EVIDENCE ON THE DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF

# Chloroform

## DRAFT

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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 one of the mechanisms by which "a chemical is known to the state to cause cancer or reproductive toxicity [is] 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" (Health and Safety Code Section 25249.8(b)). The "state's qualified experts" regarding findings of reproductive toxicity are identified as members of the Developmental and Reproductive Toxicant (DART) Identification Committee of the Office of Environmental Health Hazard Assessment's Science Advisory Board (Title 22, California Code of Regulations, Section 12301)). The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency.

Chloroform was selected for review by the DART Identification Committee because of its assignment of a final priority of "High" under the "Procedure for Prioritizing Candidate Chemicals for Consideration Under Proposition 65 by the State's Qualified Experts", adopted by OEHHA in May, 1997. A notice announcing that prioritization and initiating a 60-day data call-in period was published in the California Regulatory Notice Register on October 10, 2003.

A public meeting of the Committee will be held on November 4, 2004 in Sacramento, California. Following discussion and Committee deliberation, the Committee will determine if chloroform has been "clearly shown through scientifically valid testing according to generally accepted principles" to cause reproductive toxicity.

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# A. ABSTRACT

Chloroform (CAS No. 67-66-3) is a colorless, volatile, nonflammable liquid with a molecular formula of $CHCl_3$, and a molecular weight of 119.38. Chloroform is a high production volume chemical, with production exceeding one million pounds annually in the U.S. It is a by-product of chlorine disinfection of water.

Chloroform is readily absorbed via inhalation or oral exposure; dermal absorption requires contact with liquid, rather than vapor. Absorbed chloroform distributes widely through the body. Uptake and storage in adipose tissue can be substantial. Chloroform can cross the placenta, and is expected to appear in human colostrum and mature breast milk.

Chloroform is metabolized by cytochrome P450-dependent pathways, predominantly by oxidative metabolism to trichloromethanol, which spontaneously dehydrochlorinates to form phosgene. Reductive metabolism also results in a highly reactive product: dichloromethyl free radical. Excretion of non-metabolized chloroform is primarily through exhalation.

In long-term animal bioassays, chloroform induced renal and/or hepatic cancer in rats and mice. Long-term occupational exposure of humans to chloroform has been reported to result in neurological effects. Animal studies of long-term exposure to chloroform have reported evidence of cytotoxicity in liver, kidney, and nasal epithelium.

Epidemiologic studies have reported increased risks of adverse pregnancy outcomes associated with exposure to chloroform via tap water, as well as with total and other specific trihalomethanes (THMs). These adverse outcomes include stillbirths, intrauterine growth retardation, and reduced birth weight. A monotonic exposure-response effect has been observed for the risk of "small for gestational age" with exposure to chloroform. Possible exposure misclassification remains a major limitation in these studies. However, more recent studies, which have improved exposure estimates by including individual measurements, are also suggestive of developmental effects

Available animal data on the potential developmental toxicity of chloroform include inhalation and oral developmental toxicity studies in the rat, an inhalation study in the mouse, and an oral study in the rabbit. Findings for rats exposed to chloroform by inhalation during gestation include effects on pregnancy rate, resorption frequency, fetal weight and crown-rump length. These studies also provide some evidence for increases in the frequency of skeletal anomalies and variations. Similar results were found for rats exposed to chloroform by the oral route, as well as in mice exposed by inhalation. Rabbits exposed by the oral route showed effects on fetal viability. All of these studies also revealed some degree of maternal toxicity, from minimal decreases in food consumption to excess maternal mortality.

One study was identified that evaluated the potential of chloroform to cause developmental neurotoxicity. With the exception of lower scores for forelimb placement, no consistent, chloroform-related effects were demonstrated in tests of developmental stage, motor performance, or passive avoidance learning.

Human data on the potential male reproductive toxicity of chloroform are available from a detailed case study in which significantly reduced sperm motility was associated with increased chloroform exposure, and a limited prospective study of exposure to total THMs in tap water that reported an association with increased sperm defects.

Data from animal studies on the potential male reproductive toxicity of chloroform are available from a continuous breeding study conducted in mice, two reports of a sperm morphology study conducted in mice, a 90 day toxicity study conducted in rats, and a chronic study conducted in beagle dogs. The continuous breeding study, performed by the oral route, did not detect treatment-related changes in fertility or sperm parameters. On the other hand, abnormal sperm morphology was reported in mice exposed to chloroform by inhalation. No clearly treatment-related effects were reported on the male reproductive organs of rats or beagle dogs exposed to chloroform by the oral route.

Human data on the potential female reproductive toxicity of chloroform include a prospective study of exposure to THMs and menstrual cycle length, which reported statistically significant decreases in cycle length and follicular phase length for total trihalmethanes. One occupational study examining the effect of chloroform exposure on fertility in women found no association with time to pregnancy. Other studies of adverse pregnancy outcomes that are discussed under the developmental toxicity section include outcomes associated with exposure to chloroform in tap water that may also be indicative of female reproductive toxicity, such as spontaneous abortions and stillbirths.

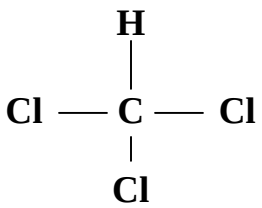
Animal data on the potential female reproductive toxicity of chloroform comes from an oral continuous breeding study conducted in mice, a 90 day toxicity study conducted by the drinking water route in rats, a chronic study conducted by the oral route in beagles, and a number of developmental toxicity studies conducted by the inhalation or oral route of exposure in rats, mice, or rabbits. The continuous breeding study reported no effects on fertility or ovarian weight. Vaginal cytology was not investigated in this study, nor in any of the other available studies. A number of developmental toxicity studies included findings related to possible female reproductive toxicity for chloroform-treated animals: decreased pregnancy rate, decreased litter size, and/or increased resorptions/whole-litter abortions. All of these effects were observed at doses or concentrations that were also associated with some degree of systemic toxicity to maternal animals.

# B. INTRODUCTION

## B.1. Chemical structure and main physical characteristics

Chloroform (CAS No. 67-66-3), or trichloromethane, is a colorless, nonflammable liquid (Merck, 1989; U.S. EPA, 2001a). The compound has a molecular formula of $CHCl_3$, and a molecular weight of 119.38. The molecular structure of chloroform is shown in Figure 1 below. Chloroform's solubility in water is 7.95 g/L at 25° C, and it is readily miscible with most organic solvents. It is relatively volatile, with a vapor pressure of 197 mm Hg at 25$^{o}$C (U.S. EPA 2001a).

**Figure 1. Structure of Chloroform**




## B.2. Use and exposure information

Workplace exposures to chloroform may occur during production, and where it is used as a solvent and/or chemical intermediate (IARC, 1999). For occupational exposure, inhalation of chloroform vapor is expected to be a primary route (see review by Davidson et al., 1982).

Chloroform is a by-product of chlorine disinfection of water, and the main exposure of the general population is via contaminated drinking water (IARC, 1999; U.S. EPA, 2001a). In addition to ingestion, dermal contact with contaminated media (such as bathing or swimming in chloroform-containing water) may also occur. Due to chloroform's volatility, exposure via inhalation of vapor is also a source of human exposure, such as when showering, bathing, or swimming in contaminated water.

Chloroform is a high production volume chemical, with production that has exceeded 500 million pounds annually in the U.S. (ATSDR, 1997). According to Toxics Release Inventory (TRI) data for total U.S. on-site and off-site environmental releases for all facilities in all industries, 1,627,824 pounds of chloroform were released to the environment in 2001 (U.S. EPA, 2003). No TRI releases were reported for California in 2001.

U.S. EPA requires that all community water systems maintain adequate residual chlorine to control the growth of microorganisms in the system, regardless of the initial

disinfection method used (U.S. EPA 2002). A consequence of the presence of residual chlorine in the distribution system is the formation of disinfection by-products (DBPs). The residual chlorine, which exists as hypochlorous acid and hypochlorite in water, reacts with naturally occurring organic matter to form a wide range of halogenated organic compounds, as first reported by Rook in the mid 1970's (Rook 1974). These DBPs include trihalomethanes (THMs), haloacetonitriles, haloketones, chloropicrin, and haloacetic acids (Table 1). The most common THMs are chloroform, bromodichloromethane (BDCM), chlorodibromomethane (CDBM), and bromoform; these THMs considered as a group are generally referred to as total THM (TTHM). The U.S. EPA has regulated TTHM at a maximum allowable annual average level of 80 ug/L.

**Table 1. Disinfection By-Products (DBPs) in drinking water**

| |
|---|
| Trihalomethanes |
| Chloroform |
| Bromodichloromethane |
| Dibromochloromethane |
| Bromoform |
| Haloacetonitriles |
| Trichloroacetonitrile |
| Dichloroacetonitrile |
| Bromochloroacetonitrile |
| Dibromoacetonitrile |
| Haloketones |
| 1,1-Dichloropropanone |
| 1,1,1-Trichloropropanone |
| Haloacids |
| Monochloroacetic acid |
| Dichloroacetic acid |
| Trichloroacetic acid |
| Monobromoacetic acid |
| Dibromoacetic acid |
| Aldehydes |
| Formaldehyde |
| Acetaldehyde |
| Miscellaneous |
| Chloropicrin |
| Chloral hydrate |
| Cyanogen chloride |
| 2,4,6-Trichlorophenol |

Chloroform is usually the most prevalent by-product formed when drinking water is chlorinated, although brominated THM can occur at high concentrations when waters with high bromide contents are chlorinated (Krasner 1989; Weisel and Chen 1994).

In drinking water, chloroform concentration has been shown to be highly correlated with concentrations of TTHM (r=0.98) and accounting for up to 90% of the TTHM (Keegan et al., 2001; King et al., 2000). Correlations between TTHM and BDCM and CDBM have been reported to be smaller (r=0.62 and -0.09, respectively (Keegan et al., 2001)). Various factors can affect the formation of chloroform in treated water including: temperature, pH, concentration of chlorine residual, reaction time, transit time within the system, and total or organic carbon. Similarly, temperature is known to increase the rate of THM formation (Smith et al., 1980 as cited in Keegan et al, 2001).

## B.3. Metabolism and pharmacokinetics

Inhalation of chloroform vapor in ambient air is a primary mode of exposure, particularly for occupational exposures (see review by Davidson et al., 1982). At anesthetic concentrations in humans at resting ventilation rate and cardiac output, total body equilibrium is reached in approximately two hours. According to the review by Davidson et al. (1982), retention value at equilibrium has been reported in different studies as 64% or 67%, the remainder representing elimination of chloroform by other routes.

Gastrointestinal absorption of chloroform in humans and animals appears to be both rapid and extensive (U.S. EPA, 2001a). Peak blood levels were found at about one hour post-dosing in animals. In humans, more than 90% of an oral dose was recovered from expired air within eight hours.

Absorption through the skin requires submersion or contact with chloroform in liquid form, rather than vapor (Davidson et al., 1982). Dermal absorption of chloroform has been studied in humans bathing in chlorinated water while breathing pure air through a facemask (Gordon et al., 1998). The facemask prevented inhalation of contaminated air, while allowing continuous monitoring of expired air. Subjects bathing in 40ºC water reached a near steady-state value after six to nine minutes; subjects at 40ºC exhaled about 30 times more chloroform than the same subjects bathing in 30ºC water.

Absorbed chloroform distributes widely through the body, with human and animal studies identifying concentrations in fat, kidney, liver, brain, and blood (U.S. EPA, 2001a). Uptake and storage of chloroform in adipose tissue can be substantial, with daily exposures potentially leading to accumulation, particularly in obese persons. There is evidence that chloroform crosses the placenta and can be expected to appear in human colostrum and mature breast milk (Davidson et al., 1982).

Distribution may be influenced by route of exposure, and physiologically-based pharmacokinetic models have been developed for the uptake and distribution of chloroform in the body. The models predict that inhalation and dermal exposure would result in higher levels of chloroform circulating throughout the body and to the bladder, while ingestion would result in a higher dose of chloroform to the liver (Blancato and Chiu, 1993).

Chloroform is metabolized by cytochrome P450-dependent pathways (U.S. EPA, 2001a). The primary enzyme involved in the metabolism of low concentrations of chloroform is cytochrome P-4502E1 (CYP2E1) (Meek et al., 2002). Both oxidative and reductive reactions take place, but oxidative metabolism is believed to strongly predominate *in vivo*. Oxidative metabolism of chloroform produces trichlormethanol, which rapidly and spontaneously dehydrochlorinates to form phosgene ($CCl_2O$). Reductive metabolism of chloroform produces $CHCl_2$, dichloromethyl free radical. The products of either oxidative or reductive chloroform metabolism are themselves highly reactive.

While most tissues are capable of metabolizing chloroform, the rate of metabolism is greatest in liver, kidney cortex, and nasal mucosa (U.S. EPA, 2001a). These tissues are also particular targets of chloroform toxicity, suggesting metabolism may have a role to play in the mechanism by which chloroform exerts its toxicity. Chloroform metabolism displays saturation kinetics; while nearly all of a low dose is metabolized, metabolic capacity may become saturated at higher doses.

Excretion of non-metabolized chloroform is primarily through exhalation (Davidson et al., 1982; U.S. EPA, 2001a). In a study reviewed by Davidson et al. (1982), eight male and female human volunteers were given $^{13}C$-chloroform dissolved in olive oil and packed in gelatin capsules. Over a period of eight hours, pulmonary excretion of unchanged $^{13}C$-chloroform ranged from 17.8-66.6% of a 0.5 g dose. As compared to overweight subjects, lean subjects tended to eliminate a greater percentage of the dose via the lungs, suggesting that adipose tissue may act as a storage site for chloroform. Data from individual subjects given 0.1 g, 0.25 g, or 1.0 g $^{13}C$-chloroform indicated that the greater the dose of chloroform, the smaller the proportion metabolized.

## B.4. Non-DART toxicities

Long-term workplace exposures to chloroform at concentrations of 100-1,000 mg/m$^3$ (20-200 ppm) have been reported to result in neurological effects, with increased incidence of symptoms such as fatigue, nausea, vomiting, lassitude, dry mouth, and anorexia, as well as effects on the liver (U.S. EPA, 2001a). Exact exposures to chloroform, however, are difficult to measure and may be confounded by simultaneous exposure to other chemicals.

The chronic toxicity and carcinogenic potential of chloroform have been investigated in experimental animals by the oral and inhalation routes of exposure (see U.S. EPA, 2001a for review). Characteristic effects of chloroform exposure include evidence of cytotoxicity in liver, kidney, and nasal epithelium. U.S. EPA's Integrated Risk Information System has established the chronic oral Reference Dose (RfD) for non-cancer effects of chloroform at 0.01 mg/kg/day (U.S. EPA, 2001b). The critical study used in developing this RfD was a chronic oral bioassay conducted in beagle dogs, which reported findings of increased fatty cyst formation in the liver and elevated serum glutamate-pyruvate transaminase (SGPT).

The International Agency for Research on Cancer (IARC) reported that no data were available on the genetic and related effects of chloroform in humans (IARC, 1999). The IARC report notes weak evidence for the genotoxicity of chloroform in experimental systems *in vivo* and in mammalian cells, fungi and yeast *in vitro*. Chloroform has not been found to be mutagenic to bacteria.

Some epidemiological studies have reported associations with small increases in relative risks between exposure to chlorinated drinking water and cancer--primarily cancers of the bladder, rectum, and possibly colon (IARC, 1999; U.S. EPA, 2001a). However, a causal inference cannot be made as observed effects cannot be certainly attributed to chloroform itself, as opposed to other disinfection byproducts (IARC, 1999; U.S. EPA, 2001a). Uncertainty is compounded by a lack of concordance in results for men and women, as well as use of surrogate indicators for chloroform exposure (IARC, 1999).

Chloroform has been tested for carcinogenicity in mice, rats, and dogs, by both the oral and inhalation routes (IARC, 1999). Renal and/or hepatic tumors have been observed in these studies, with the exception of one study performed in dogs, in which no increased frequency of tumors was observed.

IARC (1999) has assigned chloroform to category 2B: *possibly carcinogenic to humans*. Evidence from humans for the carcinogenicity of chloroform in humans was considered to be *inadequate*, while the animal evidence was considered to be *sufficient*. U.S. EPA (2001b) considers chloroform "*likely to be carcinogenic to humans by all routes of exposure* under high-exposure conditions that lead to cytotoxicity and regenerative hyperplasia in susceptible tissues...[but] *not likely to be carcinogenic to humans by any route* of exposure under conditions that do not cause cytotoxicity and cell regeneration." Chloroform is listed as a chemical "known to the state to cause cancer" under California's Proposition 65.

# C. DEVELOPMENTAL TOXICITY

## C.1. Human studies

Eight epidemiologic studies were identified which measured exposure to chloroform and developmental outcomes; an occupational study, three case-control studies, three retrospective cohort studies and one prospective cohort study. In the discussion below if these studies, all reported risk estimates are adjusted for relevant potential covariates or confounders as reported by the authors.

### C.1.1. Occupational exposure to chloroform

*Wennborg et al. (2000). Pregnancy outcome of personnel in Swedish biomedical research laboratories.*

Only one study was located which examined exposure to chloroform, not in drinking water, in association with pregnancy outcomes. This occupational study was conducted in a cohort of Swedish women identified from records of the Swedish Employee Salaries and Pension Board (Wennborg et al., 2000). The cohort included women born in 1945 or later who had worked in a laboratory or non-laboratory department for 1 year or more during 1990 –1994. Questionnaires sent to the women contained sections on reproductive history, health status, time to pregnancy, personal habits, specific work and exposure to various agents, as well as the specific periods during which the exposure occurred. Each woman was asked about her work in any laboratory, the period during which it occurred, and her exposure to various agents during the months before conception. For solvents specifically, the periods of use were requested with an accuracy margin of one month. Of the 1052 women sent questionnaires 763 responded (a response rate of 73%). After excluding 66 women according to various criteria, the final sample included 697 women. The records of these women were linked to the Swedish Medical Register, which contains information on all births after 1973; a total of 1417 singletons births were included in the cohort.

Birth outcomes examined in this study included spontaneous abortion (SAB), birth weight, preterm delivery, small for gestation age (SGA), large for gestational age, and congenital malformations. Possible confounding variables considered in the analyses included: high blood pressure, other chronic diseases, gynecological diseases (except endometriosis and sexually transmitted diseases), and sexually transmitted infectious diseases (human papilloma virus, chlamydia, genital herpes, and gonorrhea), smoking, father's work in a laboratory during the time of conception, the presence of small children in the home, previous spontaneous abortions, and the consecutive pregnancy number. The following variables were not included in the adjusted analyses due to missing values resulting from a lack of response: consumption of alcohol, coffee, tea, and cola drinks; physical activity; and stress in work. The total number of pregnancies included in the analysis was 869 (excluded were twin and in-vitro fertilization pregnancies). Various statistical methods were used to take into account the possible non-independence of pregnancies as more than one pregnancy per woman was included in the study.

No effect was reported between laboratory work in general and reported SABs. A weak association was shown between women working with chloroform during the time before conception and SABs (OR = 2.3; 95% CI, 0.9-5.9, adjusted for mother's age and previous SAB; unexposed N = 770, exposed N = 86). The OR for previous SABs was 2.2 (95% CI, 1.2-4.1) based on only two SABs among women who had worked with chloroform and had had previous SABs. No significant associations with chloroform exposure were observed for SGA or BW.

Limitations of this study include the lack of actual exposure levels, and the possible exposure to other laboratory solvents. However, the questions about laboratory agents concerned those that the women had handled personally. The response rate was low (73%), however, the responders and non-responders were similar with respect to women's health diseases, gynecological history and socioeconomic factors. Although the spontaneous abortions were self-reported, the authors noted that the reference group, female non-laboratory university personnel, had the same socioeconomic background as the laboratory personnel. This likely reduced the risk of selection bias, which would be the result of one group reporting the occurrence of spontaneous abortions differently.

### C.1.2. Exposure to chloroform as a water disinfection byproduct

Many studies to date have examined the association between THMs in drinking water and pregnancy outcomes. Of these studies seven have measured chloroform specifically (Dodds and King. 2001; Dodds et al., 2004; King et al., 2000; Kramer et al., 1992; Infante-Rivard, 2004; Waller et al., 1998; Wright et al., 2004).

*Kramer et al. (1992). The association of waterborne chloroform with intrauterine growth retardation.*

In a population-based case-control study conducted in Iowa among residents of small towns (Kramer et al., 1992), municipal water utility company records were linked to birth certificate data. Exposures to chloroform and other THMs were examined for association with low birth weight (<2500 g), prematurity (<37 weeks), and intrauterine growth retardation (IUGR) (infant weight for gestational age <5$^{th}$ percentile). The number of subjects for each pregnancy outcome included: LBW, cases = 159, controls = 795; prematurity, cases = 342, controls = 1,710; IUGR, cases = 187, controls = 935. Chloroform levels ranged from zero to 350 μg/L and were positively skewed (mean = 12.5, median = 1, standard deviation = 38.7). Exposure levels to chloroform and bromodichloromethane (BDCM) were categorized as undetectable (< 1 μg/L), low (1-9 μg/L) and high (≥ 10 μg/L). Analyses were adjusted for maternal age, parity, adequacy of prenatal care, marital status, education and maternal smoking. Reference groups for both chloroform and BDCM consisted of exposures at non-detectable levels (< 1 μg/L).

The study found exposure to chloroform at a concentration ≥10 μg/L was associated with an increased risk of IUGR (OR = 1.8, 95% CI, 1.1 – 2.9).Exposure to chloroform at a concentration of 1-9 μg/L was also associated with an increased risk of IUGR (OR = 1.3, 95% CI, 0.9 – 1.8). The OR for exposure to BDCM at a concentration of ≥10 μg/L in association with IUGR was 1.7 (95% CI, 0.9 – 2.9). No associations were observed for any other THMs or other birth outcomes studied.

A limitation of the study was the timing of the assessment of exposure. The THM data were based on a survey conducted in 1987, two years before the period of data collection for the outcome measures (1989-1990). This was done to allow for adjustment for maternal smoking since the smoking question did not appear on earlier birth certificates.

However, the survey was conducted during a drought year in Iowa and it is likely that the water quality differed from non-drought years, though possibly not uniformly across the state. As the authors point out, however, it is likely that levels of organic precursors to THMs (humic and fulvic acids) would be lower during a drought year. Thus, the absolute concentration of THMs found in the non-drought years (1989-1990) might have been higher than the levels found in the 1987 testing. The survey also did not allow for assessment of fluctuation of THM over time. In addition, no information was available on residential mobility of the mother during pregnancy or other potential maternal confounders.

*Waller et al. (1998). Trihalomethanes in drinking water and spontaneous abortion.*

A prospective cohort study (Waller et al., 1998) was conducted in Northern California from 1989-1991 to examine the association between exposure to THMs and SAB. A total of 5,144 pregnant women participating in a prepaid health plan were enrolled in the study. Individual and total THMs measurements routinely collected by 78 water utilities were used for assessing exposure. The subject's address was used to determine her residential drinking water utility. For 77% of the cohort all distribution measurements of individual and total THMs taken by the utility within each subject's first trimester were averaged. For 4% of the subjects with measurements not available within the first trimester, measurements were averaged within 30 day of the first trimester. Annual measurements were used for 9% of the cohort. Tap water consumption at home and subject information, including information on frequency and duration of showering, were obtained by telephone interview. Pregnancy outcomes were ascertained from hospital discharge or medical records (91%), follow-up interviews or matching to the California Birth Registry (8%). Subjects completed a computer-assisted telephone interview that included information on demographics, pregnancy history, employment status, consumption of tap and bottled water, alcohol, tobacco, and caffeine.

Women with a high intake of TTHMs ($\geq$5 glasses of water a day and $\geq$75 μg/L TTHMs) showed a higher risk of spontaneous abortion compared to women with a low intake of TTHMs (<5 glasses of water a day and <75 μg/L TTHMs) (OR = 1.8, 95% CI, 1.1 to 3.0). The upper quartile of chloroform was reported to be $\geq$17 μg/L. The odds ratio of SAB in association with high exposure to chloroform ($\geq$17 μg/L and drinking $\geq$5 glasses of water per day) was 0.6 (95% CI, 0.3-1.2). The odds ratio for high exposure to BDCM ($\geq$18 μg/L and drinking $\geq$5 glasses of water per day) was 2.0 (95% CI, 1.2 – 3.5) and 3.0 (95% CI, 1.4-6.6) after adjustment for other THMs. Although the study did not calculate a combined TTHM index of exposure, in a response to a letter to the editor Waller and Swan (1999) recalculated the exposure measure, which included information on ingestion, showering, and swimming. The unadjusted odds ratio for the combined TTHM exposure index was 1.1 (95% CI, 0.7 – 1.7). The authors proposed that the attenuation in the odds ratio might be a result of multiple sources of potential misclassification.

The strengths of this study include the prospective design of the study and, therefore, the lack of possible recall and selection bias. In addition, the study was able to control for

important covariates such as maternal smoking, history of pregnancy loss, maternal age, race, employment during pregnancy, etc. Although the authors incorporated better exposure assessment methods, such as information on showering, the potential for exposure misclassification remains the primary limitation for this and other THM studies. No information was collected on bathing, washing dishes, etc. Relying on one day's testing and averaging levels across several sampling sites does not take into consideration fluctuations and variations across location and days. However, since much of the possible misclassification would be non-differential, it would result in a bias towards the null; i.e., towards not detecting an existing effect.

*King et al. (2000). Relation between stillbirth and specific chlorination by-products in public water supplies.*

King et al. (2000) conducted a retrospective cohort study to determine the association between exposure to specific DBPs and the risk of stillbirths. Exposure to THMs was determined from the Nova Scotia Department of Environment's results of routine monitoring. On average four samples were measured per year. Although all four THMs were monitored, bromoform and CDBM occurred in very low concentrations and thus were not evaluated in the analysis. To provide estimates for chloroform and BDCM for each water facility for each month, least-square regression was performed for year, month and facility and predicted values were obtained. A perinatal database was used to identify all live and still births weighing greater than or equal to 500 g occurring between 1988 and 1995 (n=49,842). Data were collected prenatally, during labor, delivery and postpartum, including demographic and risk factor information. The data set was restricted to those served by a surface water source, and to municipalities where greater than 90% of the households were served by the public water facility. Potential confounders identified by backward regression (factors were eliminated at greater than 0.15 significance level) included maternal age, parity, smoking during pregnancy, infant's sex, and neighborhood family income. The latter factor was calculated based on the 1991 census data. Analyses were adjusted for smoking and maternal age.

Relative risks (95% CI) at the highest exposure categories for stillbirth from TTHM (≥ 100 μg/L), chloroform (≥ 100 μg/L) and BDCM (≥ 20 μg/L) were 1.66 (1.09-2.54), 1.56 (1.04-2.34), 1.98 (1.23-3.49), respectively. Simultaneous modeling of chloroform and BDCM suggested that the latter is the stronger independent predictor of risk. The data were analyzed by the stillbirth cause-of-death classifications of unexplained and asphyxia-related deaths. The risk estimates were higher for asphyxia-related deaths and increased with increasing levels of exposure to chloroform (compared with <50 μg/L, 50-74 μg/L - RR = 1.36 (95% CI, 0.70-2.63); 75-99 μg/L - RR =1.82 (95% CI, 0.83-3.97); ≥100 μg/L – RR = 3.15 (95% CI, 1.64-6.03). For each 10 μg/L the increase in RR was estimated to be 1.12 (95% CI, 1.05-1.20).

Limitations of this study include the lack of individual data on chloroform exposure. Mother's residence at time of delivery was assumed to be her residence during the entire pregnancy. However, data from Dodds et al. (1999) indicates that mobility rates are only approximately 10% for women in the childbearing age group (15-11 years of age) in

Nova Scotia and some of those women would have moved within the same area served by their water treatment facility.

*Dodds and King (2001). Relation between trihalomethane compounds and birth defects.*

In a retrospective cohort study Dodds and King (2001), examined the association between effect of exposure to two THMs, chloroform and BDCM, and birth defects in Nova Scotia between 1988 and 1995. This study employed the same database and exposure monitoring as in King et al. (2000), as discussed above. Exposure was determined from the Nova Scotia Department of Enviroment's results of routine monitoring. Although all four THMs were monitored bromoform and CDBM occurred in very low concentrations and thus were not evaluated in the analysis. Average chloroform and BDCM levels appropriate to the sensitive period of each specific outcome were used in the analysis. A perinatal database was used to study birth defects including neural tube defects, major cardiac defects, cleft defects, and chromosomal abnormalities (n=49,842). Information was abstracted from medical records. Information was also obtained on pregnancy terminations for a prenatally diagnosed congenital anomaly. Maternal age, parity, maternal smoking and neighborhood family income were assessed as potential confounders and were retained in the model if the coefficient for exposure to either THM changed by 5% in the presence of the confounder.

Exposure to BDCM levels $\geq$20 μg/L was associated with an increased risk of neural tube defects (RR = 2.5 (95% CI, 1.2-5.1)). An increase risk of chromosomal abnormalities was observed with exposure to chloroform at levels 75-99 μg/L (RR = 1.9, 95% CI, 1.1-3.3) and at levels $\geq$ 100 μg/L (RR = 1.4, 95% CI, 0.8-2.8). A decreased risk of cardiac abnormalities was observed for exposure to BDCM at the highest levels (RR = 0.7, 95% CI 0.2-0.7). For cleft defects the RR for exposure to chloroform at $\geq$ 100 μg/L was 1.5 (95% CI, 0.8-2.8). No information was obtained on the subjects' exposure to THMs from showering, bathing or consumption pattern.

*Dodds et al. (2004). Trihalomethanes in public water supplies and risk of stillbirth.*

A case-control study was conducted in Nova Scotia and Eastern Ontario, Canada (Dodds et al., 2004), in which stillbirths occurring between July 1999 and December 2001 were identified through population-based perinatal databases. Controls were women who delivered a liveborn infant during the same three month period as the cases and were randomly selected from the perinatal database from the same study area. Cases were contacted 6 months after delivery. Subjects completed a telephone interview that focused on water use behaviors at approximately 3-4 month's gestation as well as on other risk factors for stillbirth. An interview was completed for 60% of cases (n=112) and 68% of controls (n=398). Information collected on exposures to THMs included consumption of beverages made with tap water, description of any water filters used, consumption of bottled water, and length of time spent showering and bathing. Residential tap water samples were collected from all the subjects who lived in an area served by a public water supply. The samples were collected approximately one year later to approximate the exposure level during the same season as the critical period of exposure

(approximately 15 weeks of gestation). Subjects with a private well were assumed to have TTHM levels equal to zero. Measures of exposure were developed that included estimates of daily exposure to water through ingestion, inhalation and absorption. Adjustments were made for subjects who used filters (50% reduction in THM intake from cold tap water-based drinks), and consumed boiled water (70% reduction in THM intake). To estimate total daily THM exposure, an exposure metric was created to incorporate ingestion, showering and bathing. Information was collected to control for potentially confounding factors including pregnancy history, smoking habits, occupation, income, education, vitamin use and exposure to pesticides during pregnancy.

The odds ratio for stillbirth from chloroform was increased at the 1-49 μg/L level (OR = 1.8, 95% CI, 1.1-3.0), and at the >80 μg/L level (OR = 2.2, 95% CI, 1.0-4.8) compared with zero exposure (Table 2). Similar results were seen for TTHM and BDCM. There was no evidence of a monotonic increase. Increased risks were also seen for certain quintiles of total exposure to chloroform, which was determined using information on exposure from ingestion, showering and bathing (Table 3). Among the subjects with some THM exposure, quintiles were formed based on the distribution in the control population. Consumption of five tap water drinks per day was associated with increased risk when total THM levels were high (OR = 4.0, 95% CI, 1.4-1.1). Consuming less than five tap water drinks per day was not associated with increased risk regardless of THM level (OR = 1.1, 95% CI, 0.4-2.8 at THM levels of 50+ μg/L). Evidence was also seen of an independent effect of showering or bathing when the THM level was elevated (Table 4), with exposure at the highest residential TTHM level (50 /L) and greater than 15 minutes of showering/bathing being associated with an odds ratio of 2.6 (95% CI, 1.1-5.8).

A strength of this study was that actual residential tap water levels of chloroform and THMs were collected for all subjects with a public water supply. Information collected on exposure from showering and bathing was incorporated into an exposure metric. The limitations of this study include the possibility of recall bias. However, the subjects were not aware of the hypothesis of the study and measurement of chloroform was not subject to recall bias. The response rates of subjects were low but the authors offer that this might have been a result of having to recruit subjects through a letter from their physician rather than through more active recruitment.

**Table 2. Odds Ratios for Chloroform Level as Measured in Residential Tap Water Samples and Risk of Stillbirth**

| Residential Chloroform Level (µg/L) | Cases (n = 112) No. | (%) | Controls (n = 398) No. | (%) | Crude OR (95% CI) | Adjusted OR* (95% CI) |
|---|---|---|---|---|---|---|
| 0† | 34 | (30) | 152 | (38) | 1.0 | 1.0 |
| 1-49 | 51 | (46) | 147 | (37) | 1.6 (1.0-2.5) | 1.8 (1.1-3.0) |
| 50-79 | 14 | (13) | 73 | (18) | 0.9 (0.4-1.7) | 0.9 (0.5-1.9) |
| >80 | 13 | (12) | 26 | (7) | 2.2 (1.0-4.8) | 2.2 (1.0-4.8) |

*Adjusted for age, province of residence, and household income.
†Reference category.
Adapted from Dodds et al., 2004

**Table 3. Odds Ratios for Total Chloroform Exposure from Chlorination and Risk of Stillbirth**

| Total Exposure to chloroform† | Cases (n = 112) No. | (%) | Controls (n = 398) No. | (%) | Crude OR (95% CI) | Adjusted OR* (95% CI) |
|---|---|---|---|---|---|---|
| No Exposure‡ | 22 | (20) | 103 | (26) | 1.0 | 1.0 |
| Quintile 1 (lowest) | 21 | (19) | 59 | (15) | 1.7 (0.9-3.3) | 1.8 (0.9-3.7) |
| Quintile 2 | 13 | (12) | 59 | (15) | 1.0 (0.5-2.2) | 1.3 (0.6-3.0) |
| Quintile 3 | 21 | (19) | 58 | (15) | 1.7 (0.9-3.3) | 2.3 (1.1-4.7) |
| Quintile 4 | 13 | (12) | 60 | (15) | 1.0 (0.5-2.2) | 1.3 (0.6-2.8) |
| Quintile 5 | 22 | (20) | 59 | (15) | 1.8 (0.9-3.4) | 2.0 (1.0-4.0) |

*Adjusted for age, province of residence, and household income.
†Quintiles based on control distribution.
‡Reference category.
Adapted from Dodds et al., 2004

**Table 4. Odds Ratios for the Joint Effects Between Residential Total THM Level and Showering/Bathing***

| | No. of Minutes Showering/Bathing per Day† | | |
|---|---|---|---|
| | <10 | 10-15 | >15 |
| Residential Total THM Level (μg/L) | OR (95% CI) | OR (95% CI) | OR (95% CI) |
| 0 | 1.0 | 1.2 (0.4-3.4) | 0.7 (0.3-2.0) |
| 1-49 | 1.0 (0.5-2.1) | 1.8 (0.7-4.7) | 2.6 (1.1-5.9) |
| 50 | 1.2 (0.6-2.3) | 0.8 (0.2-3.9) | 2.6 (1.1-5.8) |

*Adjusted for age, province of residence, and household income.
†Bathing minutes divided by 3 for equivalency to showering minutes.
Adapted from Dodds et al., 2004

*Infante-Rivard (2004). Drinking water contaminants, gene polymorphism and fetal growth.*

A case-control study by Infante-Rivard (2004) examined the association between exposure to TTHM, as well as to individual THMs, including chloroform, and fetal growth. The study also tested for gene-environment interactions to determine whether effects of TTHM and chloroform exposure were modified by newborn and genetic variants (one or two variant alleles versus none). The sample included newborn cases born at a university-based mother-child center in Montréal between May 1998 and June 2000 whose birthweight was below the 10$^{th}$ percentile for gestational age and sex. Controls were selected from infants born at the same hospital whose birthweight was at or above the 10$^{th}$ percentile. Exposure to TTHMs and specific THMs were measured using data collected by municipalities (189 distribution systems) and the Ministry of Environment according to place of residence. Trihalomethane information was available for 91.6% of the study women.

The control infants (N= 426, 98.3% of those eligible) were matched to cases for gestational week, sex, and race, and usually born within a week of the matched case infant (N= 458, 97.6% of those eligible). Face to face interviews with all mothers were conducted generally within two days of delivery. The interview asked for information on residential history, source of drinking water (community, private well, bottled), use and type of domestic water filter, average number of glasses of water per day, at home or elsewhere, including those with reconstituted frozen fruit juices, usual way of consuming tap water (directly from tap, after refrigeration) and average number and usual duration of showers per week. In addition, women were asked about known risk factors for IUGR including weight gain during pregnancy, pre-pregnancy body mass index, parity, history of preeclampsia, prior history of IUGR, primiparity, and smoking during pregnancy. Exposure levels to total and individual THMs were estimated using various index measures including average exposure, cumulative exposure, as well as ones incorporating

exposure from showering and adjusting for use of filter or refrigeration before consumption.

Mothers and newborns were characterized for two genetic polymorphisms, one in the cytochrome P-4502E1 (CYP 2E1) gene (G1259C), and one in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene (C677T). The primary enzyme involved in the metabolism of low doses of chloroform is CYP 2E1. The gene variant examined in this study has been associated with increased transcriptional activity (Meek, 2002). Thus, individuals with this polymorphism would be expected to show enhanced metabolism of THMs including chloroform, that would result in more activated metabolites. The enzyme MTHFR is involved in the metabolism of methionine and homocysteine through a mechanism that is vitamin B12 dependent. It has been suggested that vitamin B12 could be inhibited by chloroform (Alston, 1991).

The only exposure variable which differed significantly between cases and controls was the use of domestic water filters (14.7% for cases vs. 9.9% for controls). However, it appeared that the consumption of bottled water might also be different (21.9% for cases vs. 26.4% for controls). It was not clear whether the analysis controlled for these differences. The average level at the tap for chloroform was 11.84 μg/L (SD = 18.19) for cases and 18.58 μg/L (SD = 16.31) for controls. The authors reported that results for cumulative exposure to specific and TTHM in drinking water were similar to those seen using average level ([sum of concentration × duration in days at level based on residence]/total number of pregnancy days). Exposure to total and specific THMs was not associated with an increased risk of IUGR (adjusted OR at the 90$^{th}$ percentile for TTHMs and chloroform = 0.97 (95% CI, 0.57 – 1.62) and 1.06 (95% CI, 0.63 –1.79), respectively). The 90$^{th}$ percentile for chloroform exposure was 23.7 (μg/L). Using a cutoff at the 95$^{th}$ percentile for average level of exposure the odds ratio for exposure to chloroform was 1.17 (95% CI, 0.60-2.29)). However, significant effect modification was observed between newborns with and without the CYP2E1 variant. Among newborns with the variant, the adjusted OR for exposure to average TTHMs above the 90$^{th}$ percentile (29.4 μg/L) was 13.20 (95% CI, 1.19 – 146.72) and for exposure to chloroform above the 90$^{th}$ percentile (23.7 μg/L) was 5.62 (95% CI, 0.82 –39.39). No indication was observed that the MTHFR C677T polymorphism modified the effect of exposure to chloroform or TTHMs. The author concluded that the findings suggest exposure to THMs at the highest levels can affect fetal growth, but only in genetically susceptible newborns.

This study improved on previous studies in that it incorporated information on exposure to THMs from showering as well as from the number of glasses of tap water consumed per day, whether a filter was used for tap water and if the water was refrigerated before consumption. However, it was reported that controlling for these additional exposure variables did not alter the findings. An additional strength of the study is that important information on potential confounding factors was obtained from interviews. It was not discernable from the study how many infants were in each cell for the analysis for gene-environment analysis. Since there were only 45 cases and 37 controls of newborns with one or two variant alleles and only a few of those would presumably have been included

in the 90th percentile of exposure, the power of the study may have been limited in its ability to detect other significant effects, if they were present. As with other studies of THMs, more accurate exposure assessment would be achieved by sampling more specifically within the distribution system.

*Wright et al. (2004). The effect of disinfection by-products and mutagenic activity on birth weight and gestational duration.*

In a retrospective cohort study, Wright et al. (2004) linked birth certificate data from 1995 – 1998 (N=282,645) with measurements of drinking water DBPs and mutagenicity for all towns in Massachusetts with populations greater than 10,000. The authors studied the effect of maternal third trimester exposure to various DBPs on mean birth weight, mean gestational age, SGA, and preterm delivery.

Exposure levels for THMs and HAAs were determined from data from the Massachusetts Department of Environmental Protection records. Maternal ZIP code and infant month of birth were used to assign third trimester town specific DBP levels. Quarterly town averages were calculated from all available samples for the various sampling locations. Preterm delivery was defined as less than 37 weeks, with very preterm being less than 34 weeks. Birth weight and SGA analyses were restricted to term births from 37-45 gestational weeks. The comparison group was composed of infants at least 37 weeks and weighing at least 2,500 g.

Mean and median chloroform were 38.2 μg/L (SD 23.6) and 33 μg/L, respectively (Table 5). Mean BW was 3,463 g. The percentage of births that were SGA and preterm were 8.9% and 5.9%, respectively. The logistic and linear regression models were adjusted for the following maternal risk factors: diabetes; lung disease; renal disease; chronic hypertension; marital status; previous preterm delivery; previous birth to an infant weighing >4,000 g; maternal education, age, and race; parity; prenatal care; maternal weight gain during pregnancy; and the number of cigarettes smoked per day. Median household income specific to ZIP code was obtained from 1990 U.S. Census data. Maternal age and race were included in all of the regression models. Weight gain during pregnancy and marital status were not available on birth certificates prior to 1997, therefore adjustment for these covariates were not done in the THM analyses. The study population included 196,000 singleton infants with recorded gestational age between 22 and 45 weeks and birthweight greater than or equal to 200 g.

Reductions in mean birth weight were observed for maternal exposures in the 50th - 90th percentile compared to less than the 50th percentile for chloroform (-14 g, 95% CI, -19 to –9). Reductions were also observed for exposures greater than the 90th percentile compared to less than the 50th percentile (-18, 95% CI, -26 to –10). Similar reductions were observed for TTHM and BDCM (Table 6). These reductions in birthweight were an order of magnitude smaller than those seen for maternal smoking and lack of prenatal care. As noted by the authors, there was no evidence of a monotonic gradient for any of the THM exposures. Reductions in mean birth weight were observed for chloroform concentrations >20 μg/L (Figure 2). Analyses of SGA found significant increases in the

odds ratios for exposure to chloroform for the 50th – 90th percentile and greater than the 90th percentile compared with less than the 50th percentile (OR = 1.05, 95% CI, 1.02 – 1.09, OR = 1.11, 95% CI, 1.08 – 1.22, respectively). Similar increases were observed in the OR for TTHM and BDCM; however, a monotonic increase was only observed for chloroform exposures at greater than >20 μg/L (Figure 2). The analyses for SGA were adjusted for the above-mentioned covariates; however, no adjustment was made for gestational age as this variable may be on the causal pathway and such adjustment may be inappropriate.

Exposure to chloroform, BDCM, and TTHMs was associated with an increase in mean gestational age (Table 6). In addition, exposure to chloroform, BDCM, and TTHMs was associated with a decreased risk of preterm delivery (Table 7). Similar reductions in risk were observed for very preterm delivery as well. The authors expressed caution concerning the interpretation of these results due to the likelihood of errors in the estimates of gestational age derived from birth certificate data.

**Table 5. Maternal third-trimester exposure to THMs for residents of Massachusetts towns with populations >10,000 during 1995-1998 (Wright et al., 2004).**

| | | | Water Concentration (μg/L) | | | | |
|---|---|---|---|---|---|---|---|
| | No. of Towns | No. of Births | 10th %ile | Median | 90th %ile | Maximum | Mean ± SD |
| TTHM | 109 | 196,000 | 8 | 33 | 74 | 163 | 38.2 ± 27.0 |
| Chloroform | 109 | 195,506 | 4 | 25 | 63 | 135 | 31.0 ± 23.6 |
| BDCM | 109 | 195,506 | 1 | 4 | 12 | 46 | 5.7 ± 5.1 |

**Table 6. The effect of third-trimester THMs exposure on mean birth weight among term births and gestational age among all births (Wright et al., 2004).**

| Exposure (µg/L) | Birth weight | | | Gestational age | | |
|---|---|---|---|---|---|---|
| | No. of births[a] | Δ Birth weight (g)[a] | 95% CI | No. of births | Δ Gestational age (days) | 95% CI |
| TTHM | | | | | | |
| 0-33 | 89,881 | Reference | --- | 95,630 | Reference | --- |
| >33-74 | 70,567 | -12 | -16 to –7 | 74,956 | 0.0 | -0.1 to 0.1 |
| >74-163 | 16,729 | -18 | -26 to –10 | 17, 627 | 0.5 | 0.3 to 0.7 |
| Per 66 µg/L | --- | -18 | -23 to –13 | --- | 0.3 | 0.1 to 0.4 |
| Chloroform | | | | | | |
| 0-26 | 91,277 | Reference | --- | 97,956 | Reference | --- |
| >26-63 | 69,285 | -14 | -19 to –9 | 73,637 | 0.0 | -0.2 to 0.1 |
| >63-135 | 16,153 | -18 | -26 to -10 | 17,054 | 0.4 | 0.2 to 0.6 |
| Per 59 µg/L | --- | -19 | -25 to –14 | --- | 0.1 | 0.0 to 0.3 |
| BDCM | | | | | | |
| 0-5 | 101,564 | Reference | --- | 108,457 | Reference | --- |
| >5-13 | 60,873 | -12 | -17 to -8 | 64,215 | 0.6 | 0.5 to 0.7 |
| >13-46 | 14,278 | -12 | -20 to –3 | 15,059 | 0.5 | 0.3 to 0.8 |
| Per 11 µg/L | --- | -9 | -13 to –4 | --- | 0.5 | 0.4 to 0.6 |

[a] among term births only

**Figure 2. Changes in birthweight and incidence of small for gestational age babies with third trimester THM exposure (Wright et al., 2004).**

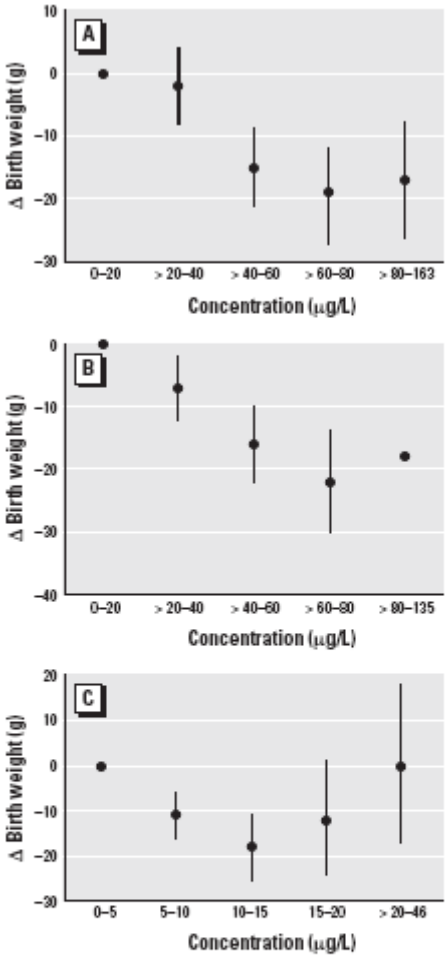



**Figure 1.** Change in mean birth weight and 95% CIs for third-trimester THM exposures: regression coefficients for (*A*) TTHM, (*B*) chloroform, and (*C*) BDCM adjusted for median household income, infant sex, adequacy of prenatal care, maternal race, maternal education, maternal cigarette consumption, maternal age, parity, previous infant weighing ≥ 4,000 g, previous preterm delivery, and maternal medical history (diabetes, chronic hypertension, lung disease, and renal disease).

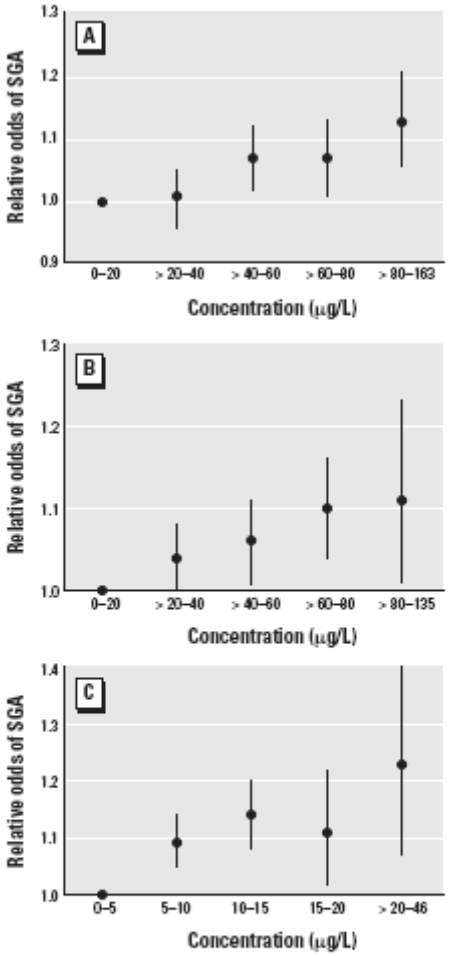



**Figure 2.** ORs and 95% CIs for SGA infancy and third-trimester THM exposures: ORs for (*A*) TTHM, (*B*) chloroform, and (*C*) BDCM adjusted for median household income, adequacy of prenatal care, maternal race, maternal education, maternal smoking, maternal age, parity, previous infant weighing ≥ 4,000 g, previous preterm delivery, and maternal medical history (diabetes, chronic hypertension, lung disease, and renal disease).

**Table 7. Adjusted odds ratios for SGA and preterm delivery for exposure to THMs in the third-trimester. (Wright et al., 2004).**

| Exposure (water conc. in μg/L) | SGA OR (95% CI) | Preterm Delivery OR (95% CI) |
|---|---|---|
| THM | | |
| 0-33 | 1.0 | 1.0 |
| >33-74 | 1.06 (1.02 to 1.10) | 0.95 (0.91 to 0.99) |
| >74-163 | 1.13 (1.07 to 1.20) | 0.88 (0.81 to 0.94) |
| Chloroform | | |
| 0-26 | 1.0 | 1.0 |
| >26-63 | 1.05 (1.02 to 1.09) | 0.95 (0.91 to 0.99) |
| >63-135 | 1.11 (1.04 to 1.17) | 0.90 (0.84 to 0.97) |
| BDCM | | |
| 0-5 | 1.0 | 1.0 |
| > 5-13 | 1.1 (1.07 to 1.14) | 0.89 (0.85 to 0.93) |
| >13-46 | 1.15 (1.08 to 1.22) | 0.92 (0.85 to 0.99) |

Limitations of this study include the misclassification of exposure due primarily to the use of aggregate municipal measures of THMs. Although residential mobility during pregnancy would be a possible source of exposure misclassification, there is some evidence that women who move during pregnancy are less likely to move during the third trimester. Thus, residential mobility may be less likely to influence third-trimester exposure estimates, the trimester most critical to the outcomes examined in this study. The lack of information concerning showering or use of bottled water is also a limitation. However, as noted by the authors, studies that have collected data on such personal habits have equivocal findings compared with the use of town average exposure measures (Klotz and Pyrch, 1999; Savitz et al., 1995; Waller et al., 1998). Although exposure misclassification is most certainly present in this study it is not likely to be associated with the outcome measures, i.e. birth outcomes. The esult of random exposure misclassification, such as this, would a biasing of the effect estimates toward the null, thus producing a non-significant effect or a more modest effect than actually exists.

### C.1.3. Exposure to other water disinfection byproducts

Numerous studies have examined the association between exposure to disinfection by -products and adverse pregnancy outcomes distinguishing exposure by source of drinking water (Aschengrau et al., 1989; Kanitz et al., 1996; Swan et al., 1998; and Yang et al., 2000), or by specifically measuring THMs, (Bove et al., 1995; Dodds et al., 1999; Dodds, 2004; Gallagher et al., 1998; Klotz and Pyrch, 1999; Savitz et al., 1995; Shaw et al., 2003; Wright et al., 2003). Although the purpose of this document is to review the potential developmental and reproductive toxicity of chloroform rather than disinfection

by-products, chloroform is the major constituent in these by-products; therefore, these studies provide supplementary data. The results of these studies are summarized in the Tables 8-10 below.

**Table 8. Odds Ratios for studies comparing pregnancy outcomes for different sources of drinking water.**

| Reference/ Study Location | Odds ratio (95% CI) | | | Source of drinking water comparison |
|---|---|---|---|---|
| | SAB [a] | PTD [b] | LBW[c] | |
| Aschengrau et al., 1989 Massachusetts | 2.2 (1.3-3.6) | | | Ground vs. surface |
| Kanitz et al., 1996 Italy | | 1.8 (0.7 – 4.7)<br>1.1 (0.3 – 3.7) | 5.9 (0.8-14.9)<br>6.0 (0.6 –12.6) | Untreated vs. chlorine dioxide or sodium hypochlorite treated |
| Swan et al., 1998 California | 4.58 (1.97-10.64) | | | Bottled vs. tap water |
| Yang et al., 2000 Taiwan | | 1.34 (1.15-1.56) | 0.90 (0.75-1.09) | Chlorinated vs. non- chlorinated municipalities |

[a] Spontaneous abortion
[b] Pre-term delivery
[c] Low birth weight

**Table 9. Odds Ratios for studies of TTHMs water concentration in association with pregnancy outcome.**

| Study authors/ Location | Odds Ratio (95% CI unless otherwise indicated) | | | | | | Exposure Level (µg/L) | Reference Level (µg/L) |
|---|---|---|---|---|---|---|---|---|
| | SAB[a] | Stillbirths | PTD[b] | SGA[c] | LBW[d] | BW[e] | | |
| Bove et al., 1995 New Jersey | | | | 1.5 (1.2-1.9)[f] | 1.4 (1.2-1.7)[g]† | -70.4 (-40.9 to -100.2)[f] | >100 | ≤20 |
| Savitz et al., 1995 Carolina | 1.7 (1.1-2.7) | | | | | | Per 50 ppb | |
| | | | 1.2 (0.8-1.8) | | 1.5 (1.0-2.3) | | 63.4-82.7 | 40.8-63.3 |
| | | | 0.9 (0.6-1.5) | | 1.3 (0.8-2.1) | | >82.8 | 40.8-63.3 |
| Gallagher et al., 1998 Colorado | | | 1.0 (0.3-2.8) | | 2.1 (1.0-4.8) | | >60 | ≤20 |
| | | | | | 5.9 (2.0-17.0) † | | >60 | ≤20 |
| Waller et al., 1998 California | 1.8 (1.1-3.0) | | | | | | ≥75 | <75 |
| Dodds et al., 1999 Nova Scotia | | 1.66 (1.09-2.52) | 0.97 (0.87-1.09) | 1.08 (0.99-1.18) | 1.04 (0.92-1.18) | | >100 | <50 |
| King et al. 2000 | | 1.66 (1.09-2.54) | | | | | | |
| Wright et al., 2003 Massachusetts | | | 0.9 (0.77-1.04) | 1.14 (1.02-1.26) | 1.05 (0.85-1.29) | -32 (-47 to –18) | >80 | ≤60 |
| Dodds et al., 2004 Nova Scotia | | 2.2 (1.1-4.4) | | | | | >80 | 0 |
| Ontario | | 2.4 (1.2-4.6) | | | | | Exposure metric-top quintile | |
| | | 2.0 (1.0-4.0) | | | | | $CHCl_3$ – top quintile | |
| | | 2.5 (1.3-4.9) | | | | | BDCM – top quintile | |

[a] Spontaneous abortion
[b] Pre-term delivery
[c] Small for gestational age
[d] Low birth weight
[e] Birth weight
[f] 90% CI
[g] 50% CI
†-Term low birth weight

**Table 10. Odds Ratios for studies of TTHMs exposure in association with congenital malformations.**

| Study | Odds ratio (95% CI) | | | | TTHM (µg/L) | |
|---|---|---|---|---|---|---|
| | CNS[a] | NTD[b] | Major Cardiac | Respiratory | Highest Level | Reference Level |
| Aschengrau et al., 1993 | | | 3.2 (1.1-9.5) | 4.1 (1.2-14.1) | chlorination vs chloramination | |
| Bove et al., 1995 | 2.59 (1.14-5.55)[c] | 2.96 (0.78-9.84)[c]<br>2.96 (1.29-6.62)[d] | 1.83 (0.68-4.37)[c]<br>1.83 (0.97-3.29)[d] | | >80 | ≤20 |
| Dodds et al., 1999 | | 1.18 (0.67-2.10) | 0.77 (0.57-1.04) | | >100 | <50 |
| Klotz & Pyrch, 1999 | | 2.1 (1.1-4.0) | | | >40 | <5 |
| Shaw et al., 2003 | | 0.62 (0.26-1.5) | | | ≥75 | 0 |

[a] Central nervous system
[b] Neural tube defect
[c] 99% CI
[d] 90% CI

## C.2. Developmental toxicity in animals

Available data on the potential developmental toxicity of chloroform include developmental toxicity studies in the rat, performed by both the inhalation and oral routes (Schwetz et al., 1974; Dilley et al., 1977; Baeder and Hoffman, 1988; Baeder and Hoffman, 1991; Thompson et al., 1974; Ruddick et al., 1983). Data from species other than the rat are represented by one study in mouse using the inhalation route of exposure (Murray et al., 1979), and one study in the rabbit using the oral route of exposure (Thompson et al., 1974). One oral study was identified that evaluated the potential of chloroform to cause developmental neurotoxicity (Burkhalter and Balster, 1979). Additional relevant information is provided by *in vitro* studies employing whole-embryo culture, discussed subsequently in Section C.3.

### C.2.1. Studies in rats

*C.2.1.1. Inhalation route*

*Schwetz et al. (1974). Embryo- and fetotoxicity of inhaled chloroform in rats*

Timed-mated Sprague-Dawley rats were exposed to chloroform by inhalation, 7 hr/day on each of gestation days 6 through 15. The initial experiment employed concentration levels of 0 or 300 ppm. Additional groups were exposed to 100 or 30 ppm chloroform in a second experiment and, since "marked anorexia" was observed in rats exposed to 300 ppm chloroform in the initial experiment, a "starved control" group (restricted to 3.7 g food/day on gestation days 6-15) was also added to the second experiment. As a means of evaluating liver function, groups of nonpregnant female rats were exposed to 100 or 300 ppm chloroform for the purpose of evaluating treatment-related changes in serum glutamic-pyruvic transaminase (SGPT). Control animals for each experiment were exposed to filtered room air. Food and water were withheld during treatment sessions.

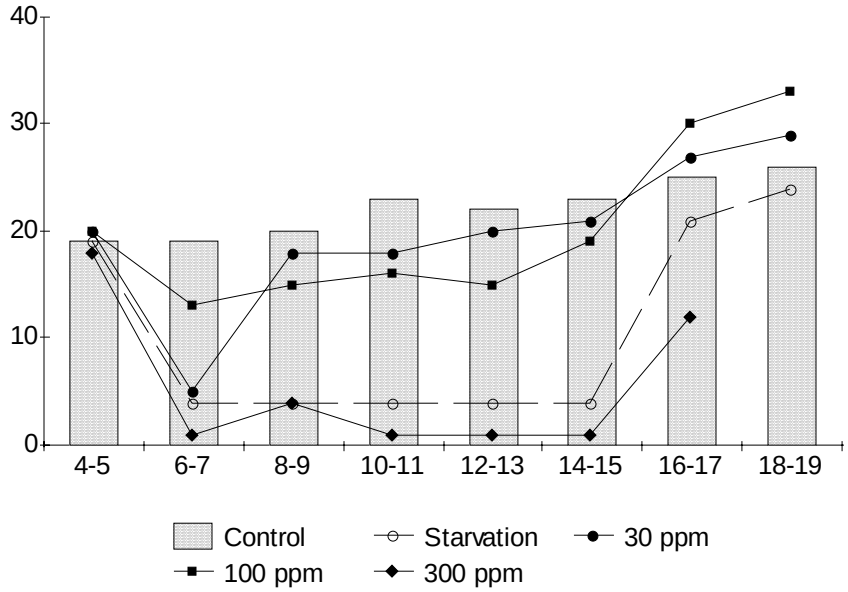
No dams died over the course of the study, but statistically-significant deficits were found for percent pregnant, as well as for maternal weight gain and food consumption (see Table 11). Maternal food consumption data are presented in detail in Figure 3 below.

**Table 11. Maternal parameters following exposure to chloroform by inhalation (Schwetz et al., 1974)**

| Parameters | Air control | Air control (starved)[1] | 30 ppm | 100 ppm | 300 ppm |
|---|---|---|---|---|---|
| N mated | 77 | 8 | 31 | 28 | 20 |
| N pregnant | 68 | 8 | 22 | 23 | 3 |
| % pregnant | 88 | 100 | 71 | 82 | 15* |
| bw (g) ± SD | | | | | |
| gd 6 | 275 ± 21 | 274 ± 13 | 266 ± 14 | 274 ± 17 | 284 ± 9 |
| gd 13 | 310 ± 17 | 223 ± 13* | 280 ± 14* | 274 ± 18* | 192 ± 9* |
| gd 21 | 389 ± 28 | 326 ± 24* | 381 ± 23 | 365 ± 22 | 241 ± 29* |
| Feed (g/d) | | | | | |
| gd 6-7 | 19 ± 3 | starved | 5 ± 3* | 13 ± 4* | 1 ± 1* |
| gd 12-13 | 22 ± 2 | starved | 20 ± 1 | 15 ± 2* | 1 ± 1* |
| gd 18-19 | 26 ± 3 | 24 ± 8* | 29 ± 5 | 33 ± 3* | not done |

[1] restricted to 3.7 g food/day on gestation days 6-15
* statistically significant difference from controls at $p < 0.05$

**Figure 3. Maternal feed consumption (g/day by gestation day) (Schwetz et al., 1974)**




All three chloroform-treated groups showed statistically significant decreases in feed consumption ($p < 0.05$) at the beginning of treatment on gestation day 6. For the 300 ppm group, feed consumption showed a slight rebound after the cessation of treatment, but remained significantly below control levels of consumption ($p < 0.05$) for all times at which the endpoint was evaluated. Animals of the "starved" group (3.7 g food on each of

gestation days 6-15) also showed a rebound increase in feed consumption once returned to ad lib feeding, but also remained significantly below control feed consumption for the post-treatment period ($p < 0.05$). For the 100 ppm group, feed consumption remained significantly below control levels during the treatment period ($p < 0.05$). After cessation of treatment, feed consumption by this group increased to levels significantly greater than that of control animals ($p < 0.05$). Animals of the 30 ppm group consumed significantly less feed than control animals only on the first day of treatment ($p < 0.05$). No significant differences from control feed-intake levels were found for any other time point for animals of this group.

Maternal body weights of the starved group were significantly lower than ad lib controls on both day 13 and day 21 ($p < 0.05$) but not on day 6. Weights of the 30 ppm chloroform group were significantly lower than controls only on gestation day 13 ($p < 0.05$). Weights of the 100 and 300 ppm chloroform groups were significantly lower than controls on both day 13 and day 21 ($p < 0.05$ in all cases).

SGPT activity was determined to assess the degree of liver toxicity in pregnant and non-pregnant rats. In nonpregnant rats, SGPT activity was not significantly affected during ten days of exposure to 100 or 300 ppm chloroform, nor at six days following cessation of exposure. Pregnant rats exposed to 300 ppm chloroform were evaluated for SGPT activity six days following cessation of exposure (gd 21). Although SGPT was lower in exposed animals as compared to controls ($61 \pm 4$ Karmen units and $72 \pm 3$ Karmen units, respectively), the difference was not statistically significant.

Changes in the appearance of the livers of nonpregnant rats immediately following the last exposure to 300 ppm chloroform were considered to be "minimal": "pale, mottled liver in 4/4 rats." Livers from pregnant and nonpregnant rats evaluated at six days following the cessation of treatment were considered to be normal in appearance.

When evaluated immediately following the cessation of treatment, absolute and relative liver weights of nonpregnant rats exposed to 100 ppm chloroform were significantly increased over control values ($p < 0.05$ in both cases). Absolute liver weights of nonpregnant rats exposed to 300 ppm chloroform, on the other hand, were significantly decreased compared to control levels ($p < 0.05$); relative liver weights of this group did not differ from controls.

When evaluated at six days following the cessation of treatment, absolute liver weights of nonpregnant animals showed no differences among treatment groups (0, 30, 100 or 300 ppm chloroform). Relative liver weights were affected only in the 300 ppm group of nonpregnant rats, showing a significant increase when compared to controls ($p < 0.05$).

For pregnant animals exposed to chloroform during gestation days 6-15, and evaluated six days following cessation of treatment (gd 21), only the 300 ppm and starved-control groups showed significant decreases in absolute liver weight relative to fed controls ($p < 0.05$ in both cases). Relative liver weights were increased over control values at 100 and 300 ppm chloroform, as well as in starved controls ($p < 0.05$ in all cases). Neither

absolute nor relative liver weights were significantly affected in dams exposed to 30 ppm chloroform.

**Table 12. Fetal parameters following exposure to chloroform by inhalation (Schwetz et al., 1974).**

| Parameters | Air control | Air control (starved) | 30 ppm | 100 ppm | 300 ppm |
|---|---|---|---|---|---|
| litters | 68 | 8 | 22 | 23 | 3 |
| fetuses/litter | 10 ± 4 | 10 ± 4 | 12 ± 2 | 11 ± 2 | 4 ± 7* |
| resorptions | 8% | 7% | 8% | 6% | 61%* |
| litters with resorptions | 57% | 25% | 68% | 52% | 100% |
| litters totally resorbed | 0/68 | 0/8 | 0/22 | 0/23 | 1/3 |
| sex ratio, M:F | 53:47 | 45:55 | 53:47 | 55:45 | 34:66* |
| mean fetal weight/ litter (g) | 5.69 ± 0.36 | 5.19 ± 0.29* | 5.51 ± 0.20 | 5.59 ± 0.24 | 3.42 ± 0.02* |
| CRL (mm) | 43.5 ± 1.1 | 42.1 ± 1.1* | 42.5 ± 0.6* | 43.6 ± 0.7 | 36.9 ± 0.2* |
| gross anomalies (% litters) | 1.5% | 0 | 0 | 13%* | 0 |
| skeletal anomalies (% litters) | 68% | 38% | 90%* | 74% | 100% |
| soft tissue anomalies (% litters) | 48% | 38% | 45% | 65% | 100% |

* statistically significant difference from controls at $p < 0.05$

Only three out of 20 mated dams (15%) in the 300 ppm group were found to be pregnant at the time of necropsy (significantly lower than controls: $p < 0.05$). For those three litters as compared to controls, litter size was significantly reduced, resorption frequency was significantly increased (both endpoints significant: $p < 0.05$), and the percentage of litters with resorptions was 100% as opposed to 57% for controls (see Table 12 above).

Fetal weights were significantly reduced relative to controls in the starved control and 300 ppm chloroform groups ($p < 0.05$ in both cases). Crown-rump length was significantly reduced in starved controls, and at 30 and 300 ppm chloroform ($p < 0.05$ in both cases), but not at 100 ppm chloroform. Also, among the three litters of the 300 ppm chloroform group, the sex ratio was skewed to an excess of females ($p < 0.05$).

Three out of 23 litters showed gross malformations at a concentration of 100 ppm chloroform; 3/23 litters had fetuses with acaudia or short tail, and 3/23 litters had fetuses with imperforate anus. This constituted a significant ($p < 0.05$) increase over the control malformation rate of one out of 68 litters. It is not stated how many fetuses were affected among the three affected litters, nor is it specified if the same fetuses in the three affected litters had both acaudia/short tail and imperforate anus. The frequency of total skeletal malformations was significantly increased at 30 ppm chloroform ($p < 0.05$), with 16/22 litters having at least one fetus showing delayed ossification of the skull (significant at $p < 0.05$), 4/22 showing wavy ribs (significant at $p < 0.05$), and 2/22 having split sternebrae. The frequency of subcutaneous edema was significantly increased in the 100 ppm chloroform group ($p < 0.05$), occurring in 14/23 litters.

*Baeder and Hoffman (1988). Initial Submission [to U.S. EPA]: Inhalation embryotoxicity study of chloroform in Wistar rats (final report) with attachments and cover letter dated February 21, 1992*

Baeder and Hoffman (1988) conducted two preliminary and one main study of the potential embryotoxicity of inhaled chloroform in Wistar rats. The two dose range-finding studies were performed on groups of four to six time-mated Wistar female rats. In the first preliminary study, the pregnant animals were exposed in the inhalation chambers for six hours daily on gestation days 7-11 and 14-16. Chloroform concentrations of 10, 30, and 100 ppm were tested. In the 10 ppm group, two dams had no fetuses and only a single implantation site. One dam in the 30 ppm group had only one fetus and three empty implantation sites. Similar effects were not seen, however, at the highest concentration of 100 ppm.

In a second preliminary experiment, dams were exposed to chloroform at concentrations of 100 and 300 ppm on each of gestation days 7-16. During the treatment period, dams in both treated groups showed a reduction in feed consumption, and a loss of body weight. In two litters of the 100 ppm group, fetal weights appeared to be somewhat reduced. In the 300 ppm group, three dams had normally developed fetuses, one dam had totally resorbed fetuses, and one dam had only empty implantation sites in the uterus.

On the basis of these results, concentrations of 0, 30, 100, and 300 ppm were chosen for the full-scale study. Groups of 20-23, time-mated Wistar rats were exposed to chloroform daily for seven hours on each of gestation days 7-16. Uterine contents were examined following cesarean section on gestation day 21. Approximately half the live fetuses from each litter and all intrauterine deaths were fixed, cleared, and stained for skeletal examinations. The remaining fetuses were preserved in Bouin's solution for free-hand sectioning in order to evaluate internal organs.

No behavioral alterations or clinical symptoms were induced in dams by treatment, and all females survived until the end of the study. During the treatment period, all chloroform-exposed females showed concentration-dependant reductions in feed consumption and body weight gain (Table 13). Following the cessation of treatment, concentration-dependent, compensatory, increased feed consumption was observed. At

the beginning of the study, on gestation day 0, groups assigned to chloroform treatment were significantly ($p < 0.05$) heavier than the control group. By the cessation of treatment on gestation day 17, maternal body weight was significantly reduced in all three treated groups ($p < 0.05$), with an apparent concentration-response relationship. Concentration-dependency was still evident on gestation day 21, but the 30 ppm group no longer showed a significant difference from controls.

**Table 13. Maternal feed consumption and body weight (mean ± SD) after inhalation exposure to chloroform (Baeder and Hoffman 1988)**

| Parameter | **0** | **30 ppm** | **100 ppm** | **300 ppm** |
|---|---|---|---|---|
| Number of live litters | 20 | 18 | 17 | 12 |
| feed, gd 14-17* | 9.20 ± 0.85 | 8.30 ± 0.79# | 8.30 ± 1.46# | 6.91 ± 1.21# |
| feed, gd 17-21* | 9.17 ± 0.67 | 9.78 ± 0.77# | 10.21 ± 0.55# | 11.34 ± 0.58# |
| body weight (g), gd 0 | 184 ± 5 | 192 ± 5# | 194 ± 11# | 191 ± 8# |
| body weight (g), gd 17 | 262 ± 14 | 240 ± 16# | 230 ± 17# | 212 ± 15# |
| body weight (g), gd 21 | 315 ± 18 | 300 ± 22 | 294 ± 20# | 284 ± 16# |
| weight gain (g)** | 131 | 107 | 99 | 93 |

* g feed consumed per 100 g body weight
** g body weight gained over gestation days 0-21; SD not provided
# significant difference from controls at $p < 0.05$

At necropsy, slight or moderate dilation of one or both renal pelves was seen in two control dams and in one dam each from the 30 and 100 ppm groups. Absolute cardiac weights appeared to be reduced in a concentration-dependent fashion, but when considered relative to body weight, only dams of the 100 ppm group appeared to show an effect on cardiac weight. Weights of other organs (liver, kidneys, spleen) did not appear to be affected by treatment.

Litters were completely resorbed in two dams at 30 ppm chloroform, in three dams at 100 ppm, and in eight dams at 300 ppm (Table 14). In dams producing live litters, neither numbers of corpora lutea nor live litter size were significantly affected by chloroform treatment. Placental weights did not differ among groups. Fetuses from treated dams showed slight stunting in terms of weight and crown-rump length (CRL). Fetal weight showed a concentration-related decrease over all treated groups, and was significantly lower than controls in the 300 ppm group ($p < 0.05$). CRL was significantly lower than controls in all three treated groups ($p < 0.05$), but did not show a concentration relationship.

**Table 14. Litter data after inhalation exposure to chloroform (Baeder and Hoffman 1988)**

| Parameters | **0** | **30 ppm** | **100 ppm** | **300 ppm** |
|---|---|---|---|---|
| N pregnant/N sperm plugs | 20/20 | 20/20 | 20/21 | 20/23 |
| N lost litters | 0 | 2 | 3 | 8 |
| N live litters | 20 | 18 | 17 | 12## |
| Resorptions/live litter* | 0.75 | 0.22 | 0.53 | 0.92 |
| Live fetuses/litter* | 12.4 | 12.8 | 12.8 | 13.4 |
| Fetal weight (g)** | 3.19 ± 0.30 | 3.16 ± 0.19 | 3.13 ± 0.21 | 3.00 ± 0.19# |
| Fetal CRL (cm)** | 3.52 ± 0.17 | 3.38 ± 0.12# | 3.39 ± 0.10# | 3.39 ± 0.12# |

* Mean per litter, no SD provided
** Mean ± SD
# significant difference from controls at $p < 0.05$
## significant difference from controls at $p < 0.005$ (calculated by OEHHA)

Examinations for external malformations, internal malformations, and skeletal abnormalities did not reveal statistically significant evidence for effects of chloroform treatment on these endpoints. One stunted control fetus had cleft palate, brachygnathia, ossification delays, a 14th rib anlage at the first lumbar vertebrae, and wavy ribs. Upon free-hand razor sectioning, a few fetuses from the 30 ppm, 100 ppm, and control groups showed blood in the abdominal cavity and distended renal pelves or ureters. One fetus in the 30 ppm group had a hematoma in the region of the left kidney. One fetus in the 100 ppm group had and undescended testis on the right side. One fetus in the 300 ppm group had blood in the left cerebral hemisphere. All groups, including controls, showed sporadic incidences of "a dark red area in the center of one or both adrenals."

Sporadic incidents of skeletal/ossification variations were also observed across all groups. These included: "longitudinally shifted and/or fragmented sternebrae, 14th thoracic vertebra with an additional pair of ribs, 14th rib anlage at 1st lumbar vertebrae, and thickened and/or wavy ribs."

*Baeder and Hoffman (1991). Initial Submission [to U.S. EPA]: Chloroform: Supplementary inhalation embryotoxicity study of chloroform in Wistar rats (final report) with attachments and cover letter dated 122491*

As a follow-on supplement to the study described above (Baeder and Hoffman, 1988), groups of 20 timed-mated Wistar rats were exposed to chloroform by inhalation at concentrations of 0, 3, 10, or 30 ppm for 7 hours daily on each of gestation days 7-16. As in the previous study, uterine contents were examined following cesarean section on gestation day 21. Approximately half the live fetuses from each litter and all intrauterine deaths were fixed, cleared, and stained for skeletal examinations. The remaining fetuses were preserved in Bouin's solution for free-hand sectioning in order to evaluate internal organs.

All dams of all groups survived until the end of the study. Localized alopecia was seen in one dam from the 30 ppm group, and two dams in the control group. No other signs of abnormal physical condition or behavior were reported. Maternal feed consumption during the first week of treatment showed statistically significant ($p < 0.05$) decreases in treated animals, with an apparent concentration-response relationship (Table 15). During the second week of treatment, only females of the 30 ppm group showed significantly reduced food consumption relative to controls ($p < 0.05$). Subsequent to the cessation of treatment until the end of the study, feed consumption by 30 ppm dams was significantly greater than that of control animals ($p < 0.05$).

Body weight and body weight gain were not affected in maternal animals exposed to 3 ppm chloroform. Body weights did not appear to differ among groups on day 0 or day 7. By day 14, following a week of treatment, body weights of the 10 and 30 ppm groups appear to be lower than those of the 0 and 3 ppm groups. By day 17, following two weeks of treatment, there appears to be a concentration-related decrease in maternal body weight. By the end of the study on gestation day 21, only the 10 and 30 ppm groups still show body weights below control values, with no difference between these two higher concentrations. Similarly, pre-treatment weight gain did not appear to vary among groups. Weight gain during the first week of treatment appeared to decrease in a concentration-dependent fashion. During the second week of treatment weight gain of treated dams in all groups exceeded control levels. After the cessation of treatment on day 17, weight gain in the 30 ppm group appeared to be somewhat greater than in control animals. When considered over the whole of gestation, weight gain for the 10 and 30 ppm groups appeared to be lower than that of controls, with a concentration-response relationship for these two concentrations.

The authors do not appear to have conducted statistical analyses of the body weight or body weight gain parameters. They did, however, tabulate and analyze a variable they called "body-weight development." It is not clear from the document how this variable was derived, though the pattern of the values appears similar to that for body weight: no effect of 3 ppm chloroform, and decreases relative to controls for 10 and 30 ppm for days 7-14, 14-17, and 17-21 (all significant at $p < 0.05$, except for 30 ppm at days 7-14).

**Table 15. Maternal feed consumption and body weight[a] after inhalation exposure to chloroform (Baeder and Hoffman 1991).**

| Parameter | **0** | **3 ppm** | **10 ppm** | **30 ppm** |
|---|---|---|---|---|
| N | 20 | 20 | 20 | 19 |
| feed, gd 7-14* | 8.03 ± 0.68 | 7.19 ± 0.66# | 6.45 ± 0.70# | 5.60 ± 0.75# |
| feed, gd 14-17* | 7.07 ± 0.32 | 7.16 ± 0.59 | 7.12 ± 0.67 | 6.52 ± 0.67# |
| feed, gd 17-21* | 6.63 ± 0.40 | 6.49 ± 0.61 | 6.91 ± 0.33 | 7.25 ± 0.52# |
| bw (g), gd 0** | 193.3 ± 12.2 | 197.5 ± 7.7 | 192.2 ± 6.4 | 200.0 ± 7.4 |
| bw (g), gd 7** | 226.0 ± 14.7 | 220.9 ± 11.0 | 222.9 ± 8.2 | 230.6 ± 10.6 |
| bw (g), gd 14** | 255.8 ± 16.2 | 253.6 ± 13.7 | 237.1 ± 10.4 | 237.3 ± 12.3 |
| bw (g), gd 17** | 269.1 ± 17.0 | 260.2 ± 13.7 | 255.2 ± 12.4 | 253.4 ± 16.3 |
| bw (g), gd 21** | 321.9 ± 22.5 | 319.1 ± 21.1 | 308.0 ± 17.5 | 308.7 ± 18.5 |
| weight gain, gd 0-7 | 32.7 ± 9.5 | 31.4 ± 9.1 | 30.7 ± 3.5 | 30.6 ± 7.3 |
| weight gain, gd 7-14*** | 29.8 ± 10.5 | 24.7 ± 6.3 | 14.3 ± 8.2 | 6.7 ± 8.8 |
| weight gain, gd 14-17*** | 13.3 ± 4.6 | 14.6 ± 5.7 | 16.1 ± 5.0 | 16.1 ± 6.7 |
| weight gain, gd 17-21*** | 52.9 ± 6.5 | 50.9 ± 11.5 | 52.9 ± 11.7 | 55.3 ± 7.8 |
| weight gain, gd 0-21*** | 120.6 ± 17.8 | 121.6 ±21.0 | 115.9 ± 16.2 | 108.7 ± 16.7 |

* g feed consumed per 100 g body weight
[a] mean ± SD
# significant difference from controls at $p < 0.05$

At necropsy of maternal animals, moderate to severe unilateral or bilateral renal pelvic dilatation was observed in one dam of the 3 ppm group, 3 dams of the 10 ppm group, and four dams of the 30 ppm group. No other organ abnormalities were noted. Heart, liver, and spleen weights did not differ among groups. Kidney weights were increased in the 30 ppm chloroform group as compared to controls (significant at $p < 0.05$).

Apart from one dam in the 30 ppm group, all dams in all groups in the study carried live fetuses to term. The one dam with no fetuses showed 13 empty implantation sites. Numbers of corpora lutea and implantations did not differ significantly among groups. Neither resorption frequency nor live litter size differed among groups.

According to the text of Baeder and Hoffman (1991), mean fetal body weights and lengths did not differ significantly among groups. Tabulated data in the report marks both fetal weight and CRL as significantly lower than controls for the 30 ppm group (see Table 16). In the case of fetal weight, however, both the mean weight and the standard deviation (SD) for all treated groups are identical, with N for the 30 ppm group being 19, rather than 20 litters. In any event, the text notes that fetuses with body weights of less than 3.0 g were more common in the 10 and 30 ppm groups than in the control and 3 ppm groups (24% and 26.9%, respectively, as opposed to 3.2% and 14.2%, respectively).

**Table 16. Litter data after inhalation exposure to chloroform (Baeder and Hoffman 1991).**

| Parameter | **0** | **3 ppm** | **10 ppm** | **30 ppm** |
|---|---|---|---|---|
| N pregnant | 20 | 20 | 20 | 20 |
| N lost litters | 0 | 0 | 0 | 1 |
| N live litters | 20 | 20 | 20 | 19 |
| Resorptions/live litter* | 0.55 ± 0.89 | 0.40 ± 0.60 | 0.75 ± 1.02 | 0.84 ± 1.42 |
| Live fetuses/litter* | 12.4 ± 2.4 | 12.4 ± 3.5 | 12.9 ± 3.0 | 12.5 ± 1.9 |
| Fetal weight (g)* | 3.4 ± 0.3 | 3.2 ± 0.3 | 3.2 ± 0.3 | 3.2 ± 0.3# |
| Fetal CRL (mm)* | 35.8 ± 2.0 | 35.5 ± 2.1 | 34.4 ± 2.6 | 34.0 ± 1.9# |

* Litter mean ± SD
# significant difference from controls at $p < 0.05$

One incident of internal hydrocephalus was observed in a live fetus of the 3 ppm group. No other gross malformations were reported in any group. In razor-sectioned fetuses, sporadic incidences were reported of findings such as: blood in the thoracic cavity, blood in the left kidney, hematoma in one liver lobe, hematoma in the right kidney, blood in the abdominal cavity, unilateral or bilateral renal pelvic distention, or unilateral or bilateral distention of the ureter.

The frequency of fetuses with poorly ossified cranial bones was significantly ($p < 0.05$) higher in the 30 ppm chloroform group than among controls (Table 17). The frequency of litters having fetuses with poorly ossified cranial bones did not differ significantly among groups. All three treated groups had significantly ($p < 0.05$) higher frequencies of poor ossification of the caudal vertebrae and sternebrae than did control fetuses, when considered as total numbers of affected fetuses per group. When considered on a per litter basis, as litters containing at least one affected fetus, sternebral ossification alone was significantly affected ($p < 0.05$). The frequency of fetuses with wavy and/or thickened ribs was greater in the 10 ppm group than among controls ($p < 0.05$). This difference was not significant when considered on a per litter basis. Other skeletal and ossification variations were observed sporadically across all groups.

**Table 17. Skeletal/ossification variations after inhalation exposure to chloroform (Baeder and Hoffman 1991).**

| Parameters | 0 | 3 ppm | 10 ppm | 30 ppm |
|---|---|---|---|---|
| N live litters | 20 | 20 | 20 | 19 |
| poorly ossified cranial bones* | 42/14 | 47/17 | 48/16 | 60#/17 |
| ossification of less than 2 caudal vertebrae* | 4/3 | 14#/5 | 16#/6 | 14#/8 |
| non- or weakly ossified sternebrae* | 7/3 | 32#/13# | 35#/14# | 18#/11# |
| wavy or thickened ribs* | 10/6 | 11/5 | 22#/10 | 15/4 |

* number affected fetuses/number litters with affected fetuses
# significant difference from controls at $p < 0.05$

*Dilley et al. (1977). Inhalation teratology studies of five chemicals in rats. [Abstract]*

Pregnant rats of an unspecified strain were exposed to chloroform vapor during gestation days 7-14. The highest concentration of chloroform was $20.1 \pm 1.2$ g/m$^3$ (approximately 4100 ppm). Two lower concentrations were also tested, but these were not specified in the abstract. Size of test groups is not reported. Animals were sacrificed on gestation day 20. Chloroform exposure was found to be associated with increased fetal mortality and decreased fetal weight gain; teratogenic effects were not observed.

*C.2.1.2. Oral route*

*Thompson et al. (1974). Teratology studies on orally administered chloroform in the rat and rabbit.*

Timed-mated Sprague-Dawley rats were given chloroform in corn oil by gavage. In a range-finding study, groups of six pregnant animals were given chloroform doses of 0, 79, 126, 300, 316, or 501 mg/kg-day on each of gestation days 6-15. Doses were initially provided once daily, but after three to six days of treatment, the doses were split into two equal halves and given approximately seven hours apart.

Results of the range-finding study were not reported in tabular form, but were discussed in the text of the paper without details of statistical analysis. The data revealed significant decrements in maternal weight gain and food consumption for animals exposed to 126 mg/kg-day chloroform or more. One rat from the 316 mg/kg group and four from the 501 mg/kg group died or were sacrificed moribund during treatment. Necropsy of the dead animals found acute toxic nephrosis, hepatitis, and gastric erosions. None of the surviving litters showed evidence of external malformations. Resorption frequency was significantly increased in the litters of dams given 316 mg/kg-day chloroform. Litter size and fetal weights were correspondingly decreased in these

animals. Neither of the females surviving exposure to 501 mg/kg chloroform had live fetuses: one was found to be not pregnant, the other had a completely resorbed litter.

In the full-scale teratology study, groups of 25 timed-mated Sprague-Dawley rats were given twice-daily gavage dosings of chloroform to total daily doses of 0, 20, 50, or 126 mg/kg-day on each of gestation days 6-15. Two females from each group were sacrificed on the last day of treatment, and tissue samples taken for histopathological examination. The remaining females were sacrificed for evaluation of their litters on gestation day 20. Two-thirds of the fetuses from each litter were preserved and prepared for skeletal staining; remaining fetuses were preserved in Bouin's fixative and examined for soft-tissue anomalies.

Data on maternal animals are not tabulated, but the text notes that most females of the high-dose group (126 mg/kg-day) displayed alopecia and rough appearance after four to five days of treatment. No spontaneous deaths occurred during the study. Dams of the 50 mg/kg-day group showed reduced weight gain over the treatment period, relative to controls. Dams from the 126 mg/kg-day group lost weight during treatment. Feed consumption was reduced during the treatment period only for the latter group. Among the females sacrificed on the last day of treatment, both high-dose animals, and one animal from the 50 mg/kg-day group, showed mild fatty changes in their livers. Such changes were not observed in other dose groups. No liver or kidney effects were observed in animals of any group necropsied at term.

Implantation frequency, numbers of corpora lutea, resorption frequency, litter size, fetal weight, and sex ratio did not differ among groups exposed to chloroform at 0, 20, or 50 mg/kg-day. In the 126 mg/kg-day group, implantation frequency was significantly increased over controls ($p < 0.05$) and fetal weight was significantly decreased ($p < 0.05$) (Table 18).

**Table 18. Litter data from rats treated with oral chloroform (litter mean ± SD) (Thompson et al. 1974).**

| Dose (mg/kg-day) | Implants | Corpora Lutea | Resorptions | Live Fetuses | Fetal Weight (g) | M:F Ratio |
|---|---|---|---|---|---|---|
| 0 | 11.5 ± 2.4 | 13.1 ± 1.4 | 1.0 ± 2.9 | 10.6 ± 3.9 | 4.0 ± 0.3 | 52:48 |
| 126 | 13.5 ± 1.1* | 14.2 ± 1.2 | 1.2 ± 2.6 | 12.3 ± 3.1 | 3.7 ± 0.4* | 56:44 |

* statistically significant difference from controls at $p < 0.05$

No major external, skeletal, or visceral fetal abnormalities were observed to result from chloroform exposure. The fetal, but not litter, incidence of bilateral extra lumbar ribs was significantly increased in fetuses exposed to chloroform at 126 mg/kg-day. Minor visceral and skeletal anomalies were observed sporadically.

*Ruddick et al. (1983). A teratological assessment of four trihalomethanes*

Chloroform was one of four trihalomethanes administered in separate experiments to pregnant Sprague-Dawley rats by oral intubation on each of gestation days 6-15. Chloroform was given at doses of 0, 100, 200, or 400 mg/kg-day, in a corn oil vehicle to a final volume of 1 ml/100 g body weight. Vehicle controls and all treated groups consisted of 15 rats each.

Females were weighed on day 1 of the experiment, on each of days 6-15, and before and after caesarean section on gestation day 22. At the time of sacrifice, maternal viscera, including the uterus, were removed for pathological examination. Two fetuses from each litter were fixed for histopathological evaluation; two-thirds of the remaining fetuses from each litter were cleared and stained for skeletal evaluation, and the rest were fixed for visceral examinations. According to the text, statistical significance was specified as $p \leq 0.05$; one table is footnoted with significance as $p \leq 0.5$, but that appears to be a typographical error.

Maternal weight gain was depressed in all chloroform-treated groups ($p \leq 0.05$) (Table 19). Maternal liver weight was significantly increased at all dose levels, and maternal kidney weight was significantly increased at 400 mg/kg-day chloroform ($p \leq 0.05$ for both endpoints). Histopathological examinations of maternal organs did not reveal any treatment-related abnormalities.

Chloroform administration at all doses was associated with depressed maternal hemoglobin and hematocrit values (both at $p \leq 0.05$). Red blood cell counts were significantly reduced at 400 mg/kg-day ($p \leq 0.05$). Sorbitol dehydrogenase in maternal serum was significantly decreased at all three doses of chloroform ($p \leq 0.05$), whereas increased serum inorganic phosphorus and cholesterol were seen at 200 and 400 mg/kg-day chloroform ($p \leq 0.05$).

**Table 19. Maternal body weight and organ weight data with oral chloroform exposure (mean ± SE) (Ruddick et al. 1983).**

| Dose (mg/kg-day) | pregnant/mated | weight gain (g) | liver weight as % bw (N) | kidney weight as % bw (N) |
|---|---|---|---|---|
| 0 | 14/15 | 58.3 ± 4.4 | 4.7 ± 0.1 (10) | 0.35 ± 0.02 (10) |
| 100 | 12/15 | 39.9 ± 4.2* | 5.2 ± 0.2* (10) | 0.39 ± 0.01 (10) |
| 200 | 10/15 | 32.1 ± 4.0* | 5.9 ± 0.1* (9) | 0.42 ± 0.02 (9) |
| 400 | 8/15 | 24.7 ± 3.9* | 5.9 ± 0.3* (6) | 0.49 ± 0.07* (6) |

* statistically significant difference from controls at $p \leq 0.05$

Resorption frequency and live litter size were unaffected by treatment, but mean fetal weight was significantly decreased at 400 mg chloroform/kg-day ($p \leq 0.05$) (Table 20). The frequencies of sternebral aberrations and runting were increased in chlorform-exposed fetuses.

**Table 20. Data from fetuses of rats exposed orally to chloroform (mean ± SE) (Ruddick et al. 1983).**

| Dose mg/kg | Number of litters | litter size | fetal weight (g) | sternebral aberrations[1] | runts[2] | runts[3] |
|---|---|---|---|---|---|---|
| 0 | 14 | 11.2 ± 0.2 | 5.4 ± 0.8 | 0/0 | 1/1 | 0/0 |
| 100 | 12 | 11.8 ± 0.6 | 5.3 ± 0.1 | 1/1 | 2/1 | 1/1 |
| 200 | 10 | 12.5 ± 0.7 | 5.0 ± 0.1 | 5/3 | 0/0 | 0/0 |
| 400 | 8 | 10.9 ± 1.1 | 4.4 ± 0.3* | 14/8 | 11/3 | 26/8 |

[1] fetuses/litters
[2] among fetuses prepared for skeletal examination; fetuses/litters
[3] among fetuses prepared for visceral examination; fetuses/litters
* statistically significant difference from controls at $p \leq 0.05$

### C.2.2. Studies in mice

*Murray et al. (1979). Toxicity of inhaled chloroform in pregnant mice and their offspring.*

Groups of 34-40 timed-mated CF-1 mice were exposed to 0 or 100 ppm chloroform by inhalation for seven hours per day on each of gestation days 6-15, 1-7, or 8-15. Chamber-exposed controls were run for each group. Dams were observed daily, and body weights, as well as food and water consumption were recorded at 1-3 day intervals. Livers of all dams were weighed at sacrifice on gestation day 18. Uteri were excised for examination of their contents.

One of the dams exposed to 100 ppm chloroform on gestation days 5-16 died on gestation day 18. The animal had not consumed food or water for several days before death, and was found to have gastric ulceration at necropsy. No clinical signs were reported for mice in other treated groups.

Data on feed and water consumption are not presented, but the text notes that feed and water consumption were reduced slightly in all treated animals of all groups relative to controls. Maternal body weight data are not presented in tabular form, but the text indicates that all chloroform-treated groups showed some reduction in body weight gain, with this change reaching statistical significance in animals treated on gestation days 1-7 or 8-15 (but not 6-15).

Absolute and relative liver weights of maternal animals were significantly ($p < 0.05$) increased over controls for animals exposed to 100 ppm chloroform on gestation days 6-16 or 8-15, but not on days 1-7. As a measure of hepatic toxicity, serum SGPT activity was assayed in additional bred mice on gestation day 16, following chloroform exposure on gestation days 6-15. Chloroform-exposed mice showed a significant ($p < 0.05$) increase in SGPT activity over controls, with activity in exposed non-pregnant animals

significantly greater ($p < 0.05$) than that of exposed pregnant animals at the end of the treatment period.

The percentage of dams in each control group that were pregnant at term ranged from 62-85%. For animals exposed to 100 ppm chloroform on either gestation days 1-7 or days 6-15, pregnancy rates were significantly decreased compared to corresponding controls ($p < 0.05$). For animals exposed on gestation days 8-15, the chloroform-treated group had a pregnancy rate of 45%, which was not a significant difference from the corresponding controls. The same pattern of results was observed for total pregnancies (those evident at term plus those detectable only by uterine staining).

Chloroform exposure did not affect the mean number of implantation sites or live fetuses per litter (Table 21). Resorption frequency was significantly ($p < 0.05$) affected only among the mice exposed to chloroform on gestation days 1-7; the effect was attributed primarily to two completely resorbed litters. As compared to corresponding controls, mean fetal body weight and CRL were reduced in all treated groups, reaching statistical significance ($p < 0.05$) for animals exposed on gestation days 1-7 or 8-15 (but not 6-15).

**Table 21. Fetal data from mice exposed to chloroform by inhalation (litter mean ± SD) (Murray et al., 1979).**

| Parameters | GD 1-7 0 ppm | GD 1-7 100 ppm | GD 6-15 0 ppm | GD 6-15 100 ppm | GD 8-15 0 ppm | GD 8-15 100 ppm |
|---|---|---|---|---|---|---|
| % pregnant[1] | 74% | 44% | 91% | 43% | 65% | 60% |
| No. litters | 22 | 11 | 29 | 12 | 24 | 18 |
| Fetuses | 10 ± 3 | 13 ± 2 | 12 ± 3 | 10 ± 4 | 12 ± 3 | 11 ± 3 |
| Resorptions | 2 ± 2 | 4 ± 5* | 2 ± 2 | 1 ± 1 | 2 ± 2 | 2 ± 2 |
| Fetal weight (g) | 1.02 ± 0.10 | 0.92 ± 0.07* | 0.99 ± 0.11 | 0.95 ± 0.13 | 1.00 ± 0.12 | 0.85 ± 0.17* |
| CRL (mm) | 24.7 ± 1.0 | 23.6 ± 1.2* | 23.7 ± 1.3 | 23.2 ± 1.1 | 24.1 ± 1.1 | 22.9 ± 2.2* |

[1] Includes females with implantation sites visible only after staining with sodium sulfide.
* Statistically significant difference from corresponding controls at $p < 0.05$.

Cleft palate was observed in three fetuses from one litter in the control group for treatment days 1-7, in one fetus from the control group for treatment days 8-15, and in 10 fetuses from four litters in the group exposed to 100 ppm chloroform on gestation days 8-15 (a significant difference from corresponding controls at $p < 0.05$). The authors note that cleft palate was seen primarily in fetuses of low weight, though it is not clear whether cleft palate showed a stronger correlation with fetal weight than with exposure.

No other external malformations were observed for animals exposed to 100 ppm chloroform on gestation days 8-15. No external malformations were observed in animals exposed to 100 ppm chloroform on gestation days 6-15. Single occurrences of exencephaly, multiple defects, inward rotation of hindlimb, and omphalocele were observed sporadically among control and exposed fetuses.

No statistically significant effects were noted among treatment and control groups in the frequencies of internal malformations. Single incidents of missing testicle were reported for the treated groups exposed on gestation days 1-7 or 8-15, but not 6-15. Missing or hypoplastic cerebellum or cerebrum were reported for single fetuses in the exposed and control groups for gestation days 6-15, in two fetuses in one litter of the control group for treatment days 8-15, and in three fetuses in two litters of the group exposed to chloroform on gestation days 8-15.

The only skeletal abnormalities noted were two cases in two litters of misshapen vertebrae among control fetuses for treatment days 6-15. Data on skeletal variations are not provided in tabular form, but the text notes that delayed ossification of skull bones was significantly increased among all chloroform-exposed groups relative to their corresponding controls. Delayed ossification of sternebrae is also stated to have occurred significantly more often among fetuses exposed to chloroform on gestation days 1-7 or 8-15, but not days 6-15.

### C.2.3. Studies in rabbits

*Thompson et al. (1974). Teratology studies on orally administered chloroform in the rat and rabbit.*

In a range-finding study, groups of five timed-mated Dutch-Belted rabbits were given chloroform in corn oil by gavage at total daily doses of 0, 25, 63, 100, 159, 251, or 398 mg/kg-day on each of gestation days 6-18. Doses were split into two treatments each day.

Three of the five dams given chloroform at 100 mg/kg-day died or were found moribund; all animals in the higher dose groups died. Severe acute hepatitis and nephrosis were found upon necropsy. Anorexia, diarrhea, weight loss, abortion, and one death were observed with 63 mg/kg-day chloroform. At 25 mg/kg-day, the only observed, overt signs of toxicity were mild diarrhea and intermittent anorexia. Among surviving animals at all doses, histopathology revealed mild fatty changes in the liver and kidneys of one female at 100 mg/kg-day.

Of the two dams surviving 100 mg chloroform/kg-day, one was not pregnant, and the other had four resorption sites but no viable conceptuses. Two of four surviving dams at 63 mg/kg-day each had six live fetuses. All five dams at 25 mg/kg-day had live fetuses, with an average of seven per litter. No gross abnormalities were found among fetuses of exposed litters.

In a full-scale teratology study, groups of 15 timed-mated Dutch-Belted rabbits were given chloroform in corn oil by gavage at total daily doses of 0, 20, 35, or 50 mg/kg-day on each of gestation days 6-18. Doses were given once each day, rather than being split into two doses as was done for the range-finding study. On gestation day 29, fetuses

were surgically removed from their dams and incubated for 24 hours to determine viability. At the end of this time, all were preserved in ethanol, examined for visceral anomalies, and then cleared and stained for skeletal examination.

Seven dams died during the course of the study: two controls, one at 20 mg/kg-day, and four at 50 mg/kg-day. Deaths in the high-dose group were attributed to hepatotoxicity. Complete abortions were seen in all groups: three in the control group, two at 20 mg/kg-day chloroform, one at 35 mg/kg-day, and four at 50 mg/kg-day. During the treatment period, some rabbits from all groups were considered to have been anorectic and had mild to severe diarrhea. Body weight gains are stated to have been significantly decreased in dams of the 50 mg/kg-day group; data and significance levels are not presented in the paper. Necropsy of dams on day 29 did not reveal treatment-related changes in the liver, kidneys, or other tissues.

There were no significant differences among groups in the numbers of aborted litters, implantation frequency, numbers of corpora lutea, mean live litter size, or sex ratio (Table 22). The 24-hour viability rate was not affected. Mean fetal weights were significantly ($p < 0.05$) lower than control values for the 20 and 50 mg/kg-day groups, but not the 35 mg/kg-day group.

Visceral examination of fetuses revealed one incident of "aortic hypertrophy, pulmonary trunk hypoplasia, single ventricle," and one incident of "aortic hypertrophy, pulmonary trunk hypoplasia, high ventricular septal defect." Both of these fetuses were in the 20 mg/kg-day chloroform group. No other visceral malformations were noted. Sporadic incidents of bilateral extra lumbar ribs and unossified or fused sternebrae were seen in all dose groups. Absent interparietal bone was noted for one control fetus, and shortened nasal bones for one fetus from the 20 mg/kg-day group; one case of cleft palate, hydrocephalus, and arthrogryposis was noted among control fetuses. Incomplete ossification of skull bones was observed in all groups, with fetal incidences reaching statistical significance ($p < 0.05$) at 20 and 35 mg/kg-day chloroform; the incidence of litters with at least one affected fetus did not differ among groups.

**Table 22. Litter data from rabbits treated orally with chloroform (litter mean ± SD) (Thompson et al., 1974).**

| Dose (mg/kg-day) | Insemination rate | Implantations | Resorptions | Live Fetuses | Fetal Weight (g) |
|---|---|---|---|---|---|
| 0 | 9/15 | 6.6 ± 2.2 | 0.1 ± 0.3 | 6.4 ± 2.2 | 34.3 ± 5.8 |
| 20 | 12/15 | 6.3 ± 2.3 | 0.7 ± 1.2 | 5.6 ± 3.0 | 31.7 ± 5.6* |
| 35 | 11/15 | 5.6 ± 2.5 | 1.1 ± 1.8 | 4.5 ± 2.9 | 32.4 ± 5.8 |
| 50 | 7/15 | 8.4 ± 1.8 | 1.0 ± 1.7 | 7.4 ± 2.2 | 30.3 ± 4.6* |

* statistically significant difference from controls at $p < 0.05$

### C.2.4. Developmental neurotoxicity studies

*Burkhalter and Balster (1979). Behavioral teratology evaluation of trichloromethane in mice*

Male and female albino ICR mice were given 31.1 mg/kg-day chloroform (trichloromethane) by gavage three weeks before being co-housed for mating. The vehicle used was a solution of one part "Emulphor" (a polyoxyethylated vegetable oil) and eight parts saline (0.9%). Treatment continued through the mating period for males, and throughout mating, gestation, and lactation for females. Five treated and five vehicle-control litters were used for the study; litters were culled to no more than eight pups by random selection on the day of birth. On postnatal day seven, and for the remainder of the study, all pups were given either 31.1 mg/kg-day chloroform, or the vehicle, by gavage (presumably according to their prenatal treatment).

Three pups from each of 10 litters were randomly selected for each day of testing. The order of testing litters was initially determined on a random basis, but subsequently was reversed on alternating days. Each pup was tested on the complete battery before the next pup was begun. The battery of tests included: righting reflex, forelimb placing response, forepaw grasp, rooting reflex, cliff drop aversion, auditory startle response, bar-holding ability, and eye opening. Motor performance was tested in 15 mice randomly selected from both groups on postnatal day 17. On days 22 and 23, 15 mice randomly selected from both groups were tested for passive avoidance learning.

Mean litter size did not differ between groups, nor did mean pup body weights (taken daily on postnatal days 7-21). Weight gain over days 7-21 was significantly lower in chloroform-exposed animals ($p < 0.01$).

Righting reflex, forelimb placing response, forepaw grasp, cliff drop aversion, auditory startle response, bar-holding ability, and eye opening all showed progressive increases in scale scores over the days of testing. Rooting reflex increased up to about days 8-10, and then was lost by day 14. While there were scattered significant differences between the chloroform and control groups on specific days, chloroform showed no overall tendency to retard neurobehavioral development of mouse pups. The one exception was forelimb placement, for which the chloroform group had lower scores on each of days 5-8, with significant differences ($p < 0.05$) on days 5 and 7.

The inverted-screen climbing test of motor performance showed no significant difference between groups. In the test of passive avoidance, all animals learned the task as demonstrated by increased latency in the second and third trials ($p < 0.05$). There were no differences between chloroform -treated animals and the control group for latencies across the three trial, nor did the groups differ with respect to the effects of shock.

## C.3. Additional Relevant Information

C.3.1. *In vitro* assays

*Brown-Woodman et al. (1998). In vitro assessment of the effect of halogenated hydrocarbons: chloroform, dichloromethane, and dibromoethane on embryonic development of the rat.*

Explanted whole rat embryos were cultured in rat serum for a total of 40 hours starting on gestation day 10.5 (Brown-Woodman et al., 1998). Embryonic growth and development were assessed following culture in the presence or absence of one of several halogenated hydrocarbons, including chloroform. There were 10-14 embryos in each treatment group.

For treated cultures, 0.125-1.00 μl chloroform per ml culture media was added at the beginning of the culture period. To account for loss of volatile solvents from the media during the procedures, actual concentrations of solvents were determined at the end of the culture period.

At the end of the culture period, embryonic viability was determined by the presence or absence of a beating heart and functional yolk-sac circulation. Crown-rump length and somite count were determined for viable embryos, and flexure and morphology were evaluated. At least two embryos per bottle (of four to five embryos) were randomly selected for total protein assay. Remaining embryos were fixed in gluteraldehyde, and a series of morphometric measurements were taken on their head regions.

Final concentrations of chloroform in treated culture media ranged from 0.043 μl/ml (0.53 μmol/ml) to 0.299 μl/ml (3.71 μmol/ml). Concentrations at or below 0.084 μl/ml (1.05 μmol/ml) had no significant effects on embryonic viability or growth. A chloroform concentration of 0.166 μl/ml (2.06 μmol/ml) did not affect viability, but caused significant reductions ($p < 0.01$) in crown-rump length, somite number, and protein content, in comparison to control embryos. Of 10 embryos exposed to the highest chloroform concentration of 0.299 μl/ml (3.71 μmol/ml), 10 had beating hearts, none had well-developed yolk-sac blood vessels, and four had attained full dorsally-convex flexure. Crown-rump length, somite number and protein content were all lower than those of the 0.166 μl/ml group, and significantly ($p < 0.01$) lower than those of control embryos.

In an additional experiment, the development of embryos exposed to 3.05 μmols/ml chloroform was followed at 1, 2, 4, 8, 16, 32, and 40 hours of culture time. By four hours of culture, the lack of developing yolk-sac vessels was an obvious difference from controls. By eight hours, significant ($p<0.05$) retardation of growth and development was apparent. All embryos had beating hearts at the end of the culture period. At the histological level, cell death was apparent in the neural tube after 16 hours; by 32 hours, the wall of the neural tube was disintegrating. Cell death became apparent throughout the

embryo continuing to become more widespread by 40 hours. Only heart cells appeared to be unaffected. Neural tube closure did occur, but the optic vesicle did not develop into the optic cup and lens.

The authors compared an embryotoxic level of chloroform exposure (2.06 μmol/ml) with reported blood levels for exposed humans: 0.034 μmol/ml, for controlled exposure until unconsciousness was about to occur; 1.01 μmol/ml, for an accidental death; and levels as high as 1.94 μmol/ml for deep, surgical anesthesia.

*Kitchin and Ebron (1984). Combined use of a water-insoluble chemical delivery system and a metabolic activation system in whole embryo culture.*

Explanted whole rat embryos were cultured for a total of 48 hours starting on gestation day 10.5. Chloroform dissolved in corn oil was added to the culture media of experimental embryos at concentrations of 0.5% or 2.5%. There were two control groups: untreated, and 2.5% corn oil. Each group consisted of six embryos.

At the end of the culture period, explants without a beating heart or functional yolk-sac circulation were classified as dead, and excluded from morphological scoring. Living explants were evaluated for criteria including: yolk-sac diameter, crown-rump length, somite number, embryonic flexion and morphology of embryonic structures. Morphogenesis was scored as normal or abnormal. Some embryos were used for quantification of DNA and total protein.

Untreated and corn oil controls did not differ for any of the parameters for which data are presented (DNA, somite number, dead embryos, or abnormal embryos). No dead embryos were identified in either control group. All embryos (6/6) died after exposure to 2.5% chloroform in culture media. Four out of six embryos died after exposure to 0.5% chloroform in culture media. Among surviving embryos in the 0.5% chloroform group, somite number and DNA content were significantly reduced relative to corn oil controls ($p < 0.05$, and $p < 0.01$, respectively).

## C.4. Integrative Evaluation for Developmental Toxicity

### C.4.1. Human data

A single study was identified that examined chloroform exposure in an occupational setting in association with pregnancy outcomes (Wennborg et al., 2000). This study reported a marginal association between women working with chloroform in laboratories during the time before conception and the occurrence of spontaneous abortions. Limitations of this study include the lack of exposure measurements, the possible exposure to other laboratory solvents, the long time between pregnancies and the administration of the questionnaire, and a moderately low response rate (73%).

However, the responders and non-responders were similar with respect to women's health diseases, gynecological history and socioeconomic factors thereby reducing the risk of selection bias. The number of women in the high exposure group was small, which could have limited the power to detect an effect.

Seven epidemiologic studies were presented which examined whether chloroform in drinking water was associated with various adverse pregnancy outcomes. Six of the studies (Dodds et al., 2004; King et al., 2000; Kramer et al., 1992; Infante-Rivard, 2004; Waller et al., 1998; Wright et al., 2004) examined pregnancy outcomes such as SAB and size at birth and are summarized in Table 23. One of these studies, the prospective study by Waller et al. (1998), found a significant association of SAB with TTHM and BDCM but not with chloroform. The remaining study (Dodds and King, 2001) examined birth defects such as neural tube defects, major cardiac defects, cleft defects, and chromosomal abnormalities.

Three studies examined fetal growth (Kramer et al., 1992; Infante-Rivard, 2004; Wright et al., 2004). Fetal growth retardation was defined as: lowest 10th percentile of BW for each week of gestational age, stratified by infant sex and maternal race (Wright et al., 2004); less than the $10^{th}$ percentile for gestational age and sex (Infante-Rivard, 2004); and less than the $5^{th}$ percentile for the particular gestational age (Kramer et al., 1992). Two of these studies, a case control study and a large retrospective cohort study, reported an increased risk of IUGR with exposure to chloroform (Kramer et al., 1992; Wright et al., 2004). The third study, a case control study (Infante-Rivard, 2004), found no effect of exposure to chloroform or TTHMs. However, following inclusion of information on genetic polymorphisms for CYP2E1 genes, exposure to TTHMs was associated with an increased risk of IUGR. The risk associated with chloroform was elevated with the inclusion of information on gene type, but not significantly so (OR = 5.6, 95% CI, 0.82 – 38.39).

Important differences among these three studies of fetal growth may explain some of the inconsistency in results. The studies differed greatly in size and in level of chloroform exposure. The retrospective cohort study by Wright et al. (2004) had the highest chloroform levels and the largest exposed population. This study observed significant adverse effects, including decreased mean BW and increased risk of IUGR, as well as evidence for a monotonic increase in risk of the latter at exposures greater than 20 μg/L. One of the two case-control studies reported a significant increase in risk of IUGR (Kramer et al., 1992). Although the Infante-Rivard study did include more case subjects, the exposure levels may have been much lower (Table 24). Not only were the measured levels in the Kramer study higher than those in the Infante-Rivard study, the exposure measurements for this study were taken two years earlier than the outcome measures, during a drought year when the amount of humic material available for the residual chlorine to react with would be lower (Kramer et al., 1992). It is, therefore, probable that the actual levels of exposure were much higher than those reported. .

**Table 23. Odds Ratios for studies of chloroform, BDCM, and TTHM exposure in drinking water in association with certain pregnancy outcomes**

| Study | Odds ratio (95% CI) | | | | | | Water Concentrations (µg/L) | |
|---|---|---|---|---|---|---|---|---|
| | SAB [a] | Stillbirth | PTD [b] | SGA [c] | LBW [d] | BW(g) [e] | Exposure Level | Reference Level |
| Kramer et al., 1992 Iowa | | | 1.1 (0.7-1.6) | 1.8 (1.1-2.9) | 1.3 (0.8-2.2) | | >10 Chloroform | ND |
| | | | 1.0 (0.6-1.5) | 1.7 (0.9-2.9) | 1.0 (0.7-1.5) | | >10 BDCM | ND |
| Waller et al., 1998 California | 0.6 (0.3-1.2) | | | | | | ≥17 Chloroform+5 glasses/d | <17+<5 glasses/d |
| | 2.0 (1.2-3.5) | | | | | | ≥18 BDCM+5 glasses/d | <18+<5 glasses/d |
| King et al., 2000 Nova Scotia | | 1.6 (1.04-2.34) | | | | | ≥100 Chloroform | <50 |
| Dodds et al., 2004 Nova Scotia and Ontario | | 1.8 (1.1-3.0) | | | | | 1-49 Chloroform | 0 |
| | | 0.9 (0.5-1.9) | | | | | 50-79 | 0 |
| | | 2.2 (1.0-4.8) | | | | | >80 | 0 |
| Wright et al., 2004 Massachusetts | | | 0.95 (0.91-0.99) | 1.05 (1.02-1.09) | | -14 (-19 to -9) | >26-63 Chloroform | 0-26 |
| | | | 0.90 (0.84-0.97) | 1.11 (1.04-1.17) | | -18 (-26 to -10) | >63-135 Chloroform | 0-26 |
| Infante-Rivard, 2004 Montréal | | | | 1.06 (0.63-1.79) | | | >23.7 Chloroform | ≤23.7 |
| | | | | 5.62 (0.82-38.39) [f] | | | >23.7 Chloroform | ≤23.7 |
| | | | | 0.97 (0.57-1.62) | | | >29.4 TTHM | ≤29.4 |
| | | | | 13.20 (1.19-146.72) [f] | | | >29.4 TTHM | ≤29.4 |

[a] Spontaneous abortion
[b] Pre-term delivery
[c] Small for gestational age
[d] Low birth weight
[e] Birth weight
[f] odds ratio for newborns or mothers carrying one or two variant alleles for CYP2E1*5 (G1259C), compared to none.

**Table 24. Chloroform water concentrations for seven epidemiologic studies of adverse developmental outcomes.**

| Study | Chloroform Level (μg/L) | | | | |
|---|---|---|---|---|---|
| | Mean (SD) | Median | Range | 90th Percentile | Highest level used for analysis |
| Kramer et al. 1992 | 12.5 (38.7) | 1 | 0-350 | | >10 |
| Waller et al. 1998 | | | | | ≥17 |
| King et al. 2000 | 64.1 | | | | ≥100 |
| Dodds and King, 2001 | 64 | | | | ≥100 |
| Dodds et al. 2004 | | | 0-315 | | >80 |
| Wright et al. 2004 | 31.0 (23.6) | 25 | 0-135 | 63 | >63 |
| Infante-Rivard 2004 | 11.84 (18.84)[a]<br>11.58 (16.31)[b] | | | 23.7 | >23.7 |

[a]Cases
[b]Controls

Two studies examining stillbirths, a population-based case control study (Dodds et al., 2004) and a retrospective cohort study (King et al., 2000) both found an increased risk of stillbirth with exposure to chloroform. King et al. (2000) observed a stronger relationship between THM factors, including chloroform, and asphyxia-related deaths than unexplained deaths. Both this study and the study by Dodds et al. (2004), were well-conducted, and controlled for known risk factors of stillbirth. The latter study is one of very few studies to have collected residential tap water samples and to have incorporated information on exposure from showering and bathing. In a study of birth defects, Dodds and King (2001) did not observe an increase risk with exposure to chloroform. They did observe an increased risk of chromosomal abnormalities associated with exposure to chloroform (RR = 1.9, 95% CI, 1.1-3.3). Exposure to BDCM was associated with an increased risk of neural tube defects (RR = 2.5, 95% CI, 1.2-5.1).

Overall the epidemiological studies of chloroform in drinking water suggest an association of chloroform exposure with reduced fetal growth, as well as stillbirth, but not spontaneous abortion or birth defects.

A major limitation of many epidemiologic studies of chloroform has been the use of water concentration as the measure of exposure, which can lead to significant exposure

misclassification. Yet some studies which have collected more extensive exposure assessment information, including exposures from showering and swimming, have not observed significant changes in effect estimates when this information has been incorporated into the analysis (Infante-Rivard, 2004; Waller et al., 1998). These additional estimates of exposure have a number of assumptions associated with them, such as shower flow, inhalation rate, temperature and dermal absorption. It has been postulated that significant effects of exposure on pregnancy outcomes may be obscured by multiple sources of potential misclassification (Swan and Waller, 1998). In many instances the measure of exposure has relied upon routinely collected drinking water measurements by water utility companies. The levels of chloroform and other chlorination by-products in tap water in individual homes may differ from the utility company measurements due to such factors as: concentration of residual chlorine; distance from the utility, i.e. transit time with the system; temperature; pH; and total or organic carbon. Thus, possible variation in actual exposure of the subjects and the estimated exposure using utility company measurements can be large. The result of this variation would most probably be, in most of these studies, a non-differential underestimation of exposure and thus a resulting bias towards the null, (i.e. not detecting an effect that may be present).

Another issue to consider in reaching conclusions from the chloroform drinking water studies is the co-occurrence of chloroform and other disinfection by-products. Studies examining adverse reproductive or development outcomes in association with exposure to TTHMs in tap water have reported significant associations of these exposures with congenital malformations, SAB, stillbirth, small for gestational age, low birth weight and decreased birth weight. Although in some studies the effects have been marginally statistically significant, important issues such as exposure assessment and sample size must be taken in consideration.

There has been limited research investigating possible mechanisms by which chloroform and other disinfection by-products may induce developmental toxicity. Chen et al. (1996), have suggested that THM (mostly chloroform) may contribute to an increase in neural tube defects through the inhibition of the use of folate in the conversion of homocysteine to methionine. One study to date has examined MTHFR in this context did not detect an indication that this enzyme modifies the effect of chloroform on fetal growth (Infante-Rivard, 2004). This study also examined the influence of gene variants of CYP2E1 and did observe an interaction with chloroform exposure and the outcome small for gestational age.

It has also been suggested, however, that THMs are actually a surrogate for other chemicals since there are numerous substances comprising DBPs. Specific to neural tube development, evidence of changes has been seen in mouse embryos when exposed to haloacetic acids, a DBP (Hunter et al., 1996 as cited in Nieuwenhuijsen et al., 2000).

### C.4.2. Animal data

Available data on the potential developmental toxicity of chloroform include developmental toxicity studies in the rat, performed by both the inhalation and oral routes (Schwetz et al., 1974; Dilley et al., 1977; Baeder and Hoffman, 1988; Baeder and Hoffman, 1991; Thompson et al., 1974; Ruddick et al., 1983). Developmental toxicity data from species other than the rat are represented by one study in the mouse using the inhalation route of exposure (Murray et al., 1979), and one study in the rabbit using the oral route of exposure (Thompson et al., 1974). One study was identified that evaluated the potential of chloroform to cause developmental neurotoxicity (Burkhalter and Balster, 1979). Additional relevant information is provided by *in vitro* studies employing whole-embryo culture (Brown-Woodman et al., 1998; Kitchen and Ebron, 1984).

Findings for rats exposed during gestation to chloroform by inhalation included effects on pregnancy rate, resorption frequency, fetal weight and CRL, as well as some evidence for increases in skeletal anomalies and variations (Schwetz et al., 1974; Dilley et al., 1977; Baeder and Hoffman, 1988; Baeder and Hoffman, 1991). Both Schwetz et al. (1974) and Baeder and Hoffman (1988) used a high concentration of 300 ppm chloroform. At this concentration in both studies, the numbers of live litters produced following successful matings were sharply reduced. Resorption frequency was significantly increased in live litters at 300 ppm in the Schwetz et al. (1974) study, with a concomitant significant decrease in live litter size; these specific changes were not found in the Baeder and Hoffman (1988) study. However, the Baeder and Hoffman (1988) study showed an apparent concentration-related increase in completely resorbed litters at all test concentrations (30, 100, 300 ppm). Dilley et al. (1977) reported their data only in abstract form, but described increased fetal mortality after inhalation exposure to 20.1 $\pm$ 1.2 g chloroform/$m^3$ (approximately 4100 ppm).

Fetal weight and fetal CRL were also significantly reduced at 300 ppm chloroform in both the Schwetz et al. (1974) and Baeder and Hoffman (1988) studies. CRL was also significantly reduced at 30 ppm in both of these studies, and at 100 ppm in the Baeder and Hoffman (1988) study. In a second Baeder and Hoffman study (1991), 30 ppm was the highest chloroform concentration used, and both fetal weight and CRL were significantly decreased at that level. Dilley et al. (1977) reported their data only in abstract form, but described reduced fetal weight gain after inhalation exposure to 20.1 $\pm$ 1.2 g chloroform/$m^3$ (approximately 4100 ppm).

Soft tissue and skeletal anomalies were observed in 100% of the three surviving litters at 300 ppm chloroform in the Schwetz et al. (1974) study, but only the 30 ppm group showed a statistically significant increase in the frequency of skeletal anomalies. Baeder and Hoffman (1988) reported only sporadic incidents of soft tissue or skeletal anomalies, but their 1991 study reported significant increases in the frequency of various skeletal anomalies at all concentrations tested (3, 10, and 30 ppm).

Schwetz et al. (1974), Baeder and Hoffman (1988), and Baeder and Hoffman (1991) all describe adverse effects on maternal animals at concentrations that were also associated

with fetal effects. Specifically, reductions in feed consumption and/or body weight were seen for at least some timepoints at all concentrations tested in all three studies, with the exception of 3 ppm in the Baeder and Hoffman (1991) study. Liver toxicity in maternal animals was evidenced in the Schwetz et al. (1974) study by a significant increase in liver weight at 300 ppm and decreased liver weight relative to body weight in both the 100 and 300 ppm groups. As a means of evaluating liver function, Schwetz et al. (1974) measured SGPT, and did not find significant differences among groups. As noted in Section B.4., the chronic oral Reference Dose (RfD) for non-cancer effects of chloroform developed by U.S. EPA (U.S. EPA, 2001b) was based on increased fatty cyst formation in the liver and elevated serum glutamate-pyruvate transaminase (SGPT) in beagle dogs. The Dilley et al. (1977) abstract did not discuss maternal endpoints.

Schwetz et al. (1974) also investigated the role of maternal influences as a determinant of fetal effects in the developmental toxicity of chloroform by including a "starved" control group, which was feed restricted in an attempt to mimic the anorexic effects seen with 300 ppm chloroform. This group was restricted to 3.7 g of feed on the days of treatment, which can be compared to the 19 g/day consumed by ad lib controls over gd 6-7, the 5 g/day consumed by the 30 ppm group, the 13 g/day consumed by the 100 ppm group, and the 1 g/day consumed by the 300 ppm group over these same days. Starved controls had significantly lower body weights than ad lib controls on gd 13 and 21, as well as significantly reduced feed intake even on gd 18-19, after they had been returned to ad lib feeding. While decreases in fetal body measurements were observed in this group, starvation alone had no effect on pregnancy rate, live litter size, or resorption frequency.

Developmental toxicity studies performed in rats using the oral route of exposure (Thompson et al., 1974; Ruddick et al., 1983), had results similar to the inhalation studies discussed above. Decreased fetal weights were seen in each reported experiment, at 400 mg/kg-day (Ruddick et al., 1983), at 316 mg/kg-day (Thompson et al., 1974), and at 126 mg/kg-day (Thompson et al., 1974). Additional fetal findings included increased implantations at 126 mg/kg-day (Thompson et al., 1974), increased resorptions and decreased litter size at 316 mg/kg-day (Thompson et al., 1974), increased fetal runting and aberrant sternebrae at 400 mg/kg-day (Ruddick et al., 1983), and complete abortion of litters at 501 mg/kg-day (Thompson et al., 1974). Maternal effects observed in all three experiments at developmentally toxic or lower doses included decreased feed consumption and weight gain. Fatty changes in maternal livers were observed at 50 and 126 mg/kg-day (Thompson et al., 1974), and increased liver weight, as well as hemoglobin, hematocrit, and red blood cell counts were seen at 100, 200, and 400 mg/kg-day (Ruddick et al., 1983). Increased maternal kidney weights were seen in this study at 400 mg/kg-day.

Studies performed with species other than the rat consist of an inhalation study performed in mice (Murray et al., 1979), and an oral study performed in rabbits, with data from both pilot and main experiments (Thompson et al., 1974). In the mouse study (Murray et al., 1979), only a single concentration of chloroform, 100 ppm, was used. Different treatment days were compared for effects observed on developing and maternal animals. Developmental effects were seen with chloroform exposure on any of the treatment days

tested: gd 1-7, 6-15, or 8-15. Although effects differed somewhat among the specific days of exposure, the overall findings were similar to those made following exposure of developing rats by the oral or inhalation routes: decreased pregnancy rate, increased resorption frequency, decreased fetal body weight and CRL, and an increased frequency of retarded ossification of sternebrae. The one unique finding was an increased frequency of cleft palate following chloroform exposure over gds 8-15. Maternal animals showed effects on weight gain with treatment on gds 1-7 or 8-15 (but not 6-15), and increases in absolute and relative liver weight with treatment on gds 6-15 or 8-15 (but not 1-7).

With oral dosing in the rabbit, reduced fetal viability was seen in a dose range-finding study at chloroform doses of 63, 100, 159, 251, or 398 mg/kg-day (Thompson et al., 1974). Observations of maternal toxicity in the pilot experiment ranged from "mild" diarrhea and anorexia at 25 mg/kg-day, to more notable weight loss at 63 mg/kg-day, to excess maternal mortality at doses of 100 mg/kg-day and above. In the main study, complete abortion of litters was observed at all test doses: 20, 35, and 50 mg/kg-day. Fetal weights were significantly decreased at 20 and 50, but not 35, mg/kg-day. Four out of 15 pregnant dams (27%) died in the 50 mg/kg-day group; these deaths were attributed to hepatotoxicity. Body weight gains were depressed in surviving dams at this dose.

Only one study of the potential developmental neurotoxicity of chloroform was identified in the literature (Burkhalter and Balster, 1979). Postnatal weight gain was significantly reduced in mice exposed to chloroform by gavage at a dose of 31.1 mg/kg-day. No consistent, chloroform-related effects were demonstrated in tests of developmental stage, motor performance, or passive avoidance learning. The one exception was forelimb placement, for which chloroform-exposed animals had consistently lower scores as compared to controls.

Explanted rat embryos grown *in vitro* in the presence of chloroform added to culture media showed evidence of adverse effects at concentrations of 2.06 μmol/ml or higher (Brown-Woodman et al., 1998), or at 0.5% or 2.5% (Kitchin and Ebron, 1984). Effects included reduced viability, decreased CRL, decreased somite number, and decreased protein and DNA contents.

**Table 25. Developmental toxicity of chloroform by inhalation in the rat**

| Reference | Study design | Maternal effects | Developmental Effects |
|---|---|---|---|
| Schwetz et al., 1974 | Sprague-Dawley rats<br>0, 30, 100, 300 ppm plus feed-restricted control; 7 hr/day; gd 6-16<br>8-77 females/group | Reduced feed consumption 100 & 300 ppm; only on gd 6-7 for 30 ppm<br>Reduced bw on gd 13 at 30, 100, & 300 ppm; on gd 21 at 300 ppm<br>Absolute liver weight increased 300 ppm<br>Relative liver weight decreased 100 & 300 ppm | Reduced pregnancy rate at 300 ppm<br>Decreased litter size, increased resorptions 300 ppm<br>Altered sex ratio 300 ppm<br>Decreased fetal weight & CRL 300 ppm<br>Increased gross anomalies 100 ppm<br>Increased skeletal anomalies 30 ppm |
| Dilley et al., 1977 (abstract) | Rats<br>0, 20.1 $\pm$ 1.2 g/m$^3$ ($\simeq$ 4100 ppm) and 2 lower conc, gd 7-14 | Not discussed | Increased fetal mortality and decreased fetal weight |
| Baeder & Hoffman, 1988 | Wistar rats<br>0, 30, 100, 300 ppm; 7 hr/day; gd 7-16<br>20 females/group | Reduced feed consumption at all concentrations<br>Higher bw on gd 0, all groups<br>Reduced bw gd 17 all concentrations<br>Reduced bw gd 21, 100 & 300 ppm | Increase in completely resorbed litters at all concentrations<br>Decreased fetal weight, 300 ppm<br>Decreased CRL, all concentrations |
| Baeder & Hoffman, 1991 | Wistar rats<br>0, 3, 10, 30 ppm; 7 hr/day; gd 7-16<br>20 females/group | Reduced feed consumption all concentrations gd 7-14<br>Reduced feed consumption at all times, 30 ppm<br>Apparent reduced bw & wt gain for 10, 30 ppm (no stats) | Decreased fetal weight & CRL at 30 ppm<br>Increased ossification variations at all concentrations |

abbreviations: bw = body weight, gd = gestation day, CRL = crown-rump length

## Table 26. Developmental toxicity of oral chloroform in the rat

| Reference | Study design | Maternal effects | Developmental Effects |
|---|---|---|---|
| Thompson et al., 1974 | Sprague-Dawley rats<br>0, 79, 126, 300, 316, 501 mg/kg-day by gavage; gd 6-15<br>6 females/group | Decreased feed consumption and weight gain at doses ≥ 126 mg/kg-day<br>Maternal deaths at 316 & 501 mg/kg-day | Increased resorptions at 316 mg/kg-day<br>Decreased litter size and fetal weights at 316 mg/kg-day<br>No live fetuses at 501 mg/kg-day |
| | Sprague-Dawley rats<br>0, 20, 50, 126 mg/kg-day by gavage; gd 6-15<br>25 females/group | Clinical symptoms at 126 mg/kg-day<br>Decreased feed consumption at 50 mg/kg-day<br>Decreased weight gain at 50 & 126 mg/kg-day<br>Fatty changes in livers at 50 & 126 mg/kg-day | Increased implantations at 126 mg/kg-day<br>Decreased fetal weights at 126 mg/kg-day |
| Ruddick et al., 1983 | Sprague-Dawley rats<br>0, 100, 200, 400 mg/kg-day by gavage; gd 6-15<br>15 females/group | Decreased weight gain all doses<br>Increased liver weight all doses<br>Increased kidney weight 400 mg/kg-day<br>Decreased hemoglobin & hematocrit all doses<br>Decreased red blood cell counts all doses | Decreased fetal weight 400 mg/kg-day<br>Apparent increases in aberrant sternebrae and in runting 400 mg/kg-day |

abbreviations: gd = gestation day

## Table 27. Developmental toxicity of chloroform by inhalation in the mouse

| Reference | Study design | Maternal effects | Developmental Effects |
|---|---|---|---|
| Murray et al., 1979 | CF-1 mice<br>0, 100 ppm; 7 hrs/day, gd 6-15, 1-7, or 8-15<br>34-40 females/group | Decreased weight gain, gd 1-7 or 8-15<br>Increased absolute & relative liver weights, gd 6-15 or 8-15 | Decreased pregnancy rate, gd 1-7 or 6-15<br>Increased resorptions, gd 1-7<br>Decreased fetal bw & CRL, gd 1-7 or 8-15<br>Increased cleft palate, gd 8-15<br>Increased retarded ossification of sternebrae, 1-7 or 8-15 |

abbreviations: bw = body weight, gd = gestation day

**Table 28. Developmental toxicity of oral chloroform in the rabbit**

| Reference | Study design | Maternal effects | Developmental Effects |
|---|---|---|---|
| Thompson et al., 1974 | Dutch-belted rabbits<br>0, 25, 63, 100, 159, 251, 398 mg/kg-day; gd 6-18; gavage<br>5 females/group | Excess maternal death at doses ≥ 100 mg/kg-day<br>Anorexia, weight loss, 63 mg/kg-day<br>Mild diarrhea and anorexia at 25 mg/kg-day | Reduced fetal viability, abortion of litters at ≥ 63 mg/kg-day |
| | Dutch-belted rabbits<br>0, 20, 35, 50 mg/kg-day; gd 6-18; gavage<br>15 females/group | Excess maternal death at 50 mg/kg-day<br>Decreased bw gains at 50 mg/kg-day | Decreased fetal weights at 20 & 50 mg/kg-day<br>Complete abortions seen in all groups |

abbreviations: bw = body weight, gd = gestation day

**Table 29. Developmental neurotoxicity of oral chloroform in the mouse**

| Reference | Study design | Maternal effects | Developmental Effects |
|---|---|---|---|
| Burkhalter & Balster, 1979 | ICR mice<br>0, 31.1 mg/kg-day, 3 weeks prior to mating, through mating, gestation and lactation, directly to weaned pups<br>5 females/group | Not discussed | Reduced postnatal weight gain<br>Lower scores for forelimb placement on postnatal days 5 and 7 |

## D. REPRODUCTIVE TOXICITY

The presentation of information on the reproductive toxicity of chloroform begins with a description of the only pair-based study available. In this study, both sexes were exposed to chloroform as breeding pairs in a continuous breeding study conducted in mice (Chapin et al., 1997; NTP, 1988). Information from human studies pertaining to the potential male reproductive toxicity of chloroform comes from a case study of a male laboratory worker exposed to chloroform (Chang et al., 2001), and from a study of semen quality in men exposed to THMs in drinking water (Fenster et al., 2003). With regard to animal data, there are two reports of a sperm morphology study conducted in mice (Land et al., 1979 and 1981), and a chronic study conducted in beagle dogs (Heywood et al., 1979).

Information from human studies pertaining to the potential female reproductive toxicity of chloroform comes from studies of occupational exposures (Dahl et al., 1999; Tylleskar-Jensen, 1967, as cited in Reprotext 2004; Wennborg et al., 2000), and from a study of women exposed to water disinfection byproducts (Windham et al., 2003). The animal data are comprised of a 90 day toxicity study conducted by the drinking water route in rats (U.S. EPA, 1980), a 7.5 year chronic study conducted by the oral route in

beagles (Heywood et al., 1979), and a number of developmental toxicity studies conducted by the inhalation or oral route of exposure in rats, mice, or rabbits (Schwetz et al., 1974; Baeder and Hoffman, 1988 and 1991; Thompson et al., 1974; Ruddick et al., 1983; Murray et al., 1979).

## D.1. Pair-based studies

*NTP (1988). Chloroform reproduction and fertility assessment in CD-1 mice when administered by gavage; Chapin et al. (1997). Chloroform [summary of full study]*

Chloroform was tested for effects on reproduction and fertility in VAF Crl:CD-1 (ICR)BR outbred albino mice (Charles River), following the National Toxicology Program's Continuous Breeding protocol (Chapin et al., 1997; NTP, 1988). Each dose group consisted of 20 mated pairs; the control group had 40 pairs. Based on results from a two-week range-finding study, exposure levels were set at 8, 20, and 50 mg/kg-day by gavage in a corn oil vehicle. Analysis of dosing solutions indicated that actual doses administered were closer to 6.6, 15.9, and 41.2 mg/kg, due to volatilization of the chloroform. Hence, these corrected doses will be used in discussing the results.

Both males and females were dosed daily for seven days prior to mating, as well as during a 98-day co-habitation period (P1). The final litters from each dose group were reared normally by their dams until weaning (F1). Pups were examined on each of postnatal days 0, 4, 7, 14, and 21. Low and middle dose pups were discarded at weaning. Control and high-dose pups were weaned on postnatal day 21, and subjected to gavage treatments commencing on postnatal day 22.

Five P1 animals died over the course of the study. The deaths were scattered across dose groups, and were not considered to be treatment related. Feed and water consumption were not affected by treatment, and group mean body weights differed by no more than 2%. At delivery, dam body weights did not differ among groups for any of five delivered litters, excepting for the 41.2 mg/kg-day group at the $4^{th}$ litter (lower than corresponding control at $p < 0.05$). Also for this group, maternal body weight on postnatal day 14 of the final ($5^{th}$) litter was significantly lower than that of the corresponding controls ($p < 0.05$).

No treatment-related changes were identified in any of the evaluated endpoints of reproductive function. A breeding pair was designated fertile if at least one litter was produced. The control, 6.6 mg/kg-day, and 41.2 mg/kg-day groups all had fertility indices of 100. The fertility index for the 15.9 mg/kg-day group was 94 (one surviving pair did not produce a litter).

No significant differences were observed among groups for the number of litters per pair, litter size, proportion of live pups, sex ratio, or pup weight at birth. Inter-litter intervals were considered to be essentially identical across all groups. Neither the proportion of

stillbirths nor postnatal survival differed among groups. Pup weights did not differ among groups at any of the time points evaluated.

Among the control and high dose group F1 pups retained postweaning, five control and two chloroform-treated mice died or were sacrificed due to gavage trauma. These animals were weighed at weaning, and then weekly during study weeks 28-31. Chloroform-exposed female mice were significantly ($p < 0.01$) heavier than corresponding controls at week 31; no significant differences between groups in body weight were noted for females at other time points, or for males at any time point.

The mating indices of mature F1 animals cohabited for one week did not differ significantly between control and exposed groups (90 and 100, respectively), though the fertility index was significantly higher in chloroform-treated animals than in controls (95 and 70, respectively; $p < 0.05$). Treated and control groups did not differ significantly with respect to the number or proportion of male pups, or the proportion of live pups of both sexes combined. The number of female pups per litter, as well as total litter size were significantly greater in the chloroform-exposed group ($p< 0.05$).

At necropsy of F1 females, terminal body weights did not differ significantly between treated and untreated groups, nor did absolute or adjusted (by analysis of covariance) right ovary or combined kidney weights. Both absolute and adjusted liver weights, on the other hand, were significantly increased in chloroform-exposed females ($P < 0.01$). Vaginal cytology was not investigated in this study. No gross lesions were noted in lung, thyroid, or kidney in either control or chloroform-treated female mice. One control female, and 13 chloroform-exposed females showed "a reticular pattern of the liver (accentuated lobular pattern)." The livers of all chloroform-treated females showed dose-related histopathologic changes, described primarily as "degeneration of hepatocytes." Two control females exhibited "minimal hepatitis." One chloroform-treated female showed "minimal tubular degeneration" in the kidney, which was not considered to be treatment-related.

Terminal body weights of F1 males did not differ significantly between treated and untreated groups, nor were between-group differences detected in absolute or adjusted weights of liver, combined kidneys, seminal vesicles, right testis, right cauda epididymis, or prostate gland. Absolute and adjusted weights of the right epididymis were significantly higher in chloroform-exposed than control animals ($p < 0.05$) (Table 30). Sperm motility, sperm density, and percent of abnormal sperm were not found to differ between chloroform-exposed and unexposed groups.

**Table 30. Absolute and adjusted epididymal weights of F1 males (mean ± SD) after exposure to chloroform by gavage (Chapin et al., 1997; NTP, 1988).**

| Dose (mg/kg-day) | Number per group | Body weight (g) | Right epididymis weight (mg) | Adjusted right epididymis weight (mg) |
|---|---|---|---|---|
| 0 | 20 | 33.686 ± 0.536 | 44.685 ± 1.087 | 44.736 ± 0.949 |
| 41.2 | 29 | 33.789 ± 0.570 | 47.725 ± 1.078* | 47.674 ± 0.949* |

* Significant difference from controls at $p < 0.05$

No gross lesions were observed in liver, lung, thyroid gland, kidneys, epididymides, seminal vesicles, coagulating glands, or prostate glands of male mice from either group. One case of enlarged spleen was not considered to be treatment related. No lesions were observed at the histological level in seminal vesicles, coagulating glands, lung, thyroid glands or prostate glands of either control or treated male mice. One case of abscessed liver, and one of hepatocellular degeneration were observed in treated mice. One treated male mouse had a cystic kidney. Epididymal lesions rated as "minimal" were identified and in 3/20 control mice, and in 6/20 treated mice; two additional treated mice had epididymal lesions classified as "mild." The nature of these lesions is described as "vacuolar degeneration of ductal epithelium in the cauda epididymis."

## D.2. Male reproductive toxicity

### D.2.1. Studies in human males

One occupational case report provides human data on the reproductive effects of exposure to chloroform, and one prospective epidemiological study provides human data on the reproductive effects of exposure to TTHMs in tap water.

*D.2.1.1. Occupational exposure to chloroform*

*Chang et al. (2001). Reduction of sperm motility in a male laboratory worker exposed to solvents: a case study.*

One case study of exposure to chloroform and male reproductive toxicity was identified (Chang et al., 2001). A 34-year-old male laboratory worker was found to suffer from asthenospermia and fertility problems. The worker had had a complete fertility test performed in May of 1996, six months after he had been married. Results showed normal semen appearance, volume and sperm count. Test results were also normal for his wife. In June 1997, test results showed a reduction in sperm motility compared to one year earlier. Since the man had been exposed to solvents at work, due to a shutdown of the ventilation system in his laboratory from August 1996 through August 1997, an investigation was conducted. An occupational physician, a urologist and an industrial hygienist conducted the investigation. Air and bulk samples were collected to determine

the worker's possible exposure level to chemical hazards. It was determined that the worker used chloroform, isooctane, and tetrahydrofuran to clean infrared spectrophotometry holders. The investigators reconstructed, in detail, the working conditions when the ventilation fan was not functioning and conducted environmental monitoring using both active and passive sampling to estimate the possible exposure levels of solvents. The exposure level to chloroform was 8.5 ppm by active sampling and 4.6 ppm by passive sampling during the shutdown of the ventilation system. However, the exposure level to chloroform was below the detection limit (<0.15 ppm) when the exhaust ventilation system was operating properly. It was also estimated that the chloroform levels in the morning could have been as high as 450 ppm due to build up through the night, since all ventilation was shut down. This indicates that the worker was exposed to levels approximately 10 times higher than the permissible exposure limit of 50 ppm, (OSHA, 1997) and 50 times higher than the threshold limit value of 10 ppm (ACGIH, 2001), for 2 hours/day, 5.5 days/week, and 4.25 weeks/month for eight months.

The semen samples from July and August of 1997 had lower percentages of total motile sperm (26% and 11%, respectively) compared to the sample from May 1996 (92%) after 30 minutes. The authors reported that drugs, drinking alcohol, smoking tobacco, or surgery probably did not cause the condition because these did not change during the period of May 1996 - July 1997. The worker had been exposed to two other chemicals, isooctane and tetrahydrofuran. No studies of male reproductive effects in association with exposure to isooctane were identified. One animal study of exposure to tetrahydrofuran found no adverse effects on male fertility. After receiving artificial insemination (from her husband) the worker's wife became pregnant.

**Table 31. Consecutive results of semen analysis[a] (Chang et al., 2001).**

| Test | July 1997 | August 1997 | October 1997 |
|---|---|---|---|
| Semen analysis | | | |
| Volume (mL) | 4.0 | 5.5 | 3.0 |
| Count (million/mL) | 68.6 | 73.8 | 90.6 |
| White blood cell | 15-20/HPF | 12-15/HPF | 1-2/HPF |
| Morphology | | | |
| Motility | | | |
| (at 30 min after ejaculation) | | | |
| Rapid | 17% | 10% | 32% |
| Medium | 6% | 1% | 6% |
| Slow | 3% | 0% | 2% |
| Static | 74% | 89% | 60% |
| Path velocity (m/sec) | 35 | 40 | 50 |

HPF - high-power field under light microscope
[a]-The patient was asked to have one ejaculation 4 days before semen collection and no ejaculation between that and the semen collection. Serum was analyzed in the fertility laboratory by computer-assisted semen analysis (Version 10 HTM-IVOS Specification, Hamilton-Thorne Research, Beverly, MA, USA)

*D.2.1.2. Exposure to water disinfection byproducts*

*Fenster et al. (2003). Trihalomethane levels in home tap water and semen quality.*

One study was found that reported on exposure to trihalomethanes (THMs) and semen quality (Fenster et al., 2003). Husbands of women enrolled in a prospective study, described in the female reproductive toxicity section (Windham et al., 2003), were asked to participate (N= 324), 164 agreed, and 157 were found to be eligible with no known risk factors for infertility. All participants completed an extensive interview. Two semen samples were collected approximately one month apart. Concentration of total trihalomethanes (TTHMs) in each subject's home tap water was estimated by utility-wide averaging after geocoding the residence to identify the water utility.

The study used a TTHM ingestion metric (TTHM concentrations times the cold home tap water consumption per day) to estimate exposure to TTHM. An association was reported between the highest category of TTHM metric (>160 μg/L) and a decrease in normal sperm morphology and an increase in head defects. The authors stated that colinearity between individual THMs limited their ability to examine the THMs independently. However the study reported a small decrease in linearity of sperm motion associated with BDCM exposure (B = -0.09, SE = 0.04 for every unit increase in BDCM). No further data were presented for this. The study was limited by several factors including: the low

participation rate; the small number of individuals in the high exposure group (N = 12 in >80 μg/L group); the large number of sperm parameters examined; the lack of measurement of exposure by inhalation and dermal routes (i.e., during showering, bathing, or swimming); and lack of measurement of exposure from consumption of tap water at work.

### D.2.2. Studies in male animals

*NTP (1988). Chloroform reproduction and fertility assessment in CD-1 mice when administered by gavage; Chapin et al. (1997) Chloroform [summary of full study]*

As described in more detail in section D.2.1. above, chloroform was tested for effects on reproduction and fertility in VAF Crl:CD-1 (ICR)BR outbred albino mice (Charles River), following the National Toxicology Program's Continuous Breeding protocol (Chapin et al., 1997; NTP, 1988).

No treatment-related changes were identified in any of the evaluated endpoints of reproductive function. Terminal body weights of F1 males did not differ significantly between treated and untreated groups, nor were between-group differences detected in absolute or adjusted weights of liver, combined kidneys, seminal vesicles, right testis, right cauda epididymis, or prostate gland. Absolute and adjusted weights of the right epididymis were significantly higher in chloroform-exposed than control animals ($p < 0.05$) (Table 30). Sperm motility, sperm density, and percent of abnormal sperm were not found to differ between chloroform-exposed and unexposed groups.

No gross lesions were observed in liver, lung, thyroid gland, kidneys, epididymides, seminal vesicles, coagulating glands, or prostate glands of male mice from either group. One case of enlarged spleen was not considered to be treatment related. No lesions were observed at the histological level in seminal vesicles, coagulating glands, lung, thyroid glands or prostate glands of either control or treated male mice. One case of abscessed liver, and one of hepatocellular degeneration were observed in treated mice. One treated male mouse had a cystic kidney. Epididymal lesions rated as "minimal" were identified and in 3/20 control mice, and in 6/20 treated mice; two additional treated mice had epididymal lesions classified as "mild." The nature of these lesions is described as "vacuolar degeneration of ductal epithelium in the cauda epididymis."

*Land et al. (1979). Mouse sperm morphology following exposure to anesthetics during early spermatogenesis*

Thirteen-week old (C57Bl/C3H)F1 male mice were exposed to an air concentration of 0.08% chloroform for four hours on each of five days. Three groups of five control mice were exposed to compressed air under test conditions. Twenty-eight days after the first day of exposure, the mice were sacrificed and both cauda epididymides removed for sperm evaluation. The epididymides were minced in physiologic buffered saline,

strained through stainless steel gauze, and stained with 1% eosin Y(H2O). Slides were prepared from this material, and evaluated by counting and evaluating 1000 sperm per slide at 400X magnification.

A statistically significant ($p < 0.05$) increase in the frequency of abnormal sperm morphology was found in animals exposed to 0.08% chloroform (see Table 32, below).

*Land et al. (1981). Morphologic changes in mouse spermatoza after exposure to inhalational anesthetics during early spermatogenesis*

In what appears to be a re-reporting and expansion of the experiment described above, nine additional mice were exposed to 0.04% chloroform for four hours on each of five days. Methods were as described above.

A statistically significant ($p < 0.01$) increase in the percent of abnormal sperm was reported for animals exposed to 0.04% chloroform, relative to controls (2.76% and 1.42%, respectively).

**Table 32. Sperm morphology in mice following inhalation exposure to chloroform (data from Land et al., 1979 and Land et al., 1981 combined).**

| Concentration | N | % abnormal sperm (SEM) |
|---|---|---|
| 0 | 15 | 1.42 (0.08) |
| 0.08 | 9[#] | 2.76 (0.31)** |
| 0.04 | 4[#] | 1.88 (0.39)* |

[#] The methods specify treatment groups of five mice per cage, but it is not clear whether chloroform-treated groups were smaller at commencement of treatment, or if one mouse from each of the two concentration groups died during the study.
* significantly different from controls at $p < 0.05$
** significantly different from controls at $p < 0.01$

*U.S. EPA (1980). Effects of chloroform in the drinking water of rats and mice*

In a 90-day subacute toxicity study, male Osborne-Mendel rats were exposed to chloroform at concentrations of 0, 200, 400, 600, 900, or 1800 ppm in drinking water. There were 30 animals in each experimental group, and 40 in the control group. Additional controls were matched to the high-concentration group for water consumption.

Body weights of all rats were taken each week for 13 weeks on the study. Ten rats were sacrificed at the beginning of the experiment for baseline data on kidney fat to kidney weight ratio, serum biochemistry, and gross and microscopic organ pathology. The same measurements were also taken on ten additional animals from each experimental group, sacrificed on test days 30, 60, and 90.

Body weights of male rats were not affected by concentrations of chloroform in drinking

water of 600 ppm or less. At 900 ppm chloroform, body weights were significantly reduced ($p < 0.05$) relative to ad lib controls only at the first week of treatment; there were no significant differences in subsequent weeks. For rats given 1800 ppm chloroform in their drinking water, or for matched controls, body weights were significantly reduced relative to ad lib controls at all time points after the initial weighing. At each time point, matched controls were heavier than animals exposed to 1800 ppm chloroform, but no tests of statistical significance were performed between these groups, nor are sufficient data presented for OEHHA staff to calculate measures of statistical significance.

No statistically significant differences among groups for water consumption were reported for rats at any of the 26 sampling periods. It is not clear whether a statistical analysis was performed. Visual inspection of the data, however, indicate that drinking water consumption was reduced with increasing concentrations of chloroform, with the greatest effect during the first week of treatment. After the first week of treatment, intake did not drop below 24.0 ml per rat per day for any group on any day; the authors considered a "normal maintenance level" of consumption to be 25 ml per rat per day. Consumed doses of chloroform were calculated on the basis of average body weights and drinking water: 0, 20, 38, 57, 81, and 160 mg/kg-day.

Data on the kidney fat to kidney weight ratio for rats exposed to 1800 ppm chloroform were compared to both ad lib controls and matched controls at 30, 60, and 90 days. Kidney fat was increased significantly ($p < 0.01$) in matched control rats at 90 days, as compared to ad lib controls.

Testes, prostates, and seminal vesicles were examined as part of a complete necropsy procedure. Examination revealed only one incident each of testicular hyperplasia and interstitial cell hyperplasia for animals of the 900 ppm chloroform group at the 30-day interim sacrifice. These may or may not have involved a single animal.

*Heywood et al. (1979). Safety evaluation of toothpaste containing chloroform III. Long-term study in beagle dogs*

Beagle dogs were given chloroform in a toothpaste base, orally in the form of gelatin capsules, six days per week for seven and a half years, followed by a 20-24 week recovery period. Eight animals of each sex were included in each of the two treated groups; chloroform doses were either 15 or 30 mg/kg-day. Eight dogs of each sex remained untreated, and additional groups of 16 males and females received the vehicle toothpaste alone. A final group of eight animals of each sex was given an alternative non-chloroform-containing toothpaste.

Of four males that died over the course of the study, one death occurred in each group excepting for the alternative toothpaste group.

Food consumption, water consumption, and group mean bodyweights were not

considered to have been influenced by chloroform treatment. Many of the dogs were classified as obese at the beginning of week 300, and their diets subsequently reduced. Findings of obesity were not related to treatment group.

Biochemical data suggested some treatment-related effects on liver function, but most dogs reverted to baseline values during the post-treatment recovery phase. Necropsies of dogs that died over the course of the study showed no obvious changes in their livers, brain, kidneys, or other organs. In animals examined at study termination, there were no treatment-related differences in absolute or relative organ weights, including weights of reproductive organs (testes and prostate).

"Ectopic testes with inhibition of spermatogenesis" was noted in two dogs in the 30 mg chloroform/kg-day group, one dog at 15 mg/kg-day, and in one untreated control animal. Other, unspecified, reproductive tract abnormalities were not considered to have any relationship to treatment.

### D.2.3. Integrative evaluation for male reproductive toxicity

#### *D.2.3.1. Human data*

One case study was identified of chloroform exposure in a laboratory worker associated with a reduction in sperm motility (Chang et al., 2001). This study was very well conducted in that the investigators re-created the exposure situation, and were able to report "baseline" sperm parameters measured before the exposure began, as well as measurements following the exposure. Test results showed reduced sperm motility following the period of exposure to chloroform as compared to the normal baseline measures taken before exposure. After exposure stopped sperm motility improved. A limited prospective study of exposure to TTHMs in tap water reported an association between exposure to >160 μg/L TTHM and increased sperm defects (Fenster et al., 2003).

#### *D.2.3.2. Animal data*

Information on the potential male reproductive toxicity of chloroform is available from a continuous breeding study conducted in mice (Chapin et al., 1997; NTP, 1988), two reports of a sperm morphology study conducted in mice (Land et al., 1979 and 1981), a 90 day toxicity study conducted in rats (U.S. EPA, 1980), and a chronic study conducted in beagle dogs (Heywood et al., 1979). The study designs and results are summarized in Table 33 below.

Under the continuous breeding protocol, male and female mice were exposed to chloroform by the gavage route for seven days prior to first mating, as well as during a subsequent 98-day cohabitation period (Chapin et al., 1997; NTP, 1988). Even at the highest dose of 41.2 mg/kg-day, systemic effects were minimal, and there was no

evidence for effects on fertility. Terminal body weights of F1 males did not differ between control and 41.2 mg/kg-day group animals, nor did absolute or adjusted weights of seminal vesicles, right testis, right cauda epididymis, or prostate gland. Absolute and adjusted weights of right epididymides, however, were significantly higher in chloroform-exposed animals.

The Chapin (1997)/NTP (1988) study did not detect any treatment-related changes in sperm motility, sperm density or percent abnormal sperm. These results contrast with a study of mouse sperm morphology reported by Land et al. (1979 and 1981). In the Land et al. (1979 and 1981) study, male mice were exposed to chloroform by inhalation, rather than by the oral route used in continuous breeding study discussed above (Chapin et al., 1997; NTP, 1988). Mice exposed to chloroform at concentrations in air of 0.08 or 0.04% showed statistically significant increases in percentages of abnormal epididymal sperm.

No clearly treatment-related effects on male reproductive organs were identified in a 90 day toxicity study conducted by the drinking water route in rats (U.S. EPA, 1980), or in a 7.5 year chronic toxicity study conducted by the oral route in beagle dogs (Heywood et al., 1979).

**Table 33. Male reproductive toxicity of chloroform**

| Reference | Study design | Systemic effects | Reproductive Effects |
|---|---|---|---|
| Chapin et al., 1997<br>NTP, 1988 | Mice, continuous breeding study<br>0, 6.6, 15.9, 41.2 mg/kg-day by gavage<br>20 pairs/group; 40 pairs control | No effects of treatment on mortality, bw, or organ weights (other than epididymis) | Increased absolute and adjusted right epididymal weight in F1<br>No adverse effects on fertility, other reproductive organs, or sperm parameters |
| Land et al., 1979<br>Land et al., 1981 | Mice, sperm morphology study<br>0, 0.04%, 0.08% by inhalation<br>4, 9, 15 animals/group | Not discussed | Increased frequency of abnormal sperm morphology at both concentrations |
| US EPA, 1980 | Rats, 90-day tox study<br>0, 20, 38, 57, 81, 160 mg/kg-day; drinking water<br>30 males/group; 40 male controls | Apparent reduced bw at 160 mg/kg-day for all timepoints; at 81 mg/kg-day for 1st week of treatment | No clear adverse effects on testes, prostate, or seminal vesicles<br>One case each of testicular hyperplasia and interstitial cell hyperplasia at 160 mg/kg-day |
| Heywood et al., 1979 | Beagles, 7.5 year chronic study<br>0, 15, 30 mg/kg-day, mixed into toothpaste and given in capsules<br>8 animals/sex/group | No effect on feed or water consumption, or bw<br>Possible, reversible, effects on liver function<br>No effects on organ weights or pathology | "Ectopic testes with inhibition of spermatogenesis" in 2 dogs at 30 mg/kg-day, 1 dog at 15 mg/kg-day, and 1 control |

abbreviations: bw = body weight

## D.3. Female reproductive toxicity

### D.3.1. Studies in human females

*D.3.1.1. Occupational exposure to chloroform*

*Dahl et al. (1999). Dental workplace exposure and effect on fertility.*

A study of the effect of workplace exposure on fertility in female dental surgeons examined exposures to chloroform in chloroform-based root canal sealers, as well as exposures to mercury, benzene and ethanol (Dahl et al., 1999). Data were collected using a self-administered mailed questionnaire. Exposure assessment was based on a "thorough examination of the work performed with specific emphasis on restorative materials and chemicals." The questionnaire was mailed to all female dental surgeons registered in the Norwegian Dental Association (N=1320) and to a random sample of female high school teachers (N=1084). The response rate was 65% for dental surgeons and 70% for teachers. Three pregnancies, the first two and the last were included if possible. The final sample of women, after applying enrollment criteria, included 834 pregnancies of 558 dental surgeons and 574 pregnancies of 450 teachers.

Assessment of exposure to chloroform in dental surgeons was based on the number of root fillings with chloroform-based root canal sealing material placed per week. Exposure assessment for teachers was based on open questions regarding the type of chemical and frequency of exposure. The questions addressed exposure especially during the six months prior to pregnancy. Fertility was measured by time-to-pregnancy, defined as the months of unprotected intercourse required to become pregnant. Survival analysis was used to determine the time-to-pregnancy distribution; discrete proportional hazard regression was used to analyze the effects of occupational exposure on fertility. The fecundability ratio produced from the regression analysis was defined as the ratio of per-cycle conception rate for the exposed women in comparison with the reference value. Since earlier analyses showed no major differences in the time-to-pregnancy distribution between any of the pregnancies, data for all three possible pregnancies were combined to increase the sample size.

In approximately 75% of the pregnancies women reported use of chloroform-based root canal sealers: 26.7 % (211) reported none, 51% (405) reported less than one per week, 15% (119) reported one-two per week, 6.7% (53) reported three-five per week, and 0.5% (4) reported more than five per week. This study found no difference in fertility with exposure to chloroform in dental surgeons. The estimated fecundability ratio for dental surgeons placing chloroform-based root fillings compared to the reference group of female high school teachers was 1.06 (95% CI = 0.95-1.10) based on 582 pregnancies. The data were adjusted for age, smoking habits and medical history.

The limitations of the study include a potential for recall bias as 75% of pregnancies occurred within 20 years of the study, with some occurring up to the previous 40 years. The potential for exposure bias also exists as the subjects were required to recall

exposures far in the past. In addition, the number of dental surgeons in the "high exposure" groups was small (53 women in the 3-5 fillings per week, and four women in the greater than five fillings per week groups), with 75% of the sample of surgeons having reported placing either zero fillings per week or <1 filling per week. No power calculations were presented, and it is possible that the study lacked the power to detect an effect if one were present.

*Tylleskar-Jensen (1967, as cited in Reprotext 2004). Chloroform – a cause of pregnancy toxemia?*

A case report published in Danish (Tylleskar-Jensen, 1967, as cited in Reprotext (2004), not translated by OEHHA), described two women with eclampsia who had worked in laboratories with measured concentrations of 100-1,000 ppm chloroform (compared with a recommended exposure limit of 50 ppm at the time). The report noted the background incidence in the population was reported to be 1 case per 4,000 pregnancies.

*Wennborg et al. (2000). Pregnancy outcome of personnel in Swedish biomedical research laboratories.*

A single study on pregnancy outcome in Swedish women occupationally exposed to chloroform has been described in detail in section C.1.1. above (Wennborg et al., 2000). The final sample included 869 singleton pregnancies among 697 women.

No effect was reported associated with laboratory work in general and reported SABs. A weak association was shown between women working with chloroform during the time before conception and SABs (OR = 2.3; 95% CI, 0.9-5.9, adjusted for mother's age and previous SAB; unexposed N = 770, exposed N = 86). The OR for previous SABs was 2.2 (95% CI, 1.2-4.1). However, there were only 2 SABs among women who had worked with chloroform and had had previous SABs.

Limitations of this study include the lack of actual exposure levels, and the possible exposure to other laboratory solvents. However, the questions about laboratory agents concerned those that the women had handled personally. The response rate was low (73%), however, the responders and non-responders were similar with respect to women's health diseases, gynecological history and socioeconomic factors. Although the spontaneous abortions were self-reported, the authors noted that the reference group, female non-laboratory university personnel, had the same socioeconomic background as the laboratory personnel. This likely reduced the risk of selection bias, which would be the result of one group reporting the occurrence of spontaneous abortions differently.

*D.3.1.2. Exposure to water disinfection byproducts*

*Windham et al. (2003). Chlorination by-products in drinking water and menstrual cycle function.*

A single study was identified that examined exposure to TTHMs and female reproductive toxicity. This prospective study by Windham et al. (2003) examined the association of exposure to TTHMs in tap water and menstrual cycle function. Married women, 18-39 years of age, who were members of the Kaiser Permanente Medical Care Program in Northern California, living within 10 miles of Santa Clara, were screened and enrolled in the study. Of the 1,092 eligible women, 553 agreed to participate but 89 dropped out and 61 became ineligible. The remaining 403 women collected daily urine samples and completed a daily diary for an average of 5.6 cycles (range 2-9). The urine samples were analyzed for metabolites of estrogen and progesterone. Women were asked the amount of usual daily consumption of unheated tap water at home, drinks made with hot tap water at home, and bottled water. The number of minutes showering per week was calculated from questions on the frequency and duration of showers. Levels of TTHMs in tap water were based on a utility-wide average of measurements. Exposure to TTHMs was calculated by two ingestion metrics (TTHM level × total cold tap water consumed, TTHM level × total cold and heated tap water).

The authors reported a monotonic decrease in mean cycle length of 1.1 days, (95% CI, –1.8 to –0.40), and in follicular phase length of 0.94 days (95% CI, –1.6 to –0.24) with increasing TTHM levels, >60 μg/L compared with levels ≤40 μg/L. Although cycle length was shorter for each of the individual THMs in the highest quartile the results for chloroform were not statistically significant. The largest decreases were evident in chlorodibromomethane, and for the sum of brominated THMs (-1.2 days, 95% CI, –2.0 to –0.38 and –1.2 days, 95% CI, -2.0 to –0.40, respectively). Similar results were reported for decreases in follicular phase length.

The authors hypothesize that an effect on the hypothalamic-pituitary system, which controls hormone secretion, could be an underlying mechanism responsible for multiple effects on both ovarian function and pregnancy, as seen in developmental studies mentioned above. This study was prospective and thus was able to collect information on potential covariates, such as smoking, which were included in the statistical analyses, and hormone levels were used to determine the outcome measure. Limitations of this study include the low participation rate and the potential for exposure misclassification due to the use of water treatment records. In addition, although the study did incorporate information about personal water use, remaining exposures, such as from swimming or from tap water at work, were not considered.

### D.3.2. Studies in female animals

*D.3.2.1. Information from pair-based studies*

*NTP (1988). Chloroform reproduction and fertility assessment in CD-1 mice when administered by gavage; Chapin et al. (1997) Chloroform [summary of full study]*

As described in detail above in section D.2.1, chloroform was tested for effects on reproduction and fertility in VAF Crl:CD-1 (ICR)BR outbred albino mice (Charles River), following the National Toxicology Program's Continuous Breeding protocol (Chapin et al., 1997; NTP, 1988).

At delivery, dam body weights did not differ among groups for any of five delivered litters, excepting for the 41.2 mg/kg-day group at the $4^{th}$ litter (lower than corresponding control: $p < 0.05$). Also for this group, maternal body weight on postnatal day 14 of the final ($5^{th}$) litter was significantly lower than that of the corresponding controls ($p < 0.05$).

No treatment-related changes were identified in any of the evaluated endpoints of reproductive function. The mating indices of mature F1 animals cohabited for one week did not differ significantly between control and exposed groups (90 and 100, respectively), though the fertility index was significantly higher in chloroform-treated animals than in controls (95 and 70, respectively: $p < 0.05$).

At necropsy of F1 females, terminal body weights did not differ significantly between treated and untreated groups, nor did absolute or adjusted (by analysis of covariance) right ovary or combined kidney weights. Both absolute and adjusted liver weights, on the other hand, were significantly increased in chloroform-exposed females ($p < 0.01$). Vaginal cytology was not investigated in this study. No gross lesions were noted in lung, thyroid, or kidney in either control or chloroform-treated female mice. One control female, and 13 chloroform-exposed females showed "a reticular pattern of the liver (accentuated lobular pattern)." The livers of all chloroform-treated females showed dose-related histopathologic changes, described primarily as "degeneration of hepatocytes." Two control females exhibited "minimal hepatitis." One chloroform-treated female showed "minimal tubular degeneration" in the kidney, which was not considered to be treatment-related.

*D.3.2.2. Information from acute and chronic toxicity studies*

*U.S. EPA (1980). Effects of chloroform in the drinking water of rats and mice*

In a 90-day subacute toxicity study, female B6C3F1 mice were exposed to concentrations of 0, 200, 400, 600, 900, 1800, and 2700 ppm in drinking water. There were 30 animals in each experimental group, and 40 in the control group. Additional controls were matched to the high-concentration group for water consumption. Ten mice were sacrificed at the beginning of the experiment for baseline data on liver fat-liver weight

ratio, serum biochemistry, and gross and microscopic pathology. The same measurements were also taken on ten additional animals from each experimental group, sacrificed on test days 30, 60, and 90.

Seven mice died during the first three weeks of treatment: one at 600 ppm, two at 900 ppm, and four at 2700 ppm. The authors report that these animals died after body weight reductions to 11g (mean initial weights ranged from 18-20 g), and attributed the deaths to refusal to drink the chloroform-treated water.

No statistically significant differences among groups for water consumption were reported for mice at any of the 26 six sampling periods. It is not clear whether a statistical analysis was performed. According to the study authors, water consumption varied considerably between individuals within groups, and no dose-dependency was evident. However, visual inspection of tabulated data indicates that water consumption of treated animals in all groups did drop off during the first three-day sampling period, gradually returning to control levels by the 5$^{th}$ sampling period (approximately 20 days after initiation of treatment).

The mice were more active and had lower body weights than expected, resulting in levels of chloroform intake (based on mean body weights and water consumption) that were 148-175% of the doses anticipated (0, 20, 40, 60, 90, 180, and 270 mg/kg-day).

Mice receiving the three highest concentrations of chloroform (900, 1800, and 2700 ppm) showed body weight loss during the first week (significant at $p \leq 0.05$). In the second week, body weights were significantly ($p \leq 0.05$) reduced at concentrations of 600, 1800, and 2700 ppm chloroform (but not at 900 ppm). In the third week, body weights were significantly ($p \leq 0.05$) reduced at concentrations of 600, and 900 ppm, but not at the higher concentrations of 1800 and 2700 ppm. In weeks 4-13, weights stabilized at levels comparable to controls. Controls matched to the 2700 ppm animals for water consumption, showed significant reductions in body weight ($p \leq 0.05$) compared to ad lib controls for weeks 1, 2, and 6.

Data on the liver fat-liver weight ratio for mice exposed to 2700 ppm chloroform were compared to both ad lib controls and matched controls at 30, 60, and 90 days. Liver fat was increased significantly ($p < 0.01$) in mice exposed to 2700 ppm at all of the time points measured. Liver fat was also found to be significantly ($p < 0.01$) increased in matched controls at 90 days. Incidents of fatty changes were observed in the livers of mice exposed to 2700 ppm chloroform at the 30, 60, and 90-day sacrifices. Similar changes were observed at all three time points in the livers of some mice exposed to 1800 ppm chloroform.

Ovaries and uteri were examined as part of necropsy procedures, and no changes were reported for these organs.

*Heywood et al. (1979). Safety evaluation of toothpaste containing chloroform III. Long-term study in beagle dogs*

In a study also described in section D.2.3.2 above, beagle dogs were given chloroform in a toothpaste base, orally in the form of gelatin capsules, six days per week for seven and a half years, followed by a 20-24 week recovery period. Eight animals of each sex were included in each of the two treated groups; chloroform doses were either 15 or 30 mg/kg-day. Eight dogs of each sex remained untreated, and additional groups of 16 males and females received the vehicle toothpaste alone. A final group of eight animals of each sex was given an alternative non-chloroform-containing toothpaste.

No chloroform-exposed females died during the study, nor did any of the alternative toothpaste females die. Of seven females that died during the study, four were in the vehicle control group and the remaining three were in the untreated control group.

Food consumption, water consumption, and group mean bodyweights were not considered to have been influenced by chloroform treatment. Many of the dogs were classified as obese at the beginning of week 300, and their diets subsequently reduced. Findings of obesity were not related to treatment group.

Biochemical data suggested some treatment-related effects on liver function, but most dogs reverted to baseline values during the post-treatment recovery phase. Necropsies of dogs that died over the course of the study showed no obvious changes in their livers, brain, kidneys, or other organs. In animals examined at study termination, there were no treatment-related differences in absolute or relative organ weights, including weights of reproductive organs (ovaries and uterus).

Changes observed in ovaries and uteri were considered to be consistent with normal estrous cyclicity, and not related to treatment. "Nodular hyperplasia of the mammary gland" was noted for three females at 15 mg/kg-day, as well as for five of the vehicle controls and one untreated control.

*D.3.2.3. Information from developmental toxicity studies*

*Schwetz et al. (1974). Embryo- and fetotoxicity of inhaled chloroform in rats*

As previously described in detail in section C.2.1.1 above, timed-mated Sprague-Dawley rats were exposed to chloroform by inhalation, 7 hr/day on each of gestation days 6 through 15. The initial experiment employed concentration levels of 0 or 300 ppm; additional groups were exposed to 100 or 30 ppm chloroform.

No dams died over the course of the study, but statistically-significant deficits were found for percent pregnant at study termination (see Table 34, below), as well as for maternal weight gain and food consumption.

**Table 34. Maternal parameters following inhalation exposure to chloroform (Schwetz et al., 1974).**

| Parameters | Air control | Air control (starved) | 30 ppm | 100 ppm | 300 ppm |
|---|---|---|---|---|---|
| N mated | 77 | 8 | 31 | 28 | 20 |
| N pregnant | 68 | 8 | 22 | 23 | 3 |
| % pregnant | 88 | 100 | 71 | 82 | 15* |

* statistically significant difference from ad lib fed controls at $p < 0.05$

Only three out of 20 mated dams (15%) in the 300 ppm group were found to be pregnant at the time of necropsy (significantly lower than controls at $p < 0.05$). For those three litters as compared to controls, litter size was significantly reduced, resorption frequency was significantly increased (both endpoints significant at $p < 0.05$), and the percentage of litters with resorptions was 100% as opposed to 57% for controls (see Table 35 below).

**Table 35. Litter parameters following inhalation exposure to chloroform (Schwetz et al., 1974).**

| Parameters | Air control | Air control (starved) | 30 ppm | 100 ppm | 300 ppm |
|---|---|---|---|---|---|
| litters | 68 | 8 | 22 | 23 | 3 |
| fetuses/litter | $10 \pm 4$ | $10 \pm 4$ | $12 \pm 2$ | $11 \pm 2$ | $4 \pm 7$* |
| resorptions | 8% | 7% | 8% | 6% | 61%* |
| litters with resorptions | 57% | 25% | 68% | 52% | 100% |
| litters totally resorbed | 0/68 | 0/8 | 0/22 | 0/23 | 1/3 |

* statistically significant difference from controls at $p < 0.05$

*Baeder and Hoffman (1988). Initial Submission [to U.S. EPA]: Inhalation embryotoxicity study of chloroform in Wistar rats (final report) with attachments and cover letter dated February 22, 1992*

As described in more detail in section C.2.1.1 above, two dose range-finding studies were performed on groups of four to six time-mated Wistar female rats. In the first preliminary study, the pregnant animals were exposed in the inhalation chambers for six hours daily on gestation days 7-11 and 14-16. Chloroform concentrations of 10, 30, and 100 ppm were tested. In the 10 ppm group, two dams had no fetuses and only a single implantation site. One dam in the 30 ppm group had only one fetus and three empty implantation sites. Similar effects were not seen, however, at the highest concentration of 100 ppm.

In a second preliminary experiment, dams were exposed to chloroform at concentrations of 100 and 300 ppm on each of gestation days 7-16. In the 300 ppm group, three dams had normally developed fetuses, one dam had totally resorbed fetuses, and one dam had only empty implantation sites in the uterus.

Concentrations of 0, 30, 100, and 300 ppm were chosen for the full-scale study. Groups of 20, time-pregnant Wistar rats were exposed to chloroform daily for seven hours on each of gestation days 7-16. Uterine contents were examined following cesarean section on gestation day 21.

Litters were completed resorbed in two dams at 30 ppm chloroform, in three dams at 100 ppm, and in eight dams at 300 ppm (Table 36). In dams producing live litters, neither corpora lutea nor live litter size were significantly affected by chloroform treatment. Placental weights did not differ among groups.

**Table 36. Litter data after inhalation exposure to chloroform (Baeder and Hoffman 1988)**

| **Parameters** | **0** | **30 ppm** | **100 ppm** | **300 ppm** |
|---|---|---|---|---|
| N pregnant | 20/20 | 20/20 | 20/21 | 20/23 |
| N lost litters | 0 | 2 | 3 | 8 |
| N live litters | 20 | 18 | 17 | 12 |
| Resorptions/live litter* | 0.75 | 0.22 | 0.53 | 0.92 |
| Live fetuses/litter* | 12.4 | 12.8 | 12.8 | 13.4 |

* Mean per litter, no SD provided

*Baeder and Hoffman (1991). Initial Submission [to U.S. EPA]: Chloroform: Supplementary inhalation embryotoxicity study of chloroform in Wistar rats (final report) with attachments and cover letter dated December 24, 1991*

As a follow-on supplement to the study described above (Baeder and Hoffman, 1988; and in more detail in section C.2.1.1), groups of 20 timed-mated Wistar rats were exposed to chloroform by inhalation at concentrations of 0, 3, 10, or 30 ppm for 7 hours daily on each of gestation days 7-16. As in the previous study, uterine contents were examined following cesarean section on gestation day 21 (Table 37).

All dams of all groups survived until the end of the study. Apart from one dam in the 30 ppm group, all dams in all groups in the study carried live fetuses to term. The one dam with no fetuses showed 13 empty implantation sites. Numbers of corpora lutea and implantations did not differ significantly among groups. Neither resorption frequency nor live litter size differed among groups.

**Table 37. Litter data after inhalation exposure to chloroform (Baeder and Hoffman, 1991).**

| Parameters | 0 | 3 ppm | 10 ppm | 30 ppm |
|---|---|---|---|---|
| N pregnant | 20 | 20 | 20 | 20 |
| N lost litters | 0 | 0 | 0 | 1 |
| N live litters | 20 | 20 | 20 | 19 |
| Resorptions/live litter* | 0.55 ± 0.89 | 0.40 ± 0.60 | 0.75 ± 1.02 | 0.84 ± 1.42 |
| Live fetuses/litter* | 12.4 ± 2.4 | 12.4 ± 3.5 | 12.9 ± 3.0 | 12.5 ± 1.9 |

* Mean per litter ± SD

*Thompson et al. (1974). Teratology studies on orally administered chloroform in the rat and rabbit.*

In a study described in more detail in section C.2.1.2 above, timed-mated Sprague-Dawley rats were given chloroform in corn oil by gavage. In a range-finding study, groups of six pregnant animals were given chloroform doses of 0, 79, 126, 300, 316, or 501 mg/kg-day on each of gestation days 6-15.

Results of the range-finding study were not reported in tabular form, but were discussed in the text of the paper without details of statistical analysis. Resorption frequency was significantly increased in the litters of dams given 316 mg chloroform/kg bw-day. Litter size was correspondingly decreased in these animals. Neither of the two females surviving exposure to 501 mg/kg chloroform had live fetuses: one was found to be not pregnant, the other had a completely resorbed litter.

In the full-scale teratology study, groups of 25 timed-mated Sprague-Dawley rats were given twice-daily gavage dosings of chloroform to total daily doses of 0, 20, 50, or 126 mg/kg-day on each of gestation days 6-15.

Implantation frequency, numbers of corpora lutea, resorption frequency, and litter size did not differ among animals exposed to chloroform at 0, 20, or 50 mg/kg-day (Table 38). In the 126 mg/kg-day group, implantation frequency was significantly increased over controls ($p < 0.05$).

**Table 38. Litter data from rats treated with oral chloroform (litter mean ± SD) (Thompson et al., 1974).**

| Dose (mg/kg-day) | Implantations | Corpora Lutea | Resorptions | Live Fetuses |
|---|---|---|---|---|
| 0 | 11.5 ± 2.4 | 13.1 ± 1.4 | 1.0 ± 2.9 | 10.6 ± 3.9 |
| 126 | 13.5 ± 1.1* | 14.2 ± 1.2 | 1.2 ± 2.6 | 12.3 ± 3.1 |

* statistically significant difference from controls at $p < 0.05$

In the rabbit portion of this study (described in more detail in section C.2.3. above) timed-mated Dutch-Belted rabbits were given chloroform in corn oil by gavage. In a range-finding study, groups of five pregnant animals were given total daily chloroform doses of 0, 25, 63, 100, 159, 251, or 398 mg/kg-day on each of gestation days 6-18. Doses were split into two treatments each day.

No animals survived treatment with 159, 251, or 398 mg/kg-day chloroform. Of the two dams surviving 100 mg chloroform/kg-day, one was not pregnant, and the other had four resorption sites but no viable conceptuses. Two of four surviving dams at 63 mg/kg-day each had six live fetuses. All five dams at 25 mg/kg-day had live fetuses, with an average of seven per litter.

In the full-scale portion of this teratology study, groups of 15 pregnant animals were given total daily chloroform doses of 0, 20, 35, or 50 mg/kg-day on each of gestation days 6-18. Doses were given once each day, rather than being split into two doses as was done for the range-finding study.

Seven dams died during the course of the study: two controls, one at 20 mg/kg-day, and four at 50 mg/kg-day. Deaths in the high-dose group were attributed to hepatotoxicity.

Complete abortions were seen in all groups: three in the control group, two at 20 mg/kg-day chloroform, one at 35 mg/kg-day, and four at 50 mg/kg-day (Table 39). There were no statistically significant differences among groups in the numbers of aborted litters, implantation frequency, numbers of corpora lutea, or mean live litter size.

**Table 39. Litter data from rabbits treated with oral chloroform (litter mean ± SD) (Thompson et al., 1974).**

| Dose (mg/kg-day) | Insemination rate | Implantations | Resorptions | Live Fetuses |
|---|---|---|---|---|
| 0 | 9/15 | 6.6 ± 2.2 | 0.1 ± 0.3 | 6.4 ± 2.2 |
| 20 | 12/15 | 6.3 ± 2.3 | 0.7 ± 1.2 | 5.6 ± 3.0 |
| 35 | 11/15 | 5.6 ± 2.5 | 1.1 ± 1.8 | 4.5 ± 2.9 |
| 50 | 7/15 | 8.4 ± 1.8 | 1.0 ± 1.7 | 7.4 ± 2.2 |

*Ruddick et al. (1983). A teratological assessment of four trihalomethanes*

In a study described in more detail in section *C.2.1.2.* above, chloroform was one of four trihalomethanes administered in separate experiments to pregnant Sprague-Dawley rats by oral intubation on each of gestation days 6-15. Chloroform was given at doses of 0, 100, 200, or 400 mg/kg-day, in a corn oil vehicle to a final volume of 1 ml/100 g body weight. Vehicle controls and all treated groups consisted of 15 rats each. Resorption frequency and live litter size were unaffected by treatment.

**Table 40. Data from fetuses of rats exposed orally to chloroform (Ruddick et al., 1983).**

| Dose (mg/kg) | Number of litters | Litter size (mean ± SE) |
|---|---|---|
| 0 | 14 | 11.2 ± 0.2 |
| 100 | 12 | 11.8 ± 0.6 |
| 200 | 10 | 12.5 ± 0.7 |
| 400 | 8 | 10.9 ± 1.1 |

*Murray et al. (1979). Toxicity of inhaled chloroform in pregnant mice and their offspring.*

In a study described in more detail in section *C.2.2.1.* above, groups of 34-40 timed-mated CF-1 mice were exposed to 0 or 100 ppm chloroform by inhalation for seven hours per day on each of gestation days 6-15, 1-7, or 8-15. Chamber-exposed controls were run for each group

The percentage of dams in each control group that were pregnant at term ranged from 62-85%. For animals exposed to 100 ppm chloroform on either gestation days 1-7 or days 6-15, pregnancy rates were significantly decreased compared to corresponding controls ($p < 0.05$). For animals exposed on gestation days 8-15, the chloroform-treated group had a pregnancy rate of 45%, which was not a significant difference from the corresponding controls. The same pattern of results were observed for total pregnancies (those evident at term plus those detectable only by uterine staining).

Chloroform exposure did not affect the mean number of implantation sites or live fetuses per litter. Resorption frequency was significantly ($p < 0.05$) affected only among the mice exposed to chloroform on gestation days 1-7 (Table 41); the effect was attributed primarily to two completely resorbed litters. As compared to corresponding controls, mean fetal body weight and crown-rump length (CRL) were reduced in all treated groups, reaching statistical significance ($p < 0.05$) for animals exposed on gestation days 1-7 or 8-15 (but not 6-15).

**Table 41. Fetal data from mice exposed to chloroform by inhalation (litter mean ± SD) (Murray et al., 1979).**

| Parameter | gd 1-7 0 ppm | gd 1-7 100 ppm | gd 6-15 0 ppm | gd 6-15 100 ppm | gd 8-15 0 ppm | gd 8-15 100 ppm |
|---|---|---|---|---|---|---|
| % pregnant[1] | 74% | 44% | 91% | 43% | 65% | 60% |
| No. litters | 22 | 11 | 29 | 12 | 24 | 18 |
| Fetuses | 10 ± 3 | 13 ± 2 | 12 ± 3 | 10 ± 4 | 12 ± 3 | 11 ± 3 |
| Resorptions | 2 ± 2 | 4 ± 5* | 2 ± 2 | 1 ± 1 | 2 ± 2 | 2 ± 2 |

[1] Includes females with implantation sites visible only after staining with sodium sulfide.

***** Statistically significant difference from corresponding controls at $p < 0.05$.

### D.3.3. Integrative evaluation for female reproductive toxicity

*D.3.3.1. Human data*

One occupational study (Dahl et al., 1999) examining the effect of chloroform exposure on fertility in women found no association with time to pregnancy. However, as mentioned earlier due to the limitations of the study, including a low participation rate and a small percentage of women exposed to higher levels of chloroform, little can be concluded from this study. Another study investigating adverse pregnancy outcome in women exposed to chloroform occupationally found a weak association between working with chloroform during the time before conception and spontaneous abortion (Wennborg et al, 2000). This study and other studies of adverse pregnancy outcome and exposure to chloroform in drinking water are also discussed in section C.1.

A prospective study (Windham et al., 2003) of exposure to THMs and menstrual cycle length reported statistically significant decreases in cycle length and follicular phase length for TTHMs. Monotonic decreases associated with exposure to the brominated THMs were also significant but not so for exposure to chloroform. This study was well conducted with improved exposure assessment, control for important potential confounders and hormonal determination of outcome measure. It is, however, the only study to examine menstrual cycle function in relation to exposure to THMs.

*D.3.3.2. Animal data*

Information on the potential female reproductive toxicity of chloroform in animals comes from a continuous breeding study conducted by the gavage route in mice (Chapin et al., 1977; NTP, 1988), a 90 day toxicity study conducted by the drinking water route in rats (U.S. EPA, 1980), a 7.5 year chronic study conducted by the oral route in beagles (Heywood et al., 1979), and a number of developmental toxicity studies conducted by the inhalation or oral route of exposure in rats, mice, or rabbits (Schwetz et al., 1974; Baeder and Hoffman, 1988 and 1991; Thompson et al., 1974; Ruddick et al., 1983; Murray et al., 1979).

Under the continuous breeding protocol, male and female mice were exposed to chloroform by the gavage route for seven days prior to first mating, as well as during a subsequent 98-day cohabitation period (Chapin et al., 1997; NTP, 1988). Even at the highest dose of 41.2 mg/kg-day, systemic effects were minimal, and there was no evidence for effects on fertility. There was no effect observed on ovarian weight. Vaginal cytology was not performed in this study, nor in any of the other available studies.

No clearly treatment-related effects on ovaries or uteri were identified in a 90 day toxicity study conducted by the drinking water route in rats (U.S. EPA, 1980), or in a 7.5 year chronic toxicity study conducted by the oral route in beagles (Heywood et al., 1979). The chronic study reported "nodular hyperplasia of the mammary gland" in three females at the low dose of 15 mg/kg-day, in five vehicle controls, and in one untreated control animal. This finding was not reported for the high dose of 30 mg/kg-day.

A number of developmental toxicity studies included findings for chloroform-treated animals of decreased pregnancy rate, decreased litter size, and/or increased resorptions (Schwetz et al., 1974; Baeder and Hoffman, 1988 and 1991; Thompson et al., 1974; and Murray et al., 1979). Only one study of this type, an oral developmental toxicity study performed in rats (Ruddick et al., 1983), gave no indication of an effect of chloroform on live litter size or resorption frequency, even at the highest dose of 400 mg/kg-day. Another oral study in the rat (Thompson et al., 1974) found effects on fetal viability, resorption frequency and litter size at doses of 501 and 316 mg/kg-day, but not at doses of 126 mg/kg-day or lower. In the pilot portion of an oral study conducted in rabbits (Thompson et al., 1974), two out of five dams survived a dose of 100 mg/kg-day; one of these dams was pregnant, the other had no live fetuses. In the full-scale portion of the same rabbit study, complete abortions were seen in all dose groups (20, 30, and 50 mg/kg-day), as well as in controls.

Inhalation developmental toxicity studies conducted in rats or mice showed effects on pregnancy rate and resorption frequency (Schwetz et al., 1974; Baeder and Hoffman, 1988 and 1991; Murray et al., 1979). In the Schwetz et al. (1974) study, only 3/20 mated female rats exposed to 300 ppm chloroform were found to be pregnant at necropsy. Those three females had smaller litters and increased resorptions relative to controls. Baeder and Hoffman (1988) showed a concentration-related increase in the frequency of totally resorbed litters among pregnant rats exposed to 30, 100, or 300 ppm chloroform. A subsequent study by the same group (Baeder and Hoffman, 1991) found only one lost litter at the high concentration of 30 ppm chloroform, and no lost litters at lower concentrations of 3 or 10 ppm. Decreased pregnancy rate and increased resorptions were also seen in pregnant mice exposed to chloroform at a concentration of 100 ppm.

All of the effects on pregnancy rate and/or fetal viability described above were observed at concentrations or doses that were also associated with systemic effects on maternal animals, such as reductions in feed consumption and some degree of alteration in maternal body weight and/or weight gain. Only the Schwetz et al. (1974) study

attempted to examine the influence of maternal effects on litter variables. That study incorporated a "starved" control group, which was feed-restricted in order to mimic the anorexic effects seen with 300 ppm chloroform. While decreases in fetal body measurements were observed in this group, starvation alone had no effect on pregnancy rate, live litter size, or resorption frequency.

**Table 42. Female reproductive toxicity of chloroform**

| Reference | Study design | Systemic effects | Reproductive Effects |
|---|---|---|---|
| Chapin et al., 1977<br>NTP, 1988 | Mice, continuous breeding study<br>0, 6.6, 15.9, 41.2 mg/kg-day by gavage<br>20 pairs/group; 40 pairs control | No effects of treatment on mortality, or organ weights<br>Reduced bw at delivery of 4th litter for 41.2 mg/kg-day group<br>Reduced bw on PND 14 of 5th litter for 41.2 mg/kg-day group<br>Histopathologic changes in the liver | No adverse effects on fertility or other reproductive indices<br>No effect on ovarian weight |
| U.S. EPA, 1980 | Mice, 90-day tox study<br>0, 20, 40, 60, 90, 180, 270 mg/kg-day; drinking water<br>30 females/group; 40 female controls | Deaths at 60, 90, 270 mg/kg-day<br>Effects on bw at $\geq$ 60 mg/kg-day<br>Fatty liver changes at 180, 270 mg/kg-day | No gross changes in ovaries or uteri |
| Heywood et al., 1979 | Beagles, 7.5 year chronic study<br>0, 15, 30 mg/kg-day, mixed into toothpaste and given in capsules<br>8 animals/sex/group | No effect on feed or water consumption, or bw<br>Possible, reversible, effects on liver function<br>No effects on organ weights or pathology | No treatment-related changes in ovaries or uteri<br>"Nodular hyperplasia of mammary gland" in 3 females at 15 mg/kg-day, in 5 vehicle controls, and 1 untreated control |
| Schwetz et al., 1974 | Sprague-Dawley rats<br>0, 30, 100, 300 ppm plus feed-restricted control; 7 hr/day; gd 6-16<br>8-77 females/group | Reduced feed consumption 100 & 300 ppm; only on gd 6-7 for 30 ppm<br>Reduced bw on gd 13 at 30, 100, & 300 ppm; on gd 21 at 300 ppm<br>Absolute liver weight increased 300 ppm<br>Relative liver weight decreased 100 & 300 ppm | Reduced pregnancy rate at 300 ppm<br>Decreased litter size, increased resorptions 300 ppm |
| Baeder & Hoffman, 1988 | Wistar rats<br>0, 30, 100, 300 ppm; 7 hr/day; gd 7-16<br>20 females/group | Reduced feed consumption at all concentrations<br>Higher bw on gd 0, all groups<br>Reduced bw gd 17 all concentrations<br>Reduced bw gd 21, 100 & 300 ppm | Increase in completely resorbed litters at all concentrations of chloroform |

**Table 42. Female reproductive toxicity of chloroform (continued)**

| Reference | Study design | Systemic effects | Reproductive Effects |
|---|---|---|---|
| Baeder & Hoffman, 1991 | Wistar rats<br>0, 3, 10, 30 ppm; 7 hr/day; gd 7-16<br>20 females/group | Reduced feed consumption all concentrations gd 7-14<br>Reduced feed consumption at all times, 30 ppm<br>Apparent reduced bw & wt gain for 10, 30 ppm (no stats) | 1 lost litter at 30 ppm<br>No effect on litter size or resorption frequency |
| Thompson et al., 1974 | Sprague-Dawley rats<br>0, 79, 126, 300, 316, 501 mg/kg-day by gavage; gd 6-15<br>6 females/group | Decreased feed consumption and weight gain at doses ≥ 126 mg/kg-day<br>Maternal deaths at 316 & 501 mg/kg-day | Increased resorptions at 316 mg/kg-day<br>Decreased litter size at 316 mg/kg-day<br>No live fetuses at 501 mg/kg-day |
| | Sprague-Dawley rats<br>0, 20, 50, 126 mg/kg-day by gavage; gd 6-15<br>25 females/group | Clinical symptoms at 126 mg/kg-day<br>Decreased feed consumption at 50 mg/kg-day<br>Decreased weight gain at 50 & 126 mg/kg-day<br>Fatty changes in livers at 50 & 126 mg/kg-day | Increased implantations at 126 mg/kg-day<br>No effects on live litter size or resorption frequency |
| | Dutch-belted rabbits<br>0, 25, 63, 100, 159, 251, 398 mg/kg-day; gd 6-18; gavage<br>5 females/group | Excess maternal death at doses ≥ 100 mg/kg-day<br>Anorexia, weight loss, 63 mg/kg-day<br>Mild diarrhea and anorexia at 25 mg/kg-day | Reduced fetal viability, abortion of litters at ≥ 63 mg/kg-day |
| | Dutch-belted rabbits<br>0, 20, 35, 50 mg/kg-day; gd 6-18; gavage<br>15 females/group | Excess maternal death at 50 mg/kg-day<br>Decreased bw gains at 50 mg/kg-day | Complete abortions seen in all groups (including controls) |
| Ruddick et al., 1983 | Sprague-Dawley rats<br>0, 100, 200, 400 mg/kg-day by gavage; gd 6-15<br>15 females/group | Decreased weight gain all doses<br>Increased liver weight all doses<br>Increased kidney weight 400 mg/kg-day<br>Decreased hemoglobin & hematocrit all doses<br>Decreased red blood cell counts all doses | No effect on live litter size or resorption frequency |
| Murray et al., 1979 | CF-1 mice<br>0, 100 ppm; 7 hrs/day, gd 6-15, 1-7, or 8-15<br>34-40 females/group | Decreased weight gain, gd 1-7 or 8-15<br>Increased absolute & relative liver weights, gd 6-15 or 8-15 | Decreased pregnancy rate, gd 1-7 or 6-15<br>Increased resorptions, gd 1-7 |

abbreviations: bw = body weight, gd = gestation day

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