EVIDENCE ON THE CARCINOGENICITY OF

CHLORAL HYDRATE (2003 UPDATE)

FINAL

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Reproductive and Cancer Hazard Assessment Section
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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).

During a public meeting held in Sacramento, California, on March 1, 1994 the Committee selected chloral hydrate as a candidate for evaluation and requested that OEHHA staff prepare a review of the scientific evidence relevant to the carcinogenic potential of this chemical. A review of pertinent information was contained in the draft document "Evidence on the Carcinogenicity of Chloral Hydrate." This document was released to the public and a notice of its availability was published in the California Regulatory Notice Register on March 10, 1995.

At the May 11, 1995 meeting of the Committee, OEHHA staff summarized scientific studies pertinent to the question of carcinogenicity available at that time. Following discussion and deliberation, the Committee did not determine that chloral hydrate "has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer." A motion to find the evidence inadequate failed (four votes for, three against, with one abstention), while a motion to find the evidence sufficient failed (three votes for, five against). The Committee unanimously decided to express to the National Toxicology Program their concern at the lack of adequate animal testing data to evaluate the carcinogenic potential of chloral hydrate. OEHHA expressed the concern of the Committee in a letter to the National Toxicology Program (NTP). Subsequently, NTP initiated and completed a series of bioassays.

At the November 16, 2000 meeting of the Committee, OEHHA staff reported on the status of these NTP studies, and the Committee requested that OEHHA update the chloral hydrate hazard identification document to include the new information. This document has been updated from that presented in 1995 to reflect the new data published since 1995 relevant to carcinogenicity.

The following is the final version of the document that was discussed by the Committee at their October 2003 meeting.

At their October 17, 2003 meeting the Committee, by a vote of one in favor, five against, and one abstention, did not find that chloral hydrate had been "clearly shown through scientifically valid testing according to generally accepted principles to cause cancer." Accordingly, chloral hydrate was not placed on the Proposition 65 list of chemicals known to the state to cause cancer.

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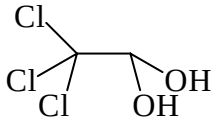
1 EXECUTIVE SUMMARY

Chloral hydrate [$CCl_3CH(OH)_2$] is used as a hypnotic drug, and also occurs as a byproduct of chlorination in drinking water. It is a metabolite of trichloroethylene, which is known to be genotoxic and carcinogenic in rodents. In two long-term studies, chloral hydrate has been shown to induce liver tumors in male mice when administered in drinking water. Chloral hydrate was also shown in a limited study to induce liver tumors in young male $B6C3F_1$ mice, after a single oral dose. Studies with female mice (long-term, stop-exposure, and single exposure groups), male mice (single exposure) and male and female rats (long-term), have not found liver cancer, however. The long-term studies in female mice did show a significant increase in the incidence of adenomas of pituitary gland pars distalis. A single exposure study in female mice also produced increases in malignant lymphoma in two dose groups. Long-term studies in male mice found increased liver carcinomas (diet restricted) and increased combined liver adenomas and carcinomas (*ad libitum* fed), although in the latter case, the increase was in a low-dose group and did not show a positive trend with dose. Like other reactive aldehydes, chloral hydrate is mutagenic and causes cytogenetic abnormalities, spindle inhibition, sister chromatid exchanges and DNA strand breaks *in vivo* and *in vitro.* Overall, there is evidence indicating the carcinogenic potential of this chemical, including the carcinogenicity findings in the animal bioassays, extensive observations of genetic toxicity, and chemical structural analogies with known carcinogens.

2 INTRODUCTION

Chloral hydrate ($C_2H_3Cl_3O_2$; CAS No. 302-17-0; molecular weight, 165.42; see Figure 1 below for structure) has been used as a sedative/hypnotic drug for over a century, and is currently used in pediatric procedures such as dentistry, CAT or MRI scans, and EEG. It also occurs as a minor contaminant of chlorinated drinking water. Smith (1990) compared the potential risks posed by trichloroethylene and chloral hydrate, and concluded that the hazards posed by the use of chloral hydrate in pediatrics were potentially high and should be reviewed.

Figure 1. Chemical Structure of Chloral Hydrate.



3 DATA ON THE CARCINOGENICITY OF CHLORAL HYDRATE

At the time of the first review by the CIC in 1995, chloral hydrate had not been subjected to the full toxicological screening received by more recently introduced drugs. However, more recent studies have increased the array of bioassays to include testing by several oral routes and for different durations of exposure in two species: rats and mice. The body of data has grown to include long-term exposure studies in which chloral hydrate was administered in drinking water to male mice (Daniel *et al.*, 1992; George *et al.*, 2000), male rats (Leuschner and Beuscher, 1998; George *et al.*, 2000) and female rats (Leuschner and Beuscher, 1998) and by oral gavage in male mice (both dietary-controlled and *ad libitum*-fed mice; NTP, 2002b) and female mice (including stop-exposure studies; NTP, 2002a). Single exposure oral route studies with long-term follow-up have been conducted in male mice (Rijhsinghani *et al.*, 1986; NTP, 2002a) and female mice (NTP, 2002a). Chloral hydrate has also been tested by the intraperitoneal route in a neonatal mouse model in both male and female mice (Von Tungeln *et al.*, 2002).There are extensive data indicating that chloral hydrate has genetic toxicity: both cytogenetic and mutational effects have been reported.

A two-year study in which male mice were treated with chloral hydrate in drinking water showed increases in hepatocellular adenomas among all treated groups (George *et al.*, 2000). Hepatocellular carcinomas and combined hepatocellular adenomas and carcinomas were increased in the high dose group. Likewise, a two-year study of tumor incidence in male B6C3F$_1$ mice receiving 1 g/l chloral hydrate in drinking water by the U.S. Environmental Protection Agency have been reported (Daniel *et al.*, 1992). Chloral hydrate was found to cause hepatocellular carcinomas, adenomas and hyperplastic nodules. Other studies in mice employing lower average daily doses have not shown such clear increases in tumors from chloral hydrate treatment. Studies conducted by the National Toxicology Program (NTP) of feed-restricted and *ad libitum* fed male mice treated with chloral hydrate by oral gavage for two years showed an increase in combined hepatocellular adenomas and carcinomas at the lowest of three doses among *ad libitum* fed mice and a significant increase in hepatocellular carcinomas among feed-restricted mice at the high dose, but no significant increases in tumors at other doses (NTP, 2002b). Female mice (fed *ad libitum*) treated with chloral hydrate by oral gavage for two years showed an increase in adenomas of the pituitary gland pars distalis at the high dose with a

significantly positive trend, and showed a positive trend for increases in malignant lymphoma, and no increase in liver tumors (NTP, 2002a). A single dose study in female mice also showed increased malignant lymphomas in two treatment groups. Stop-exposure experiments at three, six, and twelve months showed no significant increases in tumor incidence (NTP, 2002a).

In one study in which young male mice received a single oral dose of chloral hydrate, an increase in liver tumors was observed (Rijhsinghani *et al.*, 1986). This was statistically significant in spite of severe limitations on the power of the study due to small group sizes and the single dose design. However, in three studies conducted by the National Toxicology Program, early-in-life exposure of male and female mice to a single oral dose of chloral hydrate did not result in an increase in liver tumors, or tumors at any other site, after two years of follow-up (NTP, 2002a). A study of male and female neonatal mice in which chloral hydrate was administered by the intraperitoneal route produced a marginally significant increase in liver tumors in one trial, but a follow-up at higher doses did not show an increase in tumors (Von Tungeln *et al.*, 2002).

A study by Roe and Salaman (1955) of chloral hydrate tumor initiation in mouse skin, which was very limited in power, was inconclusive.

Studies in rats have not generally shown an increased tumor incidence in response to treatment with chloral hydrate (Leuschner and Beuscher, 1998; George *et al.*, 2000). A marginally significant increase in combined hepatocellular adenomas and carcinomas was observed in the low dose group of a multi-dose study of male rats (George *et al.*, 2000), although there was no indication of a dose-response, suggesting that the increase in tumors was not treatment related.

3.1 Epidemiological Studies of Carcinogenicity in Humans

Despite the extensive evidence of genotoxicity, and the continuing widespread use in pediatric medicine, direct information on the carcinogenicity of chloral hydrate in humans is not available. No epidemiological studies relating to induction of cancer in humans by chloral hydrate have been identified in the scientific literature.

3.2 Carcinogenicity Studies in Animals

3.2.1 Oral Exposure Studies

Male mice: Single oral exposure. Rijhsinghani et al., 1986

Two groups of 15-day old B6C3F_1 (C57BL × C3H F_1) male mice were given a single dose of chloral hydrate by gavage. One exposed group of 25 mice received 5 mg/kg chloral hydrate, while the other group, of 20 mice, received 10 mg/kg. All doses were dissolved in distilled water, and a control group of 35 mice received distilled water only. Mice were killed when moribund or at intervals up to 92 weeks after treatment. A number of mice in each group were killed 24 hours after the dose was given and examined for short-term pathological effects. No liver nodules were observed in eleven exposed mice (from both exposed groups, exact distribution not specified) or seven control mice killed between weaning (four weeks) and 48 weeks. The others, nineteen control mice, eight from the 5 mg/kg group and nine from the 10 mg/kg group, survived to at least 48 weeks. For the purposes of evaluating carcinogenicity, only mice killed at 48 weeks or later (the time of first appearance of a hepatic tumor, in a mouse exposed to 10 mg/kg chloral hydrate) were considered to be at risk. The liver was the only organ for which histopathological examination was reported. Hepatic nodules were examined and

classified according to the scheme of Vesselinovitch *et al.* (1978) as either hyperplastic foci, hepatocellular adenomas or trabecular carcinomas. In the group receiving 10 mg/kg, the combined incidence of adenomas and carcinomas was significantly greater than in the controls ($p = 0.002$, Fisher Exact Test). The dose-related trend was significant (Mantel-Haenszel trend test, $p = 0.0007$). The finding of a statistically significant increase in hepatic tumors persisted ($p = 0.01$) when animals killed at 48 weeks were excluded from the analysis. Hepatic tumors were observed earlier in exposed animals, occurring between 48 and 88 weeks after dosing, whereas the two tumors in control animals both occurred at week 89. Group sizes and incidences of tumors and foci are given in Table 1.

Table 1. Tumors in B6C3F$_1$ Male Mice Receiving a Single Oral Dose of Chloral Hydrate (Rijhsinghani *et al.*, 1986).

Tumor Site and Type		Dose, mg/kg[a]		
		0	**5**	**10**
Liver	Adenomas	0/19[b]	1/9	3/8
	Carcinomas	2/19	1/9	3/8
	Adenomas or Carcinomas	2/19 p=0.0007[c]	2/9 (p=0.38)[d]	6/8 p=0.002[d]

[a] Given in distilled water at 15 days of age.
[b] Number of tumor bearing animals/number of animals in the group.
[c] p value for trend (Mantel-Haenszel trend test).
[d] p values for Fisher Exact Test relative to control group. Value is given in parentheses when not significant ($p > 0.01$).

Additionally, six mice receiving 10 mg/kg chloral hydrate, ten receiving 5 mg/kg, and nine control mice were killed 24 hours after dosing and analyzed for liver mitotic index. An increase in the mean mitotic index of two- to three-fold was observed at both dose levels, although only the effect in the group receiving 5 mg/kg was significantly different from the controls ($p < 0.01$).

Male mice: 104-Week drinking water study. Daniel et al., 1992

A group of 40 male B6C3F$_1$ mice received 1 g chloral hydrate per liter drinking water for 104 weeks. Two similar control groups, totaling 33 animals, received plain water, while other groups received 2-chloroacetaldehyde or dichloroacetic acid in the drinking water. Interim sacrifices at 30 and 60 weeks (five controls and five chloral hydrate exposed mice at each time) were made for biochemical and pathological analysis. Three control animals and six exposed animals died during the study; causes of these intercurrent deaths, if established, were not presented in the publication. Gross necropsy and histopathological examination of the liver, kidneys, testes, spleen and any gross lesions were performed for all 104-week survivors: in addition a comprehensive histopathological analysis of tissues from 40 different organs was undertaken for five animals from the groups exposed to chloral hydrate or chloroacetaldehyde. At the interim sacrifice at 30 weeks, no tumors were seen. At 60 weeks two out of five exposed mice, but no controls, were found to have hepatocellular carcinomas. Only animals surviving to the final sacrifice at 104 weeks were considered in the statistical analysis. At this time, 11 exposed mice had hepatocellular carcinomas and seven had hepatocellular adenomas, out of 24 survivors. One animal had both tumor types. Hepatocellular lesions in controls included two animals with

carcinomas and one with an adenoma, out of 20 survivors (see Table 2). The incidences of adenomas ($p = 0.04$) and carcinomas ($p = 0.01$) were significantly greater than in controls. The increase in the combined incidence of the two lesion grades was highly significant ($p = 0.0002$). Other than neoplasia, only mild histopathological changes were observed in the liver, and no changes were noted in other organs.

Table 2. Tumors in B6C3F$_1$ Male Mice Receiving Chloral Hydrate (1 g/l in Drinking Water) for 104 Weeks (Daniel *et al.*, 1992).

Tumor Site and Type		Dose, mg/kg-day [a]		
		0	166	
Liver	Hyperplastic Nodules	0/20	1/24	N.S.
	Adenomas	1/20 [b]	7/24	p = 0.04 [c]
	Carcinomas	2/20	11/24	p = 0.01
	Adenomas or Carcinomas	3/20	17/24	p = 0.0002

[a] Average daily intake, based on measured consumption of drinking water.
[b] Number of lesion-bearing animals/total examined at 104 weeks.
[c] p-Values for Fisher's exact test relative to control group (N.S. = not significant).

Male mice: Two-year drinking water study. George et al., 2000

Weanling male B6C3F$_1$ mice were treated with 0, 0.12, 0.58, or 1.28 mg/L chloral hydrate in drinking water for two years. Groups of six animals per dose were killed at 26, 52, and 78 weeks. Based on water consumption data and the measured concentration of chloral hydrate in the water, doses in the treatment groups were calculated to be 13.5, 65.0, and 146.6 mg/kg-day. At sacrifice, histopathological examination was performed on the liver, kidney, spleen, and testes.

Survival was not significantly reduced by treatment with chloral hydrate, although a slight increase in survival was observed in the low-dose group relative to control animals ($p < 0.07$, according to the authors).

The incidences of liver tumors among animals surviving beyond 78 weeks (as reported by the authors) are presented in Table 3. Among all chloral hydrate-treated groups, a statistically significant increase in hepatocellular adenoma was observed relative to the control group. The incidences of hepatocellular carcinoma and combined hepatocellular adenoma and carcinoma were significantly increased in the high-dose group relative to their respective control groups. The authors reported historical control rates for hepatocellular carcinoma from lifetime exposure studies from their facility (National Health and Environmental Effects Research Laboratory) of 31.7, 26, and 23%, incidences substantially lower than that observed in the present study (23/42 = 54.7%). The authors further cited an historical control incidence of 25.5% hepatocellular carcinoma (247/968) among male mice at the National Institute of Environmental Health Sciences, with an individual study range of 11 to 48%.

Tumor incidences for animals killed at the interim sacrifices are presented in Table 4. In the publication, these results were only presented as percentage of animals with tumors. The

incidences were inferred based on the percentage presented and the authors' statement that six animals were sacrificed at each time point. Presumably, the additional one to two animals observed at the various time points either died naturally or were moribund at or near the time of the interim sacrifice. The number of animals examined (the denominator) could not be precisely determined for percentages reported as zero, but these values are presumed to number at least six.

Table 3. Liver Tumors Among Male B6C3F$_1$ Mice Treated with Chloral Hydrate in Drinking Water for Two Years and Surviving Beyond 78 Weeks (George *et al.*, 2000).

Tumor Type	Dose (mg/kg-day)				Trend [a]
	0	13.5	65.0	146.6	
Hepatocellular Adenoma	9/42	20/46 [b]	20/39 [b]	16/32 [b]	p = 0.019
Hepatocellular Carcinoma	23/42	25/46	23/39	27/32 [b]	p = 0.0018
Combined Hepatocellular Adenoma and Carcinoma	27/42	36/46	31/39	29/32 [b]	p = 0.0072

[a] Exact test for linear trend.
[b] Significantly increased above controls by pairwise comparison (Fisher's exact test, $p < 0.05$).

Table 4. Liver Tumors Among Male B6C3F$_1$ Mice Treated with Chloral Hydrate in Drinking Water and Sacrificed at 26, 52, 78, and 104 Weeks (George *et al.*, 2000).

Tumor Type	Dose (mg/kg-day)	Weeks on Study			
		26	52	78	104 [*]
Hepatocellular Adenoma	0	0	1/7	0	9/42
	13.5	0	0	0	20/46
	65.0	1/7	1/8	4/7	20/39
	146.6	0	0	3/7	16/32
Hepatocellular Carcinoma	0	0	1/6	2/7	23/42
	13.5	0	0	5/6	25/46
	65.0	0	1/8	1/7	23/39
	146.6	0	3/7	3/7	27/32

[*] 104-Week study results presented in Table 3 above. Both tumor types showed positive linear trends with dose by the exact test.

Female mice: 104-Week gavage study (Regimen A). NTP, 2002a

Twenty-eight-day old female B6C3F$_1$ mice (48/group) were treated by oral gavage five days per week with chloral hydrate in distilled water at doses of 0, 25, 50, or 100 mg/kg bodyweight for 104 weeks. These doses were calculated as average daily exposures of 0, 17.9, 35.7, and 71.4 mg/kg-day, respectively. No differences in survival were observed between treatment groups

and control animals. A statistically significant increase in adenomas of the pituitary gland pars distalis was observed in the high dose group of female mice relative to the control group ($p = 0.0237$, by the Poly-3 test) and there was a significant positive trend among all dose groups ($p = 0.0073$, dose-related trend). Positive dose-related trends were also observed for malignant lymphomas and alveolar/bronchiolar adenomas, although none of the dose groups showed a statistically significant increase in incidence above control mice by pairwise comparison. No significant increases in liver tumor incidence were observed in any treatment group, nor was there a dose-related trend in the incidences.

NTP concluded that there was "*equivocal evidence of carcinogenic activity* in female $B6C3F_1$ mice treated for two years based on increased evidence of pituitary gland pars distalis adenomas."

Table 5. Tumors among Female $B6C3F_1$ Mice Treated with Chloral Hydrate by Oral Gavage for Two Years (NTP, 2002a).

Tumor Type	Dose (mg/kg-day)				Trend [a]
	0	17.9	35.7	71.4	
Pituitary Gland Pars Distalis Adenoma	0/45	2/44	0/47	5/41 [b]	p = 0.0073
Malignant Lymphoma	9/48	7/48	8/48	15/48	p = 0.0455
Alveolar/Bronchiolar Adenoma	1/48	1/48	2/48	4/48	p = 0.0711
Hepatocellular Adenoma	1/48	2/48	3/48	2/48	N.S.
Hepatocellular Carcinoma	1/48	0/48	0/48	1/48	N.S.
Hepatocellular Adenoma or Carcinoma	2/48	2/48	3/48	3/48	N.S.

[a] P-value of test for dose-related trend (NTP, 2002). N.S. = not significant ($p > 0.05$).
[b] Significantly increased above controls by pairwise comparison (Poly-3 test, $p < 0.05$).

Female mice: Gavage stop-exposure studies with observation for two years (Regimen B). NTP, 2002a

Twenty-eight-day-old female $B6C3F_1$ mice were treated by oral gavage five days per week with zero (n = 24) or 100 mg/kg bodyweight (three groups of n = 48) for periods of three, six, and 12 months, then held until they were killed at two years. Interim sacrifices of eight mice each were performed at three, six, and 12 months. No significant increase in tumor incidence was observed among any of the stop-exposure groups relative to vehicle control animals (from Regimen A above; see Table 6 below).

Table 6. Tumors among Female B6C3F$_1$ Mice in Stop-Exposure Experiments Treated with Chloral Hydrate in Drinking Water (NTP, 2002a).

Tumor Type	Vehicle Control [a]	Dosing Period (Receiving 71.4 mg/kg-day)			
		3 Mo.	6 Mo.	12 Mo.	24 Mo. [a]
Pituitary Gland Pars Distalis Adenoma	0/45	3/36	1/36	1/33	5/41 [b]
Malignant Lymphoma	9/48	8/40	13/40	14/40	15/48
Alveolar/Bronchiolar Adenoma	1/48	2/40	4/40	7/40	4/48
Hepatocellular Adenoma	1/48	1/40	1/40	2/40	2/48
Hepatocellular Carcinoma	1/48	0/40	1/40	2/40	1/48
Hepatocellular Adenoma or Carcinoma	2/48	1/40	2/40	4/40	3/48

[a] Value from Regimen A (Table 5 above).
[b] Significantly increased above controls by pairwise comparison (Fisher's exact test, $p < 0.05$).

Female mice: Single gavage exposure of 28-day-old mice, with observation for two years (Regimen C). NTP, 2002a

Twenty-eight-day-old female B6C3F$_1$ mice (48/group) were treated by oral gavage with a single dose of 0, 10, 25, or 50 mg chloral hydrate per kilogram bodyweight, and then held until they were sacrificed at 104 weeks. The incidence of malignant lymphoma was significantly increased among chloral hydrate treated mice in the low- and high-dose groups, but not the mid-dose group (see Table 7 below). No significant changes in liver tumor incidence were reported, although there was a statistically significant negative trend for hepatocellular carcinoma.

Table 7. Tumors among Female B6C3F$_1$ Mice Treated with a Single Dose of Chloral Hydrate by Oral Gavage at 28 Days of Age, and Then Held Until Sacrifice at Two Years (Regimen C; NTP, 2002a).

Tumor Type	Dose (mg/kg)				Trend [a]
	0	10	25	50	
Malignant Lymphoma	8/48	16/48 [b]	6/48	16/48 [b]	N.S.
Hepatocellular Adenoma	3/48	3/48	2/48	2/48	N.S.
Hepatocellular Carcinoma	3/48	0/48	0/48	0/48	[p = 0.015]
Hepatocellular Adenoma or Carcinoma	6/48	3/48	2/48	2/48	N.S.

[a] P value associated with trend test (NTP, 2002). Negative trends are indicated in brackets. N.S. = not significant.
[b] Significantly increased above controls by pairwise comparison (Poly-3 test, $p < 0.05$).

Female mice: Single gavage exposure of 15-day-old mice, with observation for two years (Regimen D). NTP, 2002a

Fifteen-day-old female B6C3F_1 mice (48/group) were treated by oral gavage once with 0, 10, 25, or 50 mg chloral hydrate per kilogram body weight, and then held until 104 weeks at which time they were sacrificed. No treatment related effects on body weight or survival were observed among these mice. The incidences of liver tumors in each group are presented in Table 8 below. A significant decrease in malignant lymphoma was observed among the mice treated with 25 mg/kg chloral hydrate and an overall negative trend with dose was observed (by Poly-3 analysis).

Table 8. Tumors among Female B6C3F_1 Mice Treated by Oral Gavage with a Single Dose of Chloral Hydrate at 15 Days of Age, and Then Held Until Sacrifice at Two Years (Regimen D; NTP, 2002a).

Tumor Type	Dose (mg/kg)				Trend [a]
	0	10	25	50	
Malignant Lymphoma	14/48	10/48	5/48 [b]	7/48	[p = 0.035]
Hepatocellular Adenoma	1/48	1/48	2/48	1/48	N.S.
Hepatocellular Carcinoma	0/48	2/48	1/48	1/48	N.S.
Hepatocellular Adenoma or Carcinoma	1/48	3/48	2/48	2/48	N.S.

[a] P-value associated with trend test (NTP, 2002). N.S. = not significant. Bracket indicate negative trend.
[b] Significant decrease in incidence relative to controls ($p < 0.05$, by Poly-3 test).

Male mice: Two-year gavage study. NTP, 2002b

Male mice (120/group) were treated by oral gavage five days per week for 104-105 weeks with chloral hydrate in distilled water at doses of 0, 25, 50, or 100 mg/kg_{bw}. These were calculated to be average daily exposures of 0, 17.9, 35.7, and 71.4 mg/kg-day, respectively. The mice were divided into two groups (n=60), one of which received feed in amounts that were "calculated to maintain body weight on a previously computed idealized body weight curve." The other group received feed *ad libitum*. Of the 60 mice, 12 were sacrificed at 15 months for an interim evaluation.

Survival among each chloral hydrate dosed group of animals within a dietary group was comparable to its respective control group. Body weights also did not differ significantly from controls. Liver tumor incidences are presented in Table 9 (15 month interim sacrifice liver tumor results presented in Table 10). Among *ad libitum*-fed mice, a significant increase in combined hepatocellular adenomas and carcinomas was observed in the low-dose group ($p < 0.05$, by Poly-3 test); however, no significant positive dose-related trend was observed for these tumors. Among dietary-controlled mice, the incidence of hepatocellular carcinoma was increased in the high-dose group relative to control mice ($p < 0.05$, by Poly-3 test), and a positive dose-related trend was observed. A positive-dose related trend was also observed for combined hepatocellular adenomas and carcinomas, although there were no significant increases by pairwise comparison.

Table 9. Liver Tumor Incidence among Both *Ad Libitum*-Fed and Dietary-Controlled Male B6C3F$_1$ Mice Treated by Oral Gavage with Chloral Hydrate in Water for Two Years (NTP, 2002b).

Diet	Tumor Type	Dose (mg/kg-day)				Trend [a]
		0	17.9	35.7	71.4	
Ad Libitum-Fed	Hepatocellular Adenoma	12/48	19/48 [b]	17/47	17/48	N.S.
	Hepatocellular Carcinoma	4/48	10/48 [b]	10/47 [b]	7/48	N.S.
	Combined Hepatocellular Adenoma and Carcinoma	16/48	25/48 [c]	23/47 [b]	22/48	N.S.
Dietary-Controlled	Hepatocellular Adenoma	9/48	7/48	10/48	10/48	N.S.
	Hepatocellular Carcinoma	2/48	5/48	4/48	8/48 [c]	p = 0.029
	Combined Hepatocellular Adenoma and Carcinoma	11/48	11/48	14/48	18/48 [b]	p = 0.041

[a] Exact test for linear trend. N.S. = not significant.
[b] Pairwise comparison with controls (Poly-3 test): 0.05 < p < 0.1
[c] Significant increase above controls by pairwise comparison (Poly-3 test, p < 0.05).

Table 10. Liver Tumor Incidence among Both *Ad Libitum*-Fed and Dietary-Controlled Male B6C3F$_1$ Mice Treated by Oral Gavage with Chloral Hydrate in Water for 15 Months (NTP, 2002b).

Diet	Tumor Type	Dose (mg/kg-day)			
		0	17.9	35.7	71.4
Ad Libitum-Fed	Hepatocellular Adenoma	2/12	2/12	3/12	1/12
	Hepatocellular Carcinoma	0/12	1/12	0/12	2/12
	Combined Hepatocellular Adenoma and Carcinoma	2/12	3/12	3/12	3/12
Dietary-Controlled	Hepatocellular Adenoma	0/12	0/12	0/12	0/12
	Hepatocellular Carcinoma	0/12	0/12	0/12	0/12
	Combined Hepatocellular Adenoma and Carcinoma	0/12	0/12	0/12	0/12

Based upon the findings reported above, NTP (2002b) concluded:

> "Under the conditions used in this 2-year gavage study, there was *some evidence of carcinogenic activity* of chloral hydrate in male B6C3F$_1$ mice based on increased incidences of hepatocellular adenoma and carcinoma (combined) in *ad libitum*-fed mice and on increased incidences of hepatocellular carcinoma in dietary-controlled mice.."

Male mice: Single gavage exposure of 15-day-old mice, with observation for two years (Regimen E). NTP, 2002a

Fifteen-day-old male $B6C3F_1$ mice (48/group) were treated by oral gavage once with 0, 10, 25, or 50 mg chloral hydrate per kilogram body weight, then held until 104 weeks, at which time they were sacrificed. No treatment related effects on body weight, survival, or tumor incidence was observed among these mice. No significant increase in tumor incidence was observed in any chloral hydrate treated group relative to the control animals. The incidences of liver tumors in each group are presented in Table 11 below. A statistically significant decrease in the incidence of hepatocellular adenomas was observed in the group of male mice treated once at the low dose (10 mg/kg).

Table 11. Tumors among Male $B6C3F_1$ Mice Treated by Oral Gavage with a Single Dose of Chloral Hydrate at 15 Days of Age, and Then Held Until Sacrifice at Two Years (Regimen E; NTP, 2002a).

Tumor Type	Dose (mg/kg)				Trend [a]
	0	10	25	50	
Hepatocellular Adenoma	18/48	8/48 [b]	12/48	11/48	N.S.
Hepatocellular Carcinoma	10/48	10/48	6/48	12/48	N.S.
Hepatocellular Adenoma or Carcinoma	24/48	17/48	18/48	21/48	N.S.

[a] P-value associated with trend test (NTP, 2002). N.S. = not significant.
[b] Significantly decreased below controls by pairwise comparison (Poly-3 test, $p < 0.05$).

Male and female rats: Two-year drinking water studies. Leuschner and Beuscher, 1998

Chloral hydrate was administered to rats (50/sex/group) aged 25 to 29 days in drinking water such that the dose received was 0, 15, 45, or 135 mg/kg-day (authors' calculation) for 124 weeks (males) or 128 weeks (females). No significant effect of treatment on survival or body weight gain was observed. The high dose level produced a significant increase in the incidence of hepatocellular hypertrophy, but no evidence of carcinogenicity among the chloral hydrate treated rats was observed. The minimal toxicity observed in this study led U.S. EPA (2000) to conclude that the experiment was not conducted at the maximum tolerated dose.

Male rats: Two-year drinking water study. George et al., 2000

Weanling male F344/N rats (78/dose) were treated with drinking water containing 0, 0.12, 0.58, or 2.51 mg chloral hydrate per liter for two years. Interim sacrifices (6/group) were performed at 13, 26, 52, and 78 weeks. Based on water consumption data and the measured concentration of chloral hydrate in the water, doses in the treatment groups were calculated to be 7.4, 37.4, and 162.6 mg/kg-day. At sacrifice, histopathological examination was performed on the liver, kidney, spleen, and testes.

Survival was not significantly reduced by treatment with chloral hydrate. The minimal toxicity observed in this study led U.S. EPA (2000) to conclude that the experiment was not conducted at the maximum tolerated dose. The incidences of liver tumors among animals surviving beyond 78 weeks (as reported by the authors) are presented in Table 12.

No significant increases in the incidence or multiplicity of liver tumors among the male rats were observed. A marginally significant increase in combined hepatocellular adenomas and carcinomas was observed in the low dose group relative to controls (p = 0.055, by Fisher's exact test). No significant dose-related trends were observed. No significant increases in tumor incidence were observed at other sites.

Table 12. Liver Tumors Among Male F344/N Rats Treated with Chloral Hydrate in Drinking Water for Two Years and Surviving to 78 Weeks (George *et al.*, 2000).

Tumor Type	Dose (mg/kg-day)			
	0	**7.4**	**37.4**	**162.6**
Hepatocellular Adenoma	0/42	3/42	1/44	2/44
Hepatocellular Carcinoma	1/42	3/42	0/44	1/44
Combined Hepatocellular Adenoma and Carcinoma	1/42	6/42 [a]	1/44	3/44

[a] Marginally significant increase above controls in pairwise comparison (p = 0.055, by Fisher's exact test).

3.2.2 Intraperitoneal Exposure Studies

Male and female mice: Neonatal exposure studies. Von Tungeln et al., 2002

Twenty-four male and 21 female neonatal $B6C3F_1$ mice were administered total doses of 2000 nmol (Assay A) or 1000 nmol (Assay B) chloral hydrate in DMSO intraperitoneally. In Assay A, three-sevenths and four-sevenths of the dose was administered on the 8^{th} and 15^{th} day of life, respectively, then the animals were held until sacrifice at 12 months. In Assay B, one third and two thirds of the dose was administered on the 8^{th} and 15^{th} days of life, respectively, then the animals were held until sacrifice at 20 months. Each study included a DMSO control group and a positive control group (4-aminobiphenyl: 1000 nmol in Assay A and 500 nmol in Assay B).

An increase in liver adenomas was observed among male mice treated with chloral hydrate relative to the control group in the 12-month assay (5/24 *vs.* 1/24) (see Table 13 below), although the difference did not reach statistical significance (p = 0.094, by Fisher's exact test). No liver tumors were observed among female mice in the 12-month assay, although the positive control (4-aminobiphenyl) also did not produce any liver tumors. In the 20-month assay, there was a slight increase, though not statistically significant, in the incidence of liver adenomas (p = 0.26, by Fisher's exact test) and combined liver adenomas and carcinomas (p = 0.27, by Fisher's exact test) among the chloral hydrate treated male mice. No liver tumors were observed in female mice treated with chloral hydrate. Contrary to the 12-month study, the 20-month study with 4-aminobiphenyl produced a significant increase in liver adenomas (only) in female mice.

Table 13. Liver Tumors among Male and Female B6C3F$_1$ Mice Treated Intraperitoneally as Neonates with Chloral Hydrate in DMSO (Von Tungeln *et al.*, 2002).

Tumor Type	Sex	Control (DMSO)	Chloral Hydrate	4-Amino-biphenyl
Assay A (12 Months): 2000 nmol chloral hydrate				
Hepatocellular Adenomas	M	1/24	5/24 [a]	23/24 [b]
	F	0/24	0/21	0/24
Hepatocellular Carcinomas	M	0/24	0/24	13/24 [b]
	F	0/24	0/21	0/24
Combined Hepatocellular Adenomas and Carcinomas	M	1/24	5/24 [a]	24/24 [b]
	F	0/24	0/21	0/24
Assay B (20 Months): 1000 nmol chloral hydrate				
Hepatocellular Adenomas	M	6/23	9/23	22/22 [b]
	F	0/23	0/22	9/23 [b]
Hepatocellular Carcinomas	M	2/23	2/23	14/22 [b]
	F	0/23	0/22	0/23
Combined Hepatocellular Adenomas and Carcinomas	M	7/23	10/23	22/22 [b]
	F	0/23	0/22	9/23 [b]

[a] Pairwise comparison with controls (Fisher's exact test): $p = 0.094$.
[b] Significant increase in incidence relative to controls by pairwise comparison (Fisher's exact test, $p < 0.05$).

The marginal increase in liver tumors observed in chloral hydrate treated male mice in the 12-month assay led the investigators to conduct additional studies at higher doses in both male and female neonatal mice. Total doses of 2500 and 5000 nmol chloral hydrate were administered intraperitoneally (divided into one third and two thirds on days eight and 15 of life, respectively), and then the mice were held until sacrifice at 12 months. Among the mice in the 2500 nmol dose group, one male mouse developed a liver adenoma (1/24) and no tumors developed in female mice. Among the mice in the 5000 nmol dose group, two male mice developed liver adenomas (2/22) and one developed a lung adenoma (1/22). Female mice at 5000 nmol developed one lung adenoma (1/24), but no liver tumors. DMSO control data for this second set of experiments were not presented in the publication (Von Tungeln *et al.*, 2002).

3.2.3 Dermal Exposure Study

Male mice: Repeated dermal exposure study. Roe and Salaman, 1955

Twenty male albino "S" mice received two dermal applications of 0.3 ml of a 4% solution of chloral hydrate one week apart (total dose 24 mg), followed by 18 weekly applications of 0.5% croton oil in acetone, starting four weeks after the first chloral hydrate treatment. Of these 20 mice, 17 were still alive at 23 weeks. Four mice were observed to have skin tumors at the end of the croton oil treatment.

Another group of 20 male "S" mice received 0.3 ml of a 5% solution of chloral hydrate applied to the skin weekly for 15 weeks (total dose 225 mg). Beginning three days after the first chloral hydrate application, 18 weekly applications of 0.5% croton oil in acetone were given. All 20 exposed animals were still alive at 18 weeks. Four mice were observed to have skin tumors at the end of the croton oil treatment.

A control group of male "S" mice received 18 applications of croton oil only. There were 20 surviving controls, of which one bore skin tumors. Although the incidence of skin tumors was elevated in both exposed groups relative to that in the control group, neither of the differences was statistically significant ($p = 0.17$) individually. The elevation in tumor incidence was significant if the findings in the two exposed groups were combined and compared to the controls ($p = 0.01$). In view of the different site, limited power, and inconclusive result of this study, it cannot be regarded as either confirming or conflicting with the mouse liver results.

3.3 Other Relevant Data

In addition to the reported animal bioassays, additional evidence relating to the possible carcinogenicity of chloral hydrate is available. This includes extensive studies of genetic toxicity, observations of the pharmacokinetics and metabolism, investigations of the biochemical and pathological mechanisms of action of chloral hydrate, and structure-activity comparisons.

3.3.1 Genetic Toxicology

Chloral hydrate causes aneuploidy in various tests systems, including eukaryotic microbial organisms, mammalian cells in culture and mammalian germ cells *in vivo* (Table 14–Table 16). Positive results in the *Salmonella* point mutation assay have been reported, in both the presence and absence of microsomal activating enzymes (Waskell, 1978; NTP, 2002a). The induction of sister-chromatid exchanges and DNA strand breaks have also been reported. Sister-chromatid exchanges were observed in human lymphocytes exposed *in vitro* to chloral hydrate (Gu *et al.*, 1981).

In a review of reports on chemical-induced aneuploidy in mammalian germ cells, Allen *et al.* (1986) considered that chloral hydrate was one of only two out of 46 suspected chemicals (the other being cyclophosphamide) for which there was unequivocal evidence of aneuploidy induction. More recently, chloral hydrate was selected as a test chemical in a research project to evaluate methods for detecting aneuploidy induction and clastogenicity (Parry and Sors, 1993). Some of these methods involved direct determinations of chromosome numbers in exposed cells, whereas others used observation and characterization of micronuclei as an index of chromosome displacement during mitosis. Chloral hydrate was positive in numerous systems using mammalian cells *in vitro* (Natarajan, 1993). The detection of a positive effect of chloral hydrate in systems *in vivo* was less consistent. However, this appears to be a function of the sensitivity of the specific methods used, and details of the individual protocols. Re-examination of some apparently negative test findings showed that effects were detectable using alternative statistical analyses of the results (Adler, 1993).

Liang and Brinkley (1985) considered that the mechanism of aneuploidy induction involved interference with microtubules. Brunner *et al.* (1991) showed that chloral hydrate interferes with tubulin assembly *in vitro.* While this may account for the numerous reports of gains or losses of whole chromosomes, the observation by some authors of point mutational events and other intra-chromosomal abnormalities suggests that other genotoxic mechanisms may be involved as well.

In addition to these experimental studies, there is a single case report (Salmon *et al.*, 1991) of a 23 year old male who took repeated doses of chloral hydrate totaling approximately 35 grams over a five month period. Elevated numbers of chromosomal aberrations (including gaps), sister chromatid exchanges per cell and micronuclei were observed in peripheral blood lymphocytes. While a single report of this type cannot be interpreted in isolation, it suggests that further studies to identify and analyze such effects in exposed humans would be desirable.

Table 14. Non-Mammalian Species, Tests *In Vitro*.

Species, Strain	Results	Reference
Cytogenetic Changes:		
Aspergillus nidulans	Haploidization	Singh and Sinha (1976)
Aspergillus nidulans	Aneuploidy	Morris, NR (1981)
Aspergillus nidulans	Aneuploidy	Carere *et al.* (1984)
Aspergillus nidulans, conidia	Aneuploidy	Carere *et al.* (1985), Crebelli (1991)
Aspergillus nidulans (diploid and haploid)	Aneuploidy, polyploidy, crossing over, blocks microtubule formation	Kafer (1986)
Aspergillus nidulans, Saccharomyces cerevisiae	Aneuploidy	Reviews: Dellarco *et al.* (1986), Waters *et al.* (1986), Parry (1993)
Aspergillus nidulans	Aneuploidy	Kappas (1990)
Saccharomyces cerevisiae	Chromosome loss	Parry *et al.* (1990)
Saccharomyces cerevisiae	Mitotic chromosomal malsegregation	Albertini (1990)
Gene Mutations:		
Salmonella typhimurium	Reverse Point Mutations	Heddle and Bruce (1977)
Salmonella typhimurium	Reverse Point Mutations: TA100, + TA98, - TA104, +	Waskell (1978); Giller *et al.* (1995); Ni *et al.* (1994)
Saccharomyces cerevisiae, +S9	Gene Conversion, Point Mutation	Bronzetti *et al*. (1984)
Aspergillus nidulans, diploid conidia	Lethal/deletion	Kafer (1986)

Table 15. Mammalian Species, Tests *In Vivo*.

Species, Strain	Route	Cell Type	Results	Reference
Mouse	i.p.	Germ cells	Aneuploidy	Carere et al. (1984)
Mouse	i.p.	Spermatocytes, testicular cells	Aneuploidy	Liang and Pacchierotti (1988)
Mouse ($B6C3F_1$)	i.p.	Spermatocytes	Aneuploidy, fragments	Russo *et al.* (1984)
Mouse (BALB/c)	i.p.	Spermatids	Micronuclei	Russo and Levis (1992b, 1992a)
Mouse ($102 \times C3H\ F_1$)	i.p.	Spermatocytes	Aneuploidy	Miller and Adler (1992)
Rat (Sprague-Dawley)	oral	Liver	DNA strand breaks	Nelson and Bull (1988)
Mouse ($B6C3F_1$)	oral	Liver	DNA strand breaks	Nelson and Bull (1988)
Mouse ($101 \times C3H\ F_1$)	i.p.	Bone marrow	Mitotic abnormalities	Miller and Adler (1989a; 1989b)
Mouse (CD-1)	i.p.	Bone marrow	Micronuclei	Gudi *et al.* (1992)
Mouse (BALB/c)	i.p.	Bone marrow	Micronuclei ($CREST^+$)	Russo *et al.* (1992)
Mouse (C57Bl/Cne × C3H/Cne F_1)	i.p.	Bone marrow	Micronuclei (PCE), cell cycle delay, hyperploidy	Leopardi *et al.* (1993)
Mouse (Swiss CD-1)	i.p. & oral	Bone marrow	Micronuclei, numerical chromosomal aberrations	Marrazzini *et al.* (1994)
Mouse	i.p.	Spermatid	Spermatid micronuclei	Allen *et al.* (1994)
Mouse ($B6C3F_1$)	i.p.	Spermatid	Spermatid micronuclei	Nutley *et al.* (1996)
Mouse ($(102/E1 \times C3H/E1)F_1$ and Balb/c)	i.p.	Erythrocytes	No induced micronuclei	Grawé *et al.* (1997)

Table 16. Mammalian Species, Tests *In Vitro*.

Species (Strain)	Cell Type	Results	Reference
Human	Lymphocytes	Sister Chromatid Exchange, Strand Breaks	Gu *et al.* (1981)
Human	Lymphocytes	Aneuploidy	Vagnarelli *et al.* (1989, 1990)
Human	Fibroblasts	Micronuclei	Bonatti *et al.* (1992)
Human	Lymphocytes	Micronuclei	Van Hummelen and Kirsch-Volders (1992)
Human	Lymphocytes	Micronuclei (size changes)	Ferguson *et al.* (1993)
Human	Lymphocytes	Aneuploidy, tetraploidy	Sbrana *et al.* (1993)
Human	Lymphocytes	No micronucleus induction observed	Vian *et al.* (1995)
Hamster (Chinese)	Lung cells	Micronucleus induction	Degrassi and Tanzarella (1988)
Hamster (Chinese)	Ovary cells (primary & cell lines)	Aberrations of cell division	Parry *et al.* (1990)
Hamster (Chinese)	Embryonic diploid cells	Aneuploidy	Furnus *et al.*, (1990)
Hamster (Chinese)	Primary embryonic fibroblasts	Aneuploidy	Natarajan *et al.* (1993)
Hamster (Chinese)	V79 cell line	Micronuclei	Seelbach *et al.* (1993)
Hamster (Chinese)	DON:Wg3h, LUC2 cells	Aneuploidy, mitotic abnormalities	Warr *et al.* (1993)
Hamster (Syrian)	Embryo cells	Morphological transformation	Gibson *et al.* (1995)
Mouse (MF1)	Oocytes	Changes in spindle shape, cytokinesis, and cell cycle progression	Eichenlaub-Ritter and Betzendahl (1995)
Mouse	L5178Y/TK$^{+/-}$ –3.7.2C lymphoma cells	Mutagenic, clastogenic	Harrington-Brock *et al.* (1998)

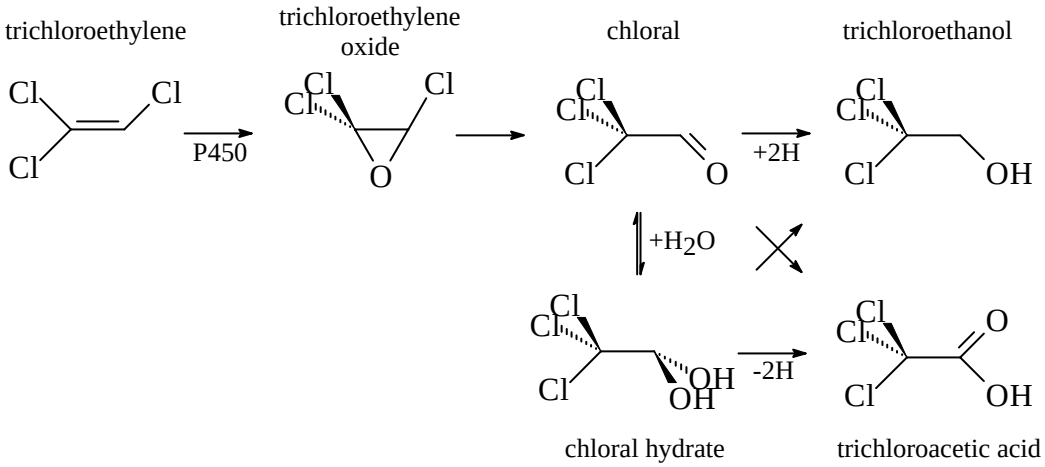
Table 17. Non-Mammalian Species, Tests *In Vivo*

Species	Results	Reference
Triturus helveticus, eggs	"Star Metaphases", spindle remnants during late mitosis, microtubule mis-orientation, chromosomal kinetochore retention	Ates (1978)
Triturus helveticus or *Pleurodeles waltii,* eggs	Star metaphase, reduced achromatic apparatus	Sentein and Ates (1978)
Pleurodeles waltii, larvae	Micronuclei (at highest dose used only)	Fernandez *et al.* (1993)
Drosophila melanogaster	Wing somatic mutation and recombination test	Zordan *et al.* (1994)
Pleurodeles waltii, larvae	Micronuclei	Giller *et al.* (1995)

3.3.2 Structure-Activity Comparisons

Chloral hydrate is a metabolite of trichloroethylene (Byington and Leibman, 1965; Miller and Guengerich, 1982; Green and Prout, 1985), as shown in Figure 2. Oxidation of trichloroethylene, like that of many compounds containing an ethylenic double bond, is catalyzed by cytochrome P-450 enzymes and generates an epoxide. In the case of trichloroethylene this epoxide is unstable, spontaneously rearranging to chloral with migration of one chlorine atom. Chloral hydrate may be one of the chemical species responsible for the genotoxic and carcinogenic effects of trichloroethylene, although the mechanism by which these effects are produced is not known and several competing theories have been advanced to explain them.

Figure 2. Metabolism of Trichlorethylene and Chloral Hydrate.



3.3.3 Pharmacokinetics and Metabolism

Chloral hydrate is metabolized by reduction to trichloroethanol (Ogino *et al.*, 1990) and oxidation to trichloroacetic acid (Sato *et al.*, 1981). Evidence from the treatment of liver microsomes from male $B6C3F_1$ mice has suggested that this conversion is likely mediated by cytochrome P450 (probably CYP2E1) and produces free radical intermediates which may result in lipid peroxidation (Ni *et al.*, 1996). Chloral hydrate's metabolism occurs mainly in the liver and kidneys. These reactions are rapid, but may be limited both by saturation of individual enzymes and by limits on the availability of reductant (NADH/NADPH) or oxidant (NAD^+/$NADP^+$) (Kawamoto *et al.*, 1987). The ratio of reduced to oxidized product excreted appears to vary with animal species and metabolic status. Because the enzymes and cofactors responsible for these reactions are also involved in ethanol metabolism, chloral hydrate and ethanol enhance the sedative effects of one another. This leads to the potency of the "Mickey Finn" cocktail (Larson and Bull, 1989).

3.3.4 Pathology

The liver tumors observed in mouse liver were considered by the authors to meet standard criteria for hepatocellular adenomas and carcinomas. These two tumor phenotypes are generally considered to be related in origin, with the possibility that adenomas may progress to carcinomas. They are normally therefore aggregated for carcinogen identification and risk assessment purposes.

The pituitary pars distalis adenomas observed in the female mice are also considered to meet standard criteria.

3.4 Mechanism

Chloral hydrate is a genotoxic compound, causing aneuploidy and micronucleus formation in various systems. A genotoxic mechanism may therefore be responsible for the observed carcinogenic effect. It has been argued for some chlorinated compounds that there is a species-specific effect on the mouse liver, involving cytotoxicity (Schumann *et al.*, 1980; Stott *et al.*, 1982), and/or peroxisome proliferation (Elcombe, 1985), which is responsible for the tumorigenicity. If that were the case, the finding of carcinogenicity of such compounds in mouse liver would not necessarily imply carcinogenicity at other sites or in other (especially non-rodent) species. NTP (2002b) conducted assays for hepatic enzymes indicative of peroxisome proliferation in a 15-month interim sacrifice during the 2-year study of both *ad libitum*-fed and dietary-controlled mice and mice in a supplemental study (also both *ad libitum*-fed and feed restricted) receiving chloral hydrate by gavage for 14 days prior to sacrifice. Cytochrome P450 CYP4A protein, lauric acid hydroxylation, and cyanide-insensitive palmitoyl CoA fatty acid hydroxylase were evaluated. An increase in CYP4A protein and lauric acid hydroxylation was observed among dietary-controlled mice at the high dose, coincident with the increase in hepatocellular carcinoma. These markers were not significantly induced among *ad libitum*-fed mice. In the George *et al.* (2000) study, 26 weeks of treatment of mice with chloral hydrate did not produce evidence of peroxisome proliferation in the liver, as indicated by a lack of increases in cyanide-insensitive palmitoyl CoA oxidase activity.

It should be noted that Daniel *et al.* (1992) reported only mild hepatocellular necrosis and hyperplasia in mice exposed to chloral hydrate (severity indices 0.79 and 0.13 respectively, where 0 = normal, 1 = mild, 4 = severe). No specific non-neoplastic lesions were described

which were considered treatment-related. This observation, and the findings of genotoxicity, indicate that it is unlikely that the carcinogenicity of chloral hydrate primarily involves cytotoxicity or peroxidative mechanisms.

The finding of genetic toxicity, *e.g.*, positive mutational or cytogenetic assay results for chloral hydrate, is therefore considered to provide strong support for the interpretation of a positive carcinogenicity finding in mouse liver as an indication of general, rather than species- or tissue-specific activity (Haseman and Lockhart, 1993; Gold *et al.*, 1991; Huff *et al.*, 1991).

4 OTHER REVIEWS

In 1995, the International Agency for Research on Cancer (IARC) reviewed the available data on the carcinogenicity of chloral hydrate and determined that the chemical was "not classifiable" (Group 3) as to its carcinogenicity to humans based upon "limited evidence" for its carcinogenicity in experimental animals and "inadequate evidence" in humans (IARC, 1995). IARC recently revisited this assessment, and reached the same conclusions regarding the evidence in experimental animals and humans and the same overall conclusion (IARC, 2002; volume unavailable pending publication; conclusions obtained from posting at http://www.iarc.fr).

The U.S. Environmental Protection Agency (2000) determined that "chloral hydrate shows suggestive evidence of human carcinogenicity by the oral route of exposure" and characterized the supporting data as follows:

> "There are no carcinogenicity data from humans. Two bioassays in rats in which chloral hydrate was administered by drinking water show no increase in tumors at any site. Because only minimal toxicity was observed in the livers of the rats in these bioassays, the tests were not conducted at the maximum tolerated dose. A chronic bioassay in female mice showed a slight increase in the severity grade of hyperplasia and a slight increase in the incidence of adenoma in the pituitary gland pars distalis at the highest exposure tested. There is some evidence that chloral hydrate causes hepatocellular tumors in male mice. An earlier study showing an increase in hepatic adenomas or trabecular carcinomas following a single bolus exposure could not be confirmed in a study using more animals and higher exposures. Three separate 2-year bioassays in male mice show an increased incidence of hepatocellular adenoma or carcinoma. There are no data identifying a lesion that is a precursor to the hepatocellular tumors. The strain of mice used has a very high spontaneous incidence of hepatocellular tumors. Two of the metabolites of chloral hydrate, trichloroacetic acid and dichloroacetic acid, have been shown to cause hepatocellular tumors in rodents. Trichloroacetic acid causes hepatocellular tumors only in mice. Dichloroacetic acid causes hepatocellular tumors in both rats and mice.
>
> There is an extensive database on genetic toxicity. A variety of results show that chloral hydrate is a weak gene mutagen and clastogen. Chloral hydrate induces aneuploidy in a wide variety of cell types. These latter effects are thought to arise by disruption of the spindle apparatus. A high concentration of chloral hydrate is required to cause observable effects. Although these data suggest that genotoxicity may play a role in the toxicity of chloral hydrate, the data indicate that these effects require concentrations that are unlikely to occur under

physiological conditions at the exposures typically encountered from the environment. Collectively, these data provide suggestive evidence of carcinogenicity, but the weight of evidence is not sufficient to conduct a risk assessment assuming a linear response at low exposure."

5 SUMMARY AND CONCLUSIONS

5.1 Summary of Evidence

Studies of chloral hydrate have been conducted in male and female $B6C3F_1$ mice and in rats, with mixed findings regarding carcinogenic effects, which include statistically significant increases in the incidences of liver tumors in several studies of male mice. In addition, extensive data on the genetic toxicity of chloral hydrate indicate that chloral hydrate causes both chromosomal and mutational changes *in vivo* and *in vitro*, including limited evidence of effects in human cells.

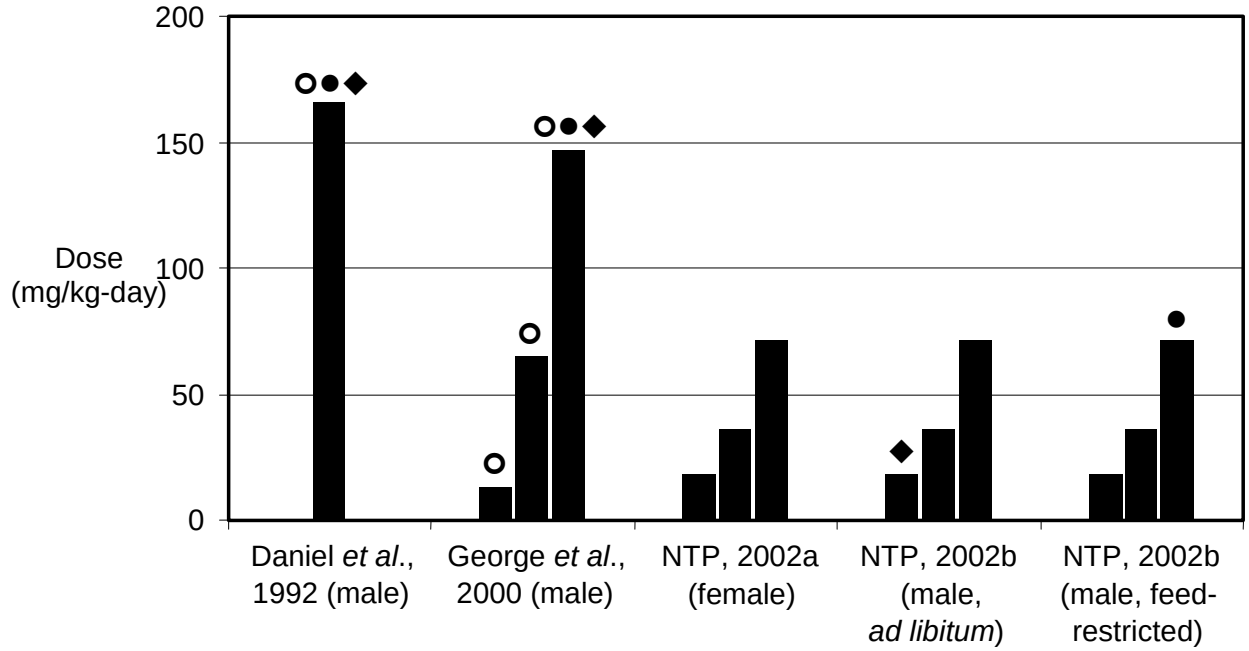
Chloral hydrate induced liver tumors in male $B6C3F_1$ mice in one of two single-dose studies, two of two drinking water studies, and in one of a pair of long-term gavage studies. In the study by Rijhsinghani *et al.* (1986), a single oral dose (5 or 10 mg/kg) was administered to small numbers of 15-day-old male $B6C3F_1$ mice and a statistically significant positive result was observed despite the low power of the study. A more recent study in which 15-day-old male $B6C3F_1$ mice were administered chloral hydrate as a single dose (10, 25, or 50 mg/kg), then evaluated after a lifetime, did not show evidence of carcinogenicity (NTP, 2002a). A second positive study was of a more standard design involving administration of chloral hydrate in drinking water at a single dose level (166 mg/kg-day) (Daniel *et al.*, 1992). The third positive study was a long-term multi-dose drinking water study (13.5, 65, or 146.6 mg/kg-day) (George *et al.*, 2000). In a set of long-term gavage studies in male $B6C3F_1$ mice employing lower average daily doses (17.9, 35.7, 71.4 mg/kg-day) in which diet was either *ad libitum* or controlled, evidence of carcinogenicity was less pronounced, although liver carcinomas were increased in the high-dose group of dietary-controlled mice and combined liver adenomas and carcinomas were increased in a low-dose group of *ad libitum* fed mice (NTP, 2002b).

The results in similar studies of female $B6C3F_1$ mice vary across studies. Single exposure studies in female $B6C3F_1$ mice aged 15 or 28 days did not produce evidence of liver carcinogenicity, although the study of mice dosed at 28 days produced significant increases in malignant lymphomas (NTP, 2002a). Long-term gavage studies in female $B6C3F_1$ mice (average daily dose: 17.9, 35.7, 71.4 mg/kg-day) showed an increase in the incidence of adenomas of the pituitary gland pars distalis and a trend for increased malignant lymphomas, but no indication of liver tumors (NTP, 2002a).

It is unclear whether the lower doses employed in the long-term exposure studies of male and female $B6C3F_1$ mice conducted by NTP (e.g., 17.9, 35.7, 71.4 mg/kg-day) contributed to the absence of carcinogenic effects observed in these studies, in contrast to the findings of hepatocarcinogenicity observed in studies employing higher average daily doses that did not exceed the maximum tolerated dose (*i.e.*, Daniel *et al.*, 1992: 166 mg/kg-day; George *et al.*, 2000: 13.5, 65, or 146.6 mg/kg-day). Figure 3 below presents graphically the average daily doses administered in the various long-term chloral hydrate administration studies (i.e., single-dose studies not shown) in mice and indicates the dose groups in which liver tumors were

statistically significantly increased compared to controls. Long-term drinking water studies in male and female rats have not produced evidence of carcinogenicity, although the maximum tolerated dose may not have been achieved in these studies (George *et al.*, 2000).

Figure 3. Average Daily Dose of Chloral Hydrate Administered in Long-Term Studies in Mice. Identification of Dose Groups with Increased Incidence of Liver Tumors.*



* Vertical bars denote different dose groups within a given study. Symbols above bars indicate the endpoints for which statistically significant increases were observed (O=hepatocellular adenomas; ●=hepatocellular carcinomas; ◆=combined hepatocellular adenomas and carcinomas).

5.2 Conclusion

There is evidence indicating carcinogenic potential of chloral hydrate, including positive findings of liver tumors in two long-term drinking water bioassays in male mice and in a lifetime study following a single oral dose in male mice. Other long-term or single exposure studies in male mice did not produce as strong evidence of liver carcinogenicity. Recent long-term studies of male mice by NTP, however, were conducted at lower doses than those associated with carcinogenic effects in other studies. Studies in female mice showed some evidence of induction of benign tumors of the pituitary gland and malignant lymphomas. Studies in male and female rats produced little evidence of carcinogenicity, although in the case of the rat studies adequate dosing may not have been achieved. There are extensive observations of the genetic toxicity of chloral hydrate, and on chemical structural analogies with known carcinogens.

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