 3%, 7%, and 5% of the total organo-soluble label, respectively. Unknown compounds represented 52% of the total ethylacetate soluble fraction. The water soluble urinary extracts were not analyzed, although they represented a major portion of total urinary radioactivity and may have been in the form of glucuronides. Analysis of total radioactivity indicated the following rank order of tissue-specific radioactivity: liver > kidney > oviduct > brain.

In vitro rat (Osborne-Mendel) liver microsomal metabolism of [^{14}C(CH$_3$)] 4-chloro-*o*-toluidine resulted in the formation of 5-chloro-2-N-hydroxylaminotoluene and 4,4'-dichloro-2,2'dimethylazobenzene (Hill *et al.*, 1979). The latter metabolite was considered to be a result of condensation between the hydroxylamine and a postulated nitroso intermediate. The

azobenzene metabolite may be analogous to metabolites detected in the metabolism studies of 2-chloro-*p*-toluidine (see above).

Xuan and Liu (1995) reported that cytochrome P450-IIB plays an important role in the metabolism of 4-chloro-*o*-toluidine, although the data suggest that cytochrome P450-IIB is not solely responsible. The potential role of non-cytochrome P450 enzymes in the metabolism of primary arylamines has been discussed by Nelson (1985).

Intraperitoneal administration of 4-chloro-*o*-toluidine has been shown to induce some cytochrome P450- as well as epoxide hydrolase- and glutathione S-transferase-dependent activities in rats (Leslie *et al*., 1988).