EVIDENCE ON THE CARCINOGENICITY OF

TRICHLOROACETIC ACID AND ITS SALTS

FINAL

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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 (OEHHA) 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).

Trichloroacetic acid was assigned a final priority of 'high' carcinogenicity concern and placed on the Final Candidate list of chemicals for Committee review on June 12, 1998. A public request for information relevant to the assessment of the evidence on the carcinogenicity of this chemical was announced in the *California Regulatory Notice Register*, also on June 12, 1998. This document reviews the available scientific evidence on the carcinogenic potential of trichloroacetic acid and its salts. It was released as the draft document *Evidence on the Carcinogenicity of Trichloroacetic Acid and Its Salts* in July 1999.

At their October 7, 1999, meeting the Committee, by a vote of zero in favor, six against, and one abstention, did not find that trichloroacetic acid and its salts had been "clearly shown through scientifically valid testing according to generally accepted principles to cause cancer."

The following document is the final version of the document that was discussed by the Committee at their October 1999 meeting.

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1 EXECUTIVE SUMMARY

Universal exposure of the population of California to low levels of trichloroacetic acid (TCA) results from its occurrence as a contaminant of drinking water disinfected by chlorination. Other minor occurrences and uses affect a much smaller number of people, but may result in high exposures to those particular individuals. Trichloroacetic acid is a major metabolite of trichloroethylene and tetrachloroethylene (perchloroethylene). These materials are widely used as industrial solvents and (in the case of tetrachloroethylene) in dry cleaning. Secondary exposures to trichloroacetic acid may therefore result from workplace exposures to these compounds, or to local environmental contamination of air or water at sites where they are used. For most exposure situations, TCA and its salts are toxicologically equivalent, since TCA is a strong organic acid that exists principally as the anion in aqueous solutions near neutral pH.

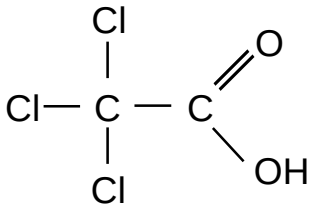
TCA caused liver tumors in male and female mice in multiple experiments by the predominant route of human exposure, i.e. drinking water. However, carcinogenicity was not observed in the only carcinogenensis study conducted in the rat. In the mouse TCA acts as a promoter of liver tumors, and promotes foci of altered hepatocytes in both the rat and the mouse.

The results of short-term tests for genotoxicity are mainly negative, although there are some marginally positive or equivocal results. Studies in mice have suggested several possible “non-genotoxic” modes of action, although genetic alterations in proto-oncogenes have been observed in TCA-induced tumors. These suggested mechanisms have included effects on peroxisome proliferation, enhanced cell proliferation as a result of receptor-mediated effects or in response to cytotoxicity, and effects on intercellular communication. However, none of these proposed mechanisms has been definitely established as a principal cause of the observed carcinogenicity. Nor have they been shown conclusively to contribute to the carcinogenicity or other toxicity of TCA.

There is sufficient evidence of TCA carcinogenicity in animals, based on results in male and female mice. Although the evaluation by the International Agency for Research on Cancer (IARC, 1995) found only limited evidence of carcinogenicity in animals, the data which they considered has since been significantly augmented, including the observation of carcinogenicity in female as well as male mice. The failure to observe carcinogenicity in the one rat study, the negative mutagenicity results in short-term tests, and other mechanistic data raise the possibility of non-genotoxic mechanisms, which might be species-specific.

2 INTRODUCTION

2.1 Identity of Trichloroacetic Acid



Trichloroacetic Acid: $C_2HCl_3O_2$
Molecular Weight = 163.39 CAS Registry No. 76-03-9

Synonyms: TCA, trichloroethanoic acid, trichloromethane carboxylic acid

TCA is a colorless or yellowish deliquescent, crystalline solid with a melting point of 58 °C (α-form) or 49.6 °C (β-form). The boiling point of the liquid is 197.5 °C.

Free TCA is a strong organic acid, which forms water-soluble salts with bases. In any medium at or near neutral pH the predominant form present is the trichloroacetate anion. The salts are therefore expected to be toxicologically equivalent to the free acid, except for the acute corrosive properties, which are at least partly determined by the very low pH of strong solutions of the acid. Because of this, the following report considers the possible carcinogenicity of trichloroacetic acid and its salts.

2.2 Occurrence and Use

Trichloroacetic acid (TCA, CAS No. 76-03-9) has a number of industrial applications, including use as a synthetic intermediate, and various other minor uses, *e.g.* as a medication, and as a reagent for albumin detection. One of the major uses of TCA (principally handled as the sodium salt) according to IARC (1995) is as a selective herbicide. However, according to the U.S. EPA Office of Pesticide Programs (1998), all registrations for herbicidal products containing this ingredient have been voluntarily cancelled; the most recently registered product had its registration cancelled in 1992. It therefore appears that TCA is not in substantial use in the United States as a herbicide at this time, although evidently it was so used previously and there may be some use of remaining stocks.

In addition to its deliberate production and use, TCA is one of the major by-products of the disinfection of water by chlorination. Concentrations measured in drinking water supplies in one study in the U.S. ranged from 4 to 103 μg/L, with a mean concentration of 38 μg/L, while another study reported quarterly median values of TCA ranging from 4.0 to 6.0 μg/L (Bull and Kopfler, 1991). IARC (1995) reported that TCA occurs in chlorinated drinking water at levels up to 200 μg/L.

TCA is formed (along with other chloroacetic acids, halomethanes, and related compounds) by reaction of chlorine or hypochlorite with organic substances such as humic acid. Studies of disinfection by-products in the distribution system of a metropolitan water district in California found that total halogenated acetic acid concentrations varied from 7.3 to 8.1 μg/L in one plant, and 13 to 21 μg/L in a second plant which differed in the timing of the treatment with chlorine and ammonia (Bull and Kopfler, 1991). In this study, total halogenated acetic acid levels were stable in the chloraminated water in the distribution system until chlorine was added prior to storage of the water in an open reservoir, after which the level of chlorination by-products increased over time.

TCA is also found in other situations where water is chlorinated, such as irrigation, swimming pools, and pulp mill effluents. Various other chlorinated compounds are also formed in the same way, and some of these are metabolized *in vivo* to TCA, resulting in additional exposure to this compound. Photodegradation of tetrachloroethylene (PCE) in water also results in the formation of TCA (IARC, 1995). Some of these precursors of TCA also have major industrial uses [*e.g.* trichloroethylene (TCE) and PCE].

Universal exposure of the population of California to low levels of TCA occurs due to its occurrence as a contaminant of drinking water disinfected by chlorination. Other occurrences and industrial or agricultural uses affect a much smaller number of people, but have the potential to result in high exposures to those particular individuals.

3 DATA ON TCA CARCINOGENICITY

The primary evidence on carcinogenicity of TCA consists of studies in mice and rats exposed to TCA in drinking water. This is supported by the findings of initiation-promotion studies, and by studies of genotoxicity, metabolism and mechanisms of toxicity. In the bioassays, and in the majority of other types of experiment reported, the dosing material was an aqueous solution adjusted to near neutral pH. As noted previously, under these conditions the principal molecular species present is the trichloroacetate anion, regardless of whether the material was originally supplied as the free acid or as a salt.

3.1 Epidemiological Studies of Carcinogenicity in Humans

No data on long-term effects of human exposure to TCA were found in an earlier search by IARC (1995), or more recently by OEHHA. However, TCA is a metabolite of TCE and PCE, and is detectable in the urine of humans exposed to those solvents. There are epidemiological studies involving occupational and community exposure to TCE and PCE. With regard to human health effects of TCA, the evidence is inadequate (IARC, 1995).

3.2 Carcinogenicity Studies in Animals

A number of bioassays have been reported which indicate that TCA is a hepatocarcinogen in the mouse. The male is more sensitive than the female. In a single study conducted in the rat, TCA was observed to be hepatotoxic but not hepatocarcinogenic. Table 1 summarizes the findings of the published studies, and indicates whether these were evaluated by IARC (1995).

Table 1. Summary of Carcinogenicity Studies of TCA.

Route	Species	Strain	Sex	Tumor site, type	IARC eval.?	Authors
oral (drinking water)	Mouse	$B6C3F_1$	M	hepatocellular adenoma (ad.) and carcinoma (ca.)	yes	Herren-Freund *et al.,* 1987
oral (drinking water)	Mouse	$B6C3F_1$	M&F	hepatocellular ca. in males only	yes	Bull *et al.,* 1990
oral (drinking water) #1	Mouse	$B6C3F_1$	M	hepatocellular ad. and ca.	no	DeAngelo and Daniel, 1990; DeAngelo, 1991
oral (drinking water) #2	Mouse	$B6C3F_1$	M	hepatocellular ad. and ca.	no	
oral (drinking water)	Mouse	$B6C3F_1$	F	hepatocellular ad. and ca.	no	
oral (drinking water)	Mouse	$B6C3F_1$	F	hepatocellular ad. and ca.	no	Pereira, 1996
oral (drinking water)	Mouse	$B6C3F_1$	F	hepatocellular ca.	no	Pereira and Phelps, 1996.
oral (drinking water)	Rat	F344	M	No increases in tumor incidence	no	DeAngelo and Daniel, 1992; DeAngelo, 1991; De Angelo *et al.,* 1997.

3.2.1 Mouse drinking water studies

Herren-Freund et al., 1987

Male $B6C3F_1$ mice received drinking water containing 0 or 5 g TCA/L neutralized with sodium hydroxide to a pH of 6.5-7.5 for 61 weeks. A complete necropsy was performed, so the appearance of any macroscopically visible tumors would have been noted, but histological examination was confined to the liver. Treated mice had a statistically

significant increase in hepatic adenomas (8/22 versus 2/22 in controls, $P < 0.05$ by Fisher's Exact test) and hepatocellular carcinomas (7/22 versus 0/22 in controls, $P < 0.01$ by Fisher's Exact test). Tumor multiplicity was also increased in TCA-treated mice, for both adenomas (0.50±0.16 adenomas per mouse, compared to 0.09±0.06 in controls) and carcinomas (0.50±0.17 carcinomas per mouse, compared to none in controls). IARC (1995) concluded that the increased incidence of hepatocellular adenomas and carcinomas was treatment related. This study was part of a larger experiment examining the carcinogenic and promoting effects of TCA, dichloroacetic acid (DCA) and trichloroethylene (TCE). A more detailed experimental description is found below, in Section 3.3.1, Tumor Initiation/Promotion Studies. See Table 5 for the study design and results.

Bull et al., 1990

Several groups of $B6C3F_1$ mice received TCA in their drinking water for a significant portion of their lifespan. The groups were as follows: a group of 11 males received 1 g TCA/L for 52 weeks ("low dose"); a group of 24 males received 2 g TCA/L for 52 weeks ("high dose"); a group of 11 males received 2 g TCA/L for 37 weeks and then water alone until week 52; two groups of females (10/group) received 0 or 2.0 g TCA/L for 52 weeks. Two groups of 35 and 11 male control mice were kept for 52 weeks. The livers and kidneys (only) were examined macroscopically, and organ weights determined: sections of all livers were prepared for histological examination. In male mice, hyperplastic or neoplastic changes were observed in livers of 2/35 controls, 5/11 low-dose ($P < 0.01$ by Fisher's Exact test, relative to controls) 19/24 high-dose ($P < 0.01$), and 4/11 high-dose exposed for 37 weeks ($P < 0.05$). Histological examination of the tumors confirmed hepatocellular carcinomas in several of the TCA exposed mice, although not all lesions or lesion-bearing mice were examined histologically in the high-dose mice exposed for 52 weeks. None of the 35 male controls had hepatocellular carcinomas. No hyperplastic nodules or neoplastic lesions were seen in either group of female mice, although the authors did report the appearance of large basophilic foci (incidence not specified) in TCA-treated female mice. This led them to hypothesize that a tumorigenic response would have been seen in the female mice if the experiment had been continued for a longer period.

The results of the study in male mice as reported by the authors are presented in Table 2. There are some difficulties in interpreting these incidence data (as noted by IARC, 1995), since the intent of the authors was clearly to study the progression and histology of the lesions rather than to report their incidence in the usual bioassay format. IARC (1995) concluded that the incidence of treatment-related liver tumors increased in male mice.

Table 2. Hyperplastic and neoplastic hepatocellular lesions in male B6C3F$_1$ mice receiving TCA in drinking water (Bull *et al.*, 1990).

Treatment			***Result: Number of lesions (number of mice)***					
TCA, g/L	Duration (weeks)	N	Total lesions	Lesions examined	Diagnosis of lesions:			
					Normal	Hyper-plastic	Adenoma	Carcin-oma
2	52	24	30 (19[b])	16 (11)	1 (1)	10 (9)	1 (1)	4 (4)
2	37	11	5 (4[a])	5 (4)	0	2 (2)	0	3 (3)
1	52	11	7 (5[b])	7 (5)	0	3 (1)	2 (2)	2 (2)
0	-	35	2 (2)	2 (2)	1 (1)	1 (1)	0	0

[a] $P < 0.05$, [b] $P<0.01$ relative to control, by Fisher's Exact Test.

DeAngelo and Daniel, 1990; DeAngelo, 1991.

DeAngelo and colleagues conducted three separate experiments in B6C3F$_1$ mice. The details of these studies appear in two reports (DeAngelo and Daniel, 1990; DeAngelo, 1991), and are described below. (In these experiments, animals were examined for macroscopic lesions, and several organ weights determined. Histology was examined for all liver tissues and any other lesions identified. The only neoplastic findings reported are those in the liver. Results were presented as prevalence percentages only, so the usual significance tests for quantal data could not be performed. The authors' judgement as to the presence of a significant effect is reported.)

a) Male B6C3F$_1$ mice received drinking water containing 0, 0.05, 0.5 or 5 g TCA/L (0, 8, 71 and 595 mg/kg bw/day mean daily dose) for 60 weeks. The number of mice/group was not specified. Prevalence of hepatocellular tumors (adenomas and carcinomas) was increased in the groups of male mice receiving 0.5 and 5 g TCA/L (37.9% and 55.2% respectively, compared to 13.3% in the control group). Tumor prevalence was not significantly increased in the group receiving 0.05 g/L TCA.

b) In a second experiment, male B6C3F$_1$ mice received drinking water containing 0 or 4.5 g TCA/L (0 and 583 mg/kg bw/day mean daily dose) for 94 weeks, which was considered to be a lifetime exposure. Prevalence of hepatocellular tumors was increased in the exposed group (86.7%, compared to 15% in the control group).

c) Female B6C3F$_1$ mice received drinking water containing 0, 0.5 or 4.5 g TCA/L (0, 71 and 583 mg/kg bw/day mean daily dose) for 104 weeks. Prevalence of hepatocellular tumors (adenomas and carcinomas) was increased in the group receiving 4.5 g TCA/L (60% respectively, compared to 7.7% in the control group). Tumor prevalence was not significantly increased in the groups receiving 0.5 g TCA/L.

The authors concluded that TCA has carcinogenic activity in male and female B6C3F$_1$ mice.

Pereira, 1996

Female B6C3F$_1$ mice received water containing 2.0, 6.67, or 20.0 mmol TCA/L for 360 or 576 days (Pereira, 1996). The livers (only) of all animals received macroscopic, biochemical, and histological examination. A statistically significant increase in hepatocellular carcinomas occurred in mice at the high dose ($P < 0.01$ compared to controls, by Fisher's Exact test) following 360 days of administration (0/40 control, 0/40 low-dose, 0/19 mid-dose, and 5/20 high dose). After 576 days of administration, there were statistically significant increases in pre-neoplastic foci of altered hepatocytes, and hepatocellular carcinomas in the mid- and high-dose groups. In high-dose mice after 576 days, the incidences of hepatocellular adenomas were significantly increased ($P < 0.01$) compared to controls. The incidences of tumors were: adenomas, 2/90, 4/53, 3/27 and 7/18; carcinomas, 2/90, 0/53, 5/27, and 5/18, in control, low- mid- and high-dose mice, respectively. Results are shown in more detail in Table 3. The authors concluded that TCA has carcinogenic activity in female B6C3F$_1$ mice.

Pereira and Phelps, 1996.

A similar but smaller and shorter experiment was reported as part of a larger study of promotion of liver tumors in female B6C3F$_1$ mice (Pereira and Phelps, 1996). This study is described in detail below, in the section on initiation and promotion studies. A significant increase in liver carcinomas (5/20 incidence and 0.5±0.18 carcinomas per mouse, $P < 0.05$ by Mann-Whitney test) was reported in mice exposed to 20 mM TCA in the drinking water for 52 weeks. The mice received no initiation treatment.

Table 3. Hepatocellular foci and tumors in female B6C3F$_1$ mice receiving TCA in drinking water (Pereira, 1996).

Treatment		*Incidence of lesions: Number of animals (percentage of animals)*			
TCA, mM	Duration (days)	N	Foci of altered hepatocytes	Hepatocellular Adenoma	Hepatocellular Carcinoma
20	360	20	0	2 (10)	5 (26.3) [a]
	576	18	11 (61.1) [a]	7 (38.9) [a]	5 (27.8) [a]
6.67	360	19	0	3 (15.8)	0
	576	27	9 (33.3) [a]	3 (11.1)	5 (18.5) [a]
2.0	360	40	3 (7.5)	3 (7.5)	0
	576	53	10 (18.9)	4 (7.6)	0
0	360	40	0	1 (2.5)	0
	576	90	10 (11.1)	2 (2.2)	2 (2.2)

[a] Significantly different from the corresponding control group, $P < 0.01$ by Fisher's exact test.

3.2.2 Rat long-term drinking water studies

DeAngelo and Daniel, 1992; DeAngelo, 1991; De Angelo et al., 1997.

Male Fischer 344 rats received water containing 0.0, 0.05, 0.5 or 5 g TCA/L (0, 3.6, 36 and 378 mg/kg bw/day mean daily dose) over a 104 week period. A complete necropsy, and histopathological examination of representative tissue samples and all lesions, was undertaken for all animals. Incidences of hepatocellular adenoma or carcinoma were 1/24 (4.2%), 3/20 (15%) and 1/22 (4.6%) for 0.05, 0.5 and 5 g TCA/L respectively, compared to 1/23 (4.4%) in the control group. Non-neoplastic histopathological effects were also examined, and there were found to be only minimal changes in the liver even at the highest dose level. Peroxisome proliferation activity was assessed by measuring cyanide-insensitive palmitoyl CoA oxidase activity. This was unaltered in all except the highest dose group, in which it was increased twofold. The authors concluded that TCA was not carcinogenic in male F344 rats.

3.3 Other Relevant Data

In addition to the reported animal bioassays, other evidence related to the possible carcinogenicity of TCA is available. This includes studies of tumor promotion, genetic

toxicity, observations of the pharmacokinetics and metabolism, and structure-activity comparisons.

3.3.1 Tumor initiation/promotion studies

TCA has been observed to act as a liver tumor promoter in several studies in which rats or mice were treated with an initiating dose of a carcinogen, followed by chronic exposure to TCA in the drinking water. (Examination of pathological changes at either macroscopic or histological level is generally confined to the liver in theses experiments, except where noted otherwise.)

Rat tumor promotion studies

Parnell et al., 1988.

Male Sprague-Dawley rats underwent two-thirds partial hepatectomy. This was followed after 24 h by an initiating treatment consisting of a single gavage dose of 10 mg N-nitrosodiethylamine (DEN)/kg bw. Two weeks later, the promotion treatment was started, and continued to the end of the experiment (*i.e.*, three or six months). The rats received drinking water containing 50, 500 or 5000 ppm TCA, neutralized with sodium hydroxide to a pH of 6.5-8.0. Promoting effect was assessed after three or six months by determining the number of γ-glutamyl transpeptidase (GGT) positive foci per cm^2 in the liver (visualized by histochemical staining of frozen sections). Negative control groups included one receiving sham operation and 500 ppm TCA promotion but no DEN initiator, one receiving partial hepatectomy but no DEN initiator or TCA promotion, and one receiving hepatectomy and DEN but no TCA promotion. A positive control group received hepatectomy and DEN followed by 500 ppm of phenobarbital (a known liver tumor promoter) in the drinking water, instead of TCA.

Results are shown in Table 4. The positive control, phenobarbital, showed a significant increase in the number of GGT positive foci in the livers at both 3 and 6 months. The number of foci was also increased in all three groups of initiated and TCA-treated rats at 6 months. At 3 months, the increases in those receiving 50 or 5000 ppm TCA were significant.

The authors concluded that TCA showed promoting activity in this test system, although this activity was not as strong as that of phenobarbital. Concurrently with the measurement of GGT positive foci in the livers, the authors also measured cyanide-insensitive palmitoyl-CoA oxidation as a measure of the induction of peroxisome proliferation. Only minimal (10 – 20%) increases were seen in this activity in TCA-treated rats, at the high dose only. Liver weights were also determined, and no evidence was found of hepatomegaly associated with TCA exposure.

Table 4. GGT-positive foci in livers of male Sprague-Dawley rats receiving DEN initiation and TCA promotion (Parnell *et al.*, 1988).

Group	Treatment[a]	Number of foci/cm^2 [b]		
		N	3 months	6 months
M	PH, DEN, PB	6	1.65 ± 0.23[c]	7.61 ± 0.72[c]
N	PH, DEN, TCA 50 ppm	6	0.71 ± 1.16[d]	1.83 ± 0.32[e]
O	PH, DEN, TCA 500 ppm	6	0.39 ± 0.16	1.63 ± 0.32[e]
P	PH, DEN, TCA 5000 ppm	6	0.70 ± 0.16[d]	2.45 ± 0.32[e]
Q	TCA 5000 ppm	6	0.23 ± 0.16	0.03 ± 0.32
R	PH	4	0.23 ± 0.20	0.41 ± 0.39
S	PH, DEN	4	0.05 ± 0.20	0.30 ± 0.39

[a] PH = partial hepatectomy. DEN = 10 mg/kg bw N-nitrosodiethylamine, single dose. PB = 500 ppm phenobarbital in drinking water, chronic exposure. TCA = trichloroacetic acid, *n* ppm in drinking water, chronic exposure.

[b] Mean number of foci per cm^2 ± standard error of least-squares mean.

[c] Significantly greater than groups N, O, P, Q, R and S by least-squares means comparisons ($P \leq 0.05$).

[d] Significantly greater than groups Q and S by least-squares means comparisons ($P \leq 0.05$). Group M excluded from comparisons.

[e] Significantly greater than groups Q, R and S by least-squares means comparisons ($P \leq 0.05$). Group M excluded from comparisons.

Parnell *et al.* (1988) also reported a parallel series of experiments to detect any initiating effect of TCA exposure. In these experiments partial hepatectomy was followed after 24 h by either a single dose of 1500 mg TCA/kg by gavage, or by exposure to 5000 ppm TCA in the drinking water for 10, 20 or 30 days. Two weeks after the initiating treatment, a promoting treatment consisting of 500 ppm phenobarbital in the drinking water was started, and continued for either three or six months. No evidence of initiating activity in this test system was found for TCA.

Mouse tumor promotion studies

Herren-Freund et al., 1987.

Male B6C3F$_1$ mice received initiating intraperitoneal doses of 0, 2.5 or 10 mg/kg body weight of ethylnitrosourea (ENU) at age 15 days. They subsequently received drinking water containing 0, 2 or 5 g TCA/L neutralized with sodium hydroxide to a pH of 6.5-7.5 for 61 weeks, starting at age 4 weeks. Similar groups received ENU treatment followed by DCA (2 or 5 g/L), TCE (3 or 40 mg/L) or phenobarbital (500 mg/L). Negative controls for initiation and promotion effects received a vehicle control injection instead of ENU, and/or drinking water containing 2 g NaCl/L, corresponding to the sodium content of the neutralized TCA solutions. A complete necropsy was performed, but histological

examination was confined to the liver. Detailed experimental design and results for the TCA groups and corresponding controls are shown in Table 5.

Table 5. Hepatocellular Adenomas and Carcinomas in male mice receiving ENU initiation and TCA promotion (Herren-Freund *et al.*, 1987)

Treatment		***Result***				
ENU, mg/kg	TCA, mg/L	N [a]	Mice with Adenomas	Adenomas / mouse [b]	Mice with Carcinomas	Carcinomas/ mouse [b]
10	5	28	11 (39%)	0.61±0.16	15 (54%)	0.93±0.22
2.5	5	23	6 (26%) [c]	0.30±0.12 [c]	11 (48%) [c]	0.57±0.21 [c]
2.5	2	33	11 (33%) [c]	0.42±0.12 [c]	16 (48%) [c]	0.64±0.14 [c]
0	5	22	8 (36%) [c]	0.50±0.16 [c]	7 (32%) [c]	0.50±0.17 [c]
10	0	23	9 (39%)	0.52±0.15	9 (39%)	0.57±0.20
2.5	0	22	1 (5%)	0.05±0.05	1 (5%)	0.05±0.05
0	0	22	2 (9%)	0.09±0.06	0 (0%)	0

[a] Number of animals examined

[b] Number of tumors per animal expressed as mean ± standard error of mean.

[c] Significantly different from the corresponding NaCl control group (no TCA): $P < 0.01$ by Fisher's exact test.

Significantly increased tumor incidences (both hepatocellular adenomas and carcinomas) and mean numbers of tumors per animal were observed in the livers of mice treated with 5 or 2 g TCA/L and 2.5 mg ENU/kg bw, compared to those receiving ENU only. In the group treated with 10 mg ENU/kg bw and 5 g TCA/L, an increased incidence of carcinomas was also seen relative to the group receiving 10 mg ENU/kg bw only. However, this was not statistically significant, due at least in part to the high tumor incidence and multiplicity in the latter group. As noted earlier, increased tumor incidence and multiplicity was noted in the group receiving TCA (5 g/L) only, compared to the corresponding control group. (All results described as statistically significant were $P < 0.01$ by Fisher's exact test, relative to the corresponding control.) Since the increases in the ENU initiated and TCA promoted groups appeared additive rather than multiplicative, compared to the non-ENU treated and non-TCA-treated groups, this experiment does not show that there is a promoting effect of TCA in addition to the observed complete carcinogenesis. A similar result was observed in the corresponding groups exposed to DCA, but the groups treated with phenobarbital showed neither promotion nor complete carcinogenesis.

Pereira and Phelps, 1996; Pereira et al., 1997; Latendresse and Pereira, 1997.

These authors studied promotion of liver tumors in female $B6C3F_1$ mice by TCA and DCA, alone or in combination, in two related, but separate, sets of experiments. In each experiment, the mice received an initiating dose by intraperitoneal injection of 25 mg N-methyl-N-nitrosourea (MNU)/kg bw when 15 days old. Promoting treatments consisted of TCA and/or DCA in the drinking water, adjusted to pH 6.5 – 7.5 with sodium hydroxide. Concentrations, starting time and duration varied between the experiments. Non-initiated controls received similar injections of saline only. Non-promoted controls received drinking water containing 20 mM NaCl (corresponding to the sodium content of the high TCA dose after neutralization). Liver foci and tumors were visualized by staining with hematoxylin and eosin and classified as either eosinophilic or basophilic. The presence of glutathione-S-transferase-π (GST-π) was evaluated by immunohistochemical staining.

a) In one set of experiments, groups of between 8 and 40 mice received the standard initiation treatment, followed by TCA or DCA in the drinking water starting at age 7 weeks (Pereira and Phelps, 1996). Groups were exposed to TCA concentrations of 20 mM, 6.67 mM or 2.0 mM TCA. At each concentration, one group received the treatment for 31 weeks prior to sacrifice and histological evaluation, while a second group received the treatment for 52 weeks. An additional "recovery" group received 20 mM TCA for 31 weeks, followed by 20 mM NaCl (control) drinking water for a further 21 weeks. Similar concentrations and exposure patterns were used for mice exposed to DCA.

   Body weights were unaffected during the course of the study by exposure to TCA. A slight increase in liver-to-body weight ratio was noted in TCA-exposed animals: this appeared to be dose-related but was much smaller than the effect seen with DCA. The numbers of foci, adenomas and carcinomas observed in TCA-exposed mice and the corresponding controls are shown in Table 6. At 31 weeks there was an increase in the mean number of adenomas per mouse in the group initiated with MNU and promoted with 20 mM TCA, but other groups showed no significant effect. At 52 weeks the number of adenomas per mouse, the number of carcinomas per mouse and the incidences of these tumors were all increased in animals initiated with MNU and promoted with 6.67 mM or 20 mM TCA. No significant effects were observed on the number of foci per mouse, or on focus or tumor incidence in mice promoted with 2.0 mM TCA. In addition, there was a significant increase in the incidence of carcinomas in mice receiving 20 mM TCA for 52 weeks without MNU initiation. The incidence was 5/20 (25%), with 0/40 incidence in the controls. There was an average of 0.5 ± 0.18 carcinomas per mouse ($P < 0.05$).

   The authors concluded that TCA acted as a tumor promoter in this test system. They also noted that the tumors in TCA-treated animals were predominantly basophilic and negative for GST-π, whereas foci or tumors in DCA-treated animals were

predominantly eosinphilic and GST-π positive. Dose-response for tumor promotion appeared to be linear with dose for TCA, in contrast to the sharp upward curvature in the dose-response for DCA. The authors suggested that these observations indicated a difference in the mechanisms by which TCA and DCA acted as tumor promoters.

Table 6. Foci and tumors in livers of female B6C3F$_1$ mice receiving MNU initiation and TCA promotion (Pereira and Phelps, 1996). [a]

Treatment		*31 weeks*			*52 weeks*			
MNU mg/kg	TCA mM	N[b]	Foci/mouse	Adenomas/ mouse	N	Foci/mouse	Adenomas/ mouse	Carcinomas/ mouse
25	20	10	0.2±0.13 (20)	1.3±0.45 [c] (60)	23 +1	0.13±0.07 (12.5)	1.29±0.24 [c] (66.7)	2.79±0.48 [d] (83.3)
25	20 R	-	-	-	11	0.45±0.31 (18.2)	0.91±0.28 [c] (63.6)	0.73±0.33 [c] (36.4)
25	6.67	8	0.13±0.13 (12.5)	0.50±27 (37.5)	6	0 (0)	2.0±0.82 [d] (83.3)	1.33±0.42 [d] (83.3)
25	2.0	8	0.25±0.25 (12.5)	0.13±0.13 (12.5)	10	0.2±0.13 (20)	0.6±0.34 (30)	0 (0)
25	0	10	0.3±0.21 (20)	0 (0)	39 +1	0.1±0.05 (10)	0.28±0.11 (17.5)	0.1±0.05 (10)
0	20	10	0 (0)	0 (0)	19 +1	0 (0)	0.15±0.11 (10)	0.5±0.18 [e] (25)
0	6.67	10	0 (0)	0 (0)	19	0 (0)	0.21±0.12 (15.8)	0 (0)
0	2.0	15	0 (0)	0 (0)	40	0.08±0.04 (7.5)	0.08±0.04 (7.5)	0 (0)
0	0	15	0.13±0.13 (6.7)	0.13±0.13	40	0 (0)	0.03±0.03 (2.5)	0 (0)

[a] Treatment schedule is described in the text above. Results are given as the mean number of lesions per mouse ± standard error, with the incidence expressed as a percentage in parentheses.

[b] Number of animals examined following terminal sacrifice. In cases where animals were found dead or moribund during the course of the experiment these are noted as additions (+*n*) and are included in the incidence calculation.

[c, d] Significantly different from both the control groups (MNU initiation but no TCA promotion, and the corresponding dose of TCA without MNU initiation) by Mann-Whitney test: [c] $P < 0.05$, [d] $P < 0.01$.

[e] Significantly different from the corresponding control group (no TCA promotion or MNU initiation) by Mann-Whitney test: $P < 0.05$.

Latendresse and Pereira (1997) reported further immunohistochemical analysis of the proliferative lesions observed in this study. DCA-induced lesions, in addition to the previously noted eosinophilia and positive reaction for GST-π, were positive for the growth regulator TGF-α, gene products of proto-oncogenes *c-jun* and *c-myc*, and the cytochrome P-450 isoenzymes 2E1 and 4A1. DCA-induced lesions were consistently negative for *c-fos* gene product and TGF-β, although normal hepatocytes are positive for TGF-β. TCA-induced proliferative lesions were, as noted previously, basophilic and GST-π negative. Reactions for the other biomarkers studied were variable, but usually more than 50% of the hepatocytes in TCA-induced lesions were negative for

these biomarkers (TGF-α and -β, *c-fos, c-jun* and *c-myc* gene products, cytochrome P-450 2E1 and 4A1).

b) In the second series of experiments, groups of between 20 and 45 mice received the standard initiation treatment, followed by TCA and DCA alone or in combination in the drinking water for 44 weeks, starting at age 6 weeks (Pereira *et al.*, 1997). Groups exposed to TCA received drinking water containing 6.0 or 25 mM TCA: one series received this treatment alone while a second series received these doses of TCA combined with 15.6 mM DCA. Groups exposed to DCA received drinking water containing 7.8, 15.6 or 25 mM DCA, either alone or along with 6.0 mM TCA.

   The results were similar to those reported by Pereira and Phelps (1996) for MNU-initiated mice exposed to chloroacetic acids. A significant increase in hepatocellular proliferative lesions, primarily adenomas, was noted after exposure to TCA, and this showed a linear dose-response. DCA showed a large increase in proliferative lesions at 25 mM, but a much smaller increase at the lower doses. An incidence of 4/29 carcinomas was reported in the group receiving 25 mM TCA, whereas no such lesions were reported in the (initiated) controls. Carcinomas were also observed in animals exposed to the high dose of DCA, and in one of the animals receiving both compounds. In the case of DCA the proliferative lesions included both adenomas and altered foci. In animals exposed to both compounds, the response was synergistic, and both altered foci and adenomas were reported. Lesions (adenomas and foci) were primarily eosinophilic and GST-π positive in animals exposed to DCA alone or in combination with TCA, whereas the lesions (adenomas) in animals exposed to TCA alone were predominantly basophilic and GST-π negative. (The results of this study were primarily reported in a graphical format, and exact tabular results were not available).

3.3.2 Genetic Toxicology

Bacterial mutagenicity assays.

Most mutagenicity studies in bacterial systems with TCA have been negative. TCA did not induce λ prophage in *Escherichia coli* and was not mutagenic in *Salmonella typhimurium* strain TA 100 (De Marini *et al.*, 1994; Rapson *et al.*, 1980). Giller *et al.* (1997) reported that TCA did not induce DNA damage in *Escherichia coli* PQ37 but that TCA was mutagenic in *Salmonella typhimurium* TA 100 at concentrations greater than 1750 μg/mL in a modified Ames assay. Mutagenicity was found to decrease in the presence of rat liver S9 homogenate.

Assays in mammalian cells in vitro.

Harrington-Brock *et al.* (1998) reported that TCA was mutagenic only in the presence of S9 activation in L5178Y/TK$^{+/-}$ -3.7.2C mouse lymphoma cells. The authors noted that TCA was one of the least potent mutagens that they had evaluated.

MacKay *et al.* (1995) reported that TCA (as the free acid) caused an increase in chromosomal aberrations in incubations of cultured human lymphocytes (500, 2000, 3500 µL in the presence and absence of S9 activation) but that these aberrations were associated with a TCA-induced decrease in pH. When incubations were conducted with neutralized TCA at these concentrations, significant increases in the incidence of aberrant cells were not observed.

Assays in mammals in vivo: chromosomal effects.

In a mouse bone marrow micronucleus test *in vivo* (MacKay *et al.,* 1995), neutralized TCA (336, 675, 1080 mg/kg in males; 405, 810, 1300 mg/kg in females) was administered i.p. (two doses, 24 hours apart) to male or female C57BL mice. Mice were sacrificed 6 or 24 hours after the second dose, and bone marrow samples were analyzed for the presence of micronuclei. A small but significant increase in micronuclei was seen at 24 hours in males in the mid-dose group. This was not considered to be biologically significant by the authors. No other increases in micronuclei were observed.

Bhunya and Behera (1987) found that TCA was genotoxic in Swiss mice in three cytogenetic studies *in vivo*. In a bone marrow chromosomal aberration assay, TCA-exposed animals (125, 250 or 500 mg/kg, i.p. or p.o.), sacrificed 6, 24 or 48 hours after treatment, were found to have significantly greater scored chromosomal aberrations than vehicle control animals (treated with distilled water). Increases in chromosomal aberrations were greater after i.p. exposure. To evaluate increases in bone marrow micronuclei, animals received two TCA injections (0, 125, 250 or 500 mg/kg, i.p.) 24 hours apart and were then sacrificed 6 hours after the second dose. TCA treatment resulted in significant increases in micronuclei in polychromatic and normochromatic erythrocytes at all dose levels. Significant increases in micronuclei were observed in nucleated cells only at the 500 mg/kg dose. Examination of sperm collected from mice given i.p. injections of TCA revealed numerous sperm-head abnormalities which were significantly greater than controls at all dose levels.

DNA strand breaks.

Nelson and Bull (1988) studied the ability of TCA and other compounds to induce DNA strand breakage in male Sprague-Dawley rats and male $B6C3F_1$ mice. TCA was administered to animals, by gavage, in three dose groups (mice, 0.001 – 0.1 mmmol/kg; rats, 0.6 - 23 mmol/kg), with control animals receiving an equal volume of vehicle. Animals were sacrificed 4 h after treatment, and liver suspensions were analyzed for DNA strand breakage by the alkaline unwinding assay. TCA induced dose-dependent increases in single strand breaks in both mice and rats, which were significantly greater than vehicle control values in the highest dose groups. Mice were markedly more susceptible to DNA strand breakage than rats; the lowest effective dose in mice was 0.006 mmol/kg, whereas in rats the lowest effective dose was 0.6 mmol/kg. Nelson and Bull (1988) also assessed serum enzyme levels and reported DNA strand breakage was induced at doses which produced no hepatotoxic effects.

In contrast, Styles *et al.* (1991) and Chang *et al.* (1992) did not find significant increases in DNA strand breaks. Styles *et al.* (1991) treated $B6C3F_1$ mice with 1, 2, or 3 daily doses of TCA (500 mg/kg, p.o.) either as the free acid or as a neutralized aqueous solution. Animals were sacrificed 4 hours after treatment, and DNA strand breakage was measured by the rate of alkaline unwinding of DNA in a 10% (w/v) liver homogenate.

Chang *et al.* (1992) treated male F344 rats and male $B6C3F_1$ with TCA (0, 1, 5, or 10 mmol/kg, p.o.). Four hours after treatment, animals were sacrificed and homogenized liver tissue was analyzed for alkaline unwinding. No detectable DNA damage was found in rats. In mice, at 10 mmol/kg, TCA produced a small amount of DNA strand breakage (7%). The authors interpreted these results to be without biological relevance. Chang *et al.* (1992) analyzed primary cultures of isolated rat or mouse hepatocytes, and CCRF-CEM cells (a human lymphoblastic leukemia cell line), for DNA strand breakage following incubation with TCA. No increases in DNA strand breaks were observed in any cell cultures.

Oxidative DNA damage.

Austin *et al.* (1996) found that TCA treatment resulted in increases in 8-hydroxy-2-deoxyguanosine, a marker for oxidative stress. Male $B6C3F_1$ mice were given a single acute oral dose of TCA (30, 100 or 300 mg/kg). Animals were sacrificed at various time points (1-12 hours) after dosing and liver nuclear DNA was isolated in order to assess increases in 8-hydroxydeoxyguanosine. TCA induced a significant increase in guanine hydroxylation in the highest dose group from 8 hours after treatment to the end of the experiment (12 hours post-dosing). Compared to brominated haloacetates tested in the same study, the TCA-induced response took longer to become apparent in the post-exposure period, was less marked, and occurred only at the highest dose level.

Parrish *et al.* (1996), in contrast, did not find significant increases in 8-hydroxydeoxy-guanosine after prolonged exposure to TCA (or DCA). In this study, male $B6C3F_1$ mice were exposed to TCA (0, 0.1, 0.5, or 2.0 g/L) in drinking water for 3 or 10 weeks. (No water consumption data were reported, but if an average 35g mouse drinks 6 ml/day, 2 g/L = 343 mg/kg/day) In the same study, Parrish *et al.* (1996) found that TCA (unlike DCA or their brominated analogs) exposure resulted in significant increases in 12-hydroxylation of lauric acid and in cyanide-insensitive acyl-CoA oxidase activity, both indicators of peroxisome proliferation.

Effects on proto-oncogenes and oncoproteins.

Ferreia-Gonzalez *et al.* (1995) evaluated *ras* gene mutations in spontaneous hepatocellular tumors from $B6C3F_1$ mice and in tumors of both TCA-treated and DCA-treated $B6C3F_1$ mice. Treated mice were exposed to TCA (4.5 g/L) or DCA (3.5 or 1.0 g/L) in drinking water for 104 weeks; control mice were exposed to drinking water neutralized to pH 6.8-7.2. After sacrifice, DNA was extracted from sections of liver carcinomas and analyzed for *ras* point mutations by single-stranded conformation polymorphism studies. The incidence of mutations at codon 61 in the H-*ras* gene was

similar in spontaneously induced and TCA-induced (and DCA-induced) carcinomas. The incidence in spontaneous tumors was 58% and was not significantly different from that in TCA or DCA induced tumors. Sequence analysis revealed that TCA-induced tumors showed the same mutational spectrum as spontaneous tumors: a change at codon 61 from CAA to AAA in 80% and CAA to CGA in 20%. In contrast, a different mutational spectrum was observed in DCA-induced tumors.

These differences are consistent with those of Stauber and Bull (1997) who compared TCA- and DCA-induced tumors in male $B6C3F_1$ mice for immunoreactivity to antibodies of c-*jun* and c-*fos* oncoproteins. After exposure to TCA or DCA in drinking water, DCA-induced liver tumors were immunoreactive to anti-c-*jun* and anti-c-*fos* antibodies. In contrast, TCA-induced tumors did not display immunoreactivity to either antibody.

In a study which examined TCA-induced tumor promotion, Tao *et al.* (1996) treated female $B6C3F_1$ mice with *N*-methyl-*N*-nitrosourea (25 mg/kg, i.p.) and thereafter exposed them to TCA (20.0 mM in drinking water, neutralized with NaOH to pH 6.5-7.5) for 52 weeks. After sacrifice, DNA of diagnosed hepatocellular adenomas and carcinomas was extracted and examined for loss of heterozygosity (LOH) at four polymorphic loci on chromosome 6. LOH for at least two loci occurred in almost 30% of tumors promoted by TCA. The authors noted that 20% of hepatic tumors promoted by PCE have been previously shown to exhibit a LOH on chromosome 6, suggesting the presence of a tumor suppressor gene. Interestingly, LOH on chromosome 6 was not observed in tumors promoted by DCA.

DNA Synthesis.

Sanchez and Bull (1990) reported significant increases in [^{3}H] thymidine incorporation into hepatic DNA in mice treated with TCA. Male $B6C3F_1$ mice were exposed to TCA (0, 0.3, 1.0 and 2.0 g/l) in drinking water (adjusted to pH 6.8-7.2) for 2, 5 or 14 days. Two hours prior to sacrifice, animals received a single injection of [^{3}H]thymidine (1μCi/g body weight). DNA was extracted from excised livers, and radioactivity was measured by scintillation counting and autoradiography. [^{3}H]Thymidine incorporation into hepatic DNA increased compared to control animals in a dose-dependent manner and was statistically significant at the highest dose level at 5 and 14 days of administration. Autoradiographs of liver sections did not demonstrate differences between TCA-treated and control animals in percent of labeled hepatocytes. In contrast, in experiments with DCA undertaken in the same study, dose related increases in radiolabeled hepatocytes were identified near areas of necrosis. Significant increases in [^{3}H]thymidine incorporation without evidence of cell division in hepatocytes of TCA-treated mice suggested to the authors an increased rate of DNA repair synthesis.

Dee and Travis (1994), however, reported that increased [^{3}H]thymidine uptake in hepatic DNA of TCA-treated mice was associated with increased cell replication. In this study, male and female $B6C3F_1$ mice received 11 daily doses of TCA (0, 100, 250, 500 or 1000 mg/kg in corn oil) by gavage followed by a single intraperitoneal injection of [^{3}H]thymidine twenty-four hours after the final TCA dose. After sacrifice, DNA was

extracted from excised livers and [^{3}H]thymidine incorporation was determined by autoradiography and scintillation counting. Autoradiographic analysis of liver sections from TCA-treated mice showed increased mitotic figures in both male and female mice whereas no mitotic figures were observed in untreated mice. In TCA-treated animals, label incorporation was observed predominantly in intermediate zone cells compared to control animals where label was found in peri-sinusoidal cells. Increases in [^{3}H]thymidine incorporation in hepatic DNA from TCA-treated mice were dose dependent and statistically significant for all dose groups in males and in all but the low dose group in females.

Stauber and Bull (1997) studied the tumor phenotype and cell replicative behavior in TCA-induced hepatic tumors. In the study, male B6C3F$_1$ mice were treated with TCA (2.0 g/ml, in drinking water) for 50 weeks. Pretreated animals were then given different doses of TCA (0, 0.02, 0.5, 1.0 or 2.0 g/l in drinking water) for two additional weeks to determine whether cell proliferation was dependent on continued treatment. Dividing cells were labeled by implantation, 5 days prior to sacrifice, of mini-osmotic pumps which continuously delivered 5-bromo-2'-deoxyuridine (BrdU). In TCA-induced tumors and altered hepatic foci, rates of cell division were very high and appeared to be independent of continued TCA treatment. In normal hepatic tissue, high doses (1.0 or 2.0 g/l) of TCA during the last two weeks of treatment significantly depressed cell replication. The authors suggest that inhibitory growth factors may be responsible for the decrease in normal hepatocytes, while tumor cells appeared to be resistant.

3.3.3 Structure-Activity Comparisons

The other chlorinated acetic acids have also been tested for carcinogenicity in rodents. Oral exposure to dichloroacetic acid (DCA) is observed to cause liver cancer in mice, as described in related components of several of the studies detailed above. DCA is listed under Proposition 65 as causing cancer. Monochloroacetic acid however is generally not carcinogenic to mice or rats (NTP, 1992), although this compound has sufficiently severe non-neoplastic toxic effects that any such response might be difficult to observe (DeAngelo *et al.*, 1997). A number of other chlorinated aliphatic compounds, including TCE and PCE (of which TCA is identified as a metabolite, see below) are identified as carcinogens for the purposes of Proposition 65.

3.3.4 Pharmacokinetics and Metabolism

The metabolism and pharmacokinetics of TCA have been studies both in animals dosed with TCA, and in animals dosed with halogenated compounds metabolized to TCA. There is an extensive literature dealing with the pharmacokinetics of haloalkenes and their metabolites: the reader is referred to two recent papers (Stenner *et al.*, 1998; Abbas and Fisher, 1997) as examples.

The majority (60 – 70 %) of an oral dose of TCA given to rats and mice was found to appear in the urine, and at least 60% of that was in the form of unchanged TCA (Larson and Bull, 1992). Oxalic and thiodiacetic acids were also observed. Some reductive

dechlorination to DCA and chloroacetic acid was implied by the findings of these authors. DCA is known to be metabolized via glucolic and glyoxylic acids to oxalic acid and carbon dioxide, whereas chloroacetic acid may undergo glutathione conjugation to dithioacetic acid (Larson and Bull, 1992: IARC, 1995).

Recent pharmacokinetic models for chloroalkenes have accommodated formation of TCA as a major metabolite, which is mostly excreted unchanged. Although some reduction of TCA to DCA is usually assumed, recent work suggests that artifacts of the sampling and analysis techniques employed in some of the earlier experiments may have overestimated the amount of DCA formed (Ketcha *et al.*, 1996). Brashear *et al.* (1997) used a mass spectrometry based technique designed to avoid this artifact and found extremely low levels of DCA in plasma of human volunteers exposed to 100 ppm TCE vapor. After a 4 hour exposure the average peak level of TCA (seen four hours after the exposure stopped) was 10 μg/ml, whereas peak DCA levels, observed during the course of exposure, were 12 to 14 ng/ml. This suggests that at least in humans under the conditions observed DCA is an extremely minor metabolite of TCE and/or TCA. Similarly, Merdink *et al.* (1998) measured DCA in blood of male $B6C3F_1$ mice following intravenous dosing with TCA, DCA, TCE, trichloroethanol or chloral hydrate. Pharmacokinetic models were used to predict metabolite levels following treatment with TCA or chloral hydrate, and to estimate the extent of conversion of TCA to DCA. They found, using appropriate sampling techniques which avoided post-sampling conversion of TCA to DCA, that DCA levels in blood were extremely low, being as much as 2 or 3 orders of magnitude lower than those reported from earlier studies. They also showed that conversion of DCA to other metabolites was extremely rapid, so if a small amount of DCA was formed metabolically it would be further transformed before significant concentrations could appear in blood or tissues.

Some formation of DCA from TCA may occur in the gut as a result of the activity of the microflora there: Moghaddam *et al.* (1996) showed that this conversion was performed by rat or mouse cecal contents under anaerobic conditions. However, a study (Moghaddam *et al.*, 1997) of this process *in vivo* suggested that the intestinal microflora contributed only minimally, if at all, to the formation of DCA, via TCA, from trichloroethylene in $B6C3F_1$ mice.

3.3.5 Pathology

Several of the authors of carcinogenicity studies with TCA in mice have reported a succession of hyperplastic and neoplastic lesions in the liver, ranging from apparently pre-neoplastic changes to fully malignant lesions. In particular Bull *et al.* (1990) and Pereira (1996) reported in detail the relative incidences of preneoplastic foci of altered hepatocytes, hepatocellular adenomas, and hepatocellular adenomas, which were identified by standard morphological criteria. Bull *et al.* (1990), DeAngelo (1991), and DeAngelo and Daniel (1992) also commented on the non-neoplastic lesions observed, noting that whereas treatment with DCA resulted in extensive occurrence of focal necrotic lesions, the frequency of such lesions was low in TCA treated mice. Pereira (1996) also noted extensive vacuolation of hepatocytes in mice treated with DCA, which

was not a feature of the pathology observed with TCA. The neoplastic lesions seen in the livers of TCA-treated mice have also characterized with regard to various enzyme activity markers and proto-oncogene mutations (Stauber and Bull, 1997; Latendresse and Pereira, 1997; Pereira, 1996; Parnell *et al.,* 1986), as described in sections 3.3.1 and 3.3.2.

3.4 Mechanism

TCA is inactive in most standard gene mutation assays; positive reports described activity which was weak or only at very high concentrations (Giller *et al.*, 1997; Harrington-Brock *et al.*, 1998). This, and the lack of obvious chemical reactivity of TCA or of metabolism to reactive products, suggests that TCA may not induce carcinogenesis by a "genotoxic" mechanism (*i.e*. TCA does not appear to be directly reactive to DNA). In addition, the liver tumor promoting effect of TCA demonstrated by some experimental protocols is often seen with agents generally presumed to work by non-genotoxic mechanisms. On the other hand, there are some reports of TCA inducing other types of genotoxic effects, including clastogenicity (Bhunya and Behera, 1987). DNA strand breakage (Nelson and Bull, 1988) and oxidative DNA damage (Austin *et al.*, 1996) have also been reported. Other investigators (Styles *et al.,* 1991; Chang *et al.,* 1992; Parrish *et al.*, 1996) have failed to confirm these results, as described in Section 3.3.2 (Genotoxicity). However, some authors have found evidence for DNA damage and repair after TCA exposure (Sanchez and Bull, 1990), and have argued from this in favor of a direct genotoxic mode of action. Neither this possibility, nor the alternative non-genotoxic hypotheses so far proposed for TCA's mode of action have been established.

TCA, DCA and other compounds of which TCA is a metabolite, induce peroxisome proliferation in mouse liver (Elcombe *et al*., 1985; Larson and Bull, 1992; De Angelo *et al.*, 1989). It has been hypothesized that the response to peroxisome proliferators (a miscellaneous class of compounds also including di(2-ethylhexyl)phthalate [DEHP] and various hypolipidemic drugs) is important in the mechanism of carcinogenesis by these compounds (Moody *et al.,* 1991). The peroxisome proliferation response is much greater in rodent liver than in other tissues or other species (Kurata *et al.,* 1998). This suggests that non-rodent species might be less sensitive, or perhaps even insensitive, to carcinogenesis by peroxisome proliferation inducing agents. However, this has not been established, and the true significance of these observations is unclear with regard to both TCA and other peroxisome proliferation inducers.

TCA is more effective in the mouse than the rat in inducing peroxisome proliferation-related responses (De Angelo *et al.*, 1989). However, the extent of these responses even in the mouse is not large compared to the responses seen for other carcinogenic inducers of peroxisome proliferation such as DEHP or the fibric acids (Cohen and Grasso, 1981). Although the peroxisome proliferation responses in rodents to TCA and DCA appear qualitatively similar, the studies by Pereira and colleagues (*e.g.* Latendresse and Pereira, 1997) have consistently suggested that there is a basic mechanistic difference between the neoplastic response in mice to TCA and that to DCA. This was evidenced by differences

in pathology, enzyme activities, and proto-oncogene mutations in liver tumors induced by TCA or DCA. Taken together, these observations call into question the proposed link between peroxisome proliferation induction and the mechanism of carcinogenesis for both compounds.

Similar doubts about the existence of a direct link between peroxisome proliferation and rodent carcinogenesis have been raised in discussion of the carcinogenicity of other peroxisome proliferation inducers (Marsman *et al.,* 1988; Conway *et al.,* 1989). Recent research suggests that peroxisome proliferation and carcinogenesis are parallel (rather than sequential or co-dependent) responses to binding of the inducing agent to a specific peroxisome proliferator activated receptor, PPARα (Green, 1992; Melnick *et al*., 1996). TCA and DCA have been shown to bind to PPARα (Zhou and Waxman, 1998). This appears to be the mechanism of the observed peroxisome proliferation and increase in oxidative enzymes. However, it is not established that this peroxisome proliferation response is closely related to the carcinogenic response to TCA.

Oxidative damage to DNA as a result of peroxisome proliferation may be a contributor to the overall spectrum of hepatotoxicity by peroxisome proliferators, but it has not been demonstrated to play a primary or unique role in carcinogenesis by these agents. Austin *et al.* (1996) reported increases in oxidative damage to DNA (8-hydroxydeoxyguanosine formation) in response to single doses of halogenated acetic acids. These were more substantial for bromine-containing haloacids than for TCA. Subsequently these authors reported (Parrish *et al.,* 1996) that there was no increase in oxidative damage after chronic treatment with TCA. They speculated that whereas oxidative damage may be important in haloacetate carcinogenicity, it may in some cases be at least partly independent of peroxisome proliferation.

Other studies have found that TCA has an effect on growth-related parameters in the mouse liver. Thus Dees and Travis (1994) observed that oral treatment of mice with TCA results in stimulation of DNA synthesis and mitosis in mature hepatocytes. These authors suggested that the observed increase in liver cell mitosis (suggestive of cytotoxicity-induced cell turnover) might contribute to the tumorigenic effects of TCA. Such a mechanism can be readily understood as contributing to the observed tumor promoting effects of TCA, but the basis for proposing that the complete carcinogenic effect could arise in this way is less secure. If the carcinogenic effect were to be explained solely by amplification of background mutation rates (similar for instance to that proposed to explain the induction of bladder carcinomas by saccharin), a large and sustained increase in the total cell turnover in the liver would be implied. Although DNA synthesis and proliferation increased at certain times following single doses or during chronic dosing with TCA, it is not clear that the observed extent of this effect (Stauber and Bull, 1997; Dee and Travis, 1994; Sanchez and Bull, 1990) is sufficient to explain the carcinogenic effect. On the other hand, Ferreira-Gonzalez *et al.* (1995) found that TCA-induced liver tumors showed a spectrum of mutations in the H-*ras* proto-oncogene similar to that observed in spontaneously arising tumors. This observation is supportive of, or at least does not contradict, the cell turnover hypothesis.

Other effects of TCA have been reported that may be related to cell growth regulation. Benane *et al.* (1996) reported that TCA treatment of cultured liver cells resulted in interference with gap junction intercellular communication, as assessed by a dye-transfer assay. TCA produced a more severe effect (based on the concentration required to reduce by 50% over 24 h the number of rows of cells showing dye transfer) than DCA, chloral hydrate or trichloroethanol. Interestingly, PCE produced an even stronger effect than TCA. A number of carcinogens thought to act by non-genotoxic mechanisms have been found to alter intercellular communication, and this function is often observed to be reduced or absent in tumor cells. However, the details and implications of this effect are unclear, and the relevance of this finding to tumorigenicity is far from established, as Benane *et al.* (1996) acknowledge.

Any or all of these effects may contribute to the observed carcinogenic activity of TCA in mouse liver. They may combine with the well-known high sensitivity of the $B6C3F_1$ mouse liver to the consequences of disruption (by both genotoxic and supposedly non-genotoxic carcinogens) of cellular growth regulation. In the male $B6C3F_1$ mouse the background rate of appearance of liver tumors is notably high, whereas the female mouse liver shows a considerably lower background tumor incidence both in the TCA studies described here and in other studies. Some alleged "mouse-liver-specific carcinogens" actually appear specific to the male $B6C3F_1$ mouse. TCA, on the other hand, is clearly established as a carcinogen in both sexes of this strain of mice. Additionally, although the single reported bioassay of TCA in the rat (De Angelo *et al.,* 1997) was negative, evidence of a promoting effect in rat liver was reported (Parnell *et al.*, 1988), possibly similar to that observed in the mouse (Pereira and Phelps, 1996; Pereira *et al.*, 1997).

Overall, these observations add up to a less than complete account of the mechanisms by which TCA induces mouse liver tumors. In the absence of a more complete mechanistic understanding, it cannot be confidently asserted that the observation of tumors in mouse liver, but not in the rat, implies that TCA would not have tumorigenic effects in other untested rodents, non-rodent mammals, or humans.

4 OTHER REVIEWS

IARC has classified TCA in Group 3, not classifiable as to its carcinogenicity to humans, based on inadequate evidence in humans and limited evidence in animals (IARC, 1995). As noted previously in Table 1, IARC (1995) considered the bioassays reported by Herren-Freund *et al.* (1987) and Bull *et al*. (1990). Other bioassays of TCA have been performed (Table 1; Section 3.2) which provide additional evidence of carcinogenicity, including observations of carcinogenicity in female as well as male mice.

5 SUMMARY AND CONCLUSIONS

5.1 Summary of Evidence

TCA caused liver tumors in male and female mice in multiple experiments, by a common route of human exposure (*via* drinking water). However, carcinogenicity was not observed in a study in the rat. In rodents, TCA acts as a promoter of liver tumors and foci of altered hepatocytes initiated by known carcinogenic initiators. The results of short-term tests for mutagenicity are mainly negative, although results in some other tests for genotoxic effects are conflicting, including both positive and negative results. Studies of the possible mode of action in mice have suggested possible roles for "non-genotoxic" mechanisms. These suggested mechanisms have included effects on peroxisome proliferation, enhanced cell proliferation as a result of receptor-mediated effects or in response to cytotoxicity, and effects on intercellular communication. However, none of these proposed mechanisms have been established as a principal cause of the observed carcinogenicity, or even as a contributor to the observed phenomena. A number of chlorinated aliphatic compounds, including TCE and PCE, of which TCA is a metabolite, and DCA, are listed as causing cancer for the purposes of Proposition 65.

5.2 Conclusion

There is sufficient evidence of the carcinogenicity of TCA and its salts in animals, based on multiple independent observations of liver tumors in male and female mice. Although the evaluation by IARC (1995) found only limited evidence of carcinogenicity in animals, the data which they considered have since been significantly augmented, including observations of carcinogenicity in female as well as male mice. This evidence is supported by the carcinogenicity of related compounds (DCA, TCE, PCE). On the other hand, the failure to observe carcinogenicity in the rat, and the mainly negative or conflicting findings of genotoxicity in short-term tests, raise the question of possible non-genotoxic mechanisms, which might be species-specific. Current knowledge as to the validity and importance of the various mechanisms proposed is not sufficient to determine whether or not the observations of carcinogenicity in mice are relevant to human health risks.

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