National Toxicology Program
Toxicity Report Series
Number 59

NTP Technical Report on the Toxicity and Metabolism Studies of

Chloral Hydrate

(CAS No. 302-17-0)

Administered by Gavage to F344/N Rats and $B6C3F_1$ Mice

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U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health

FOREWORD

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The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

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This NTP report on the toxicity studies of chloral hydrate is based primarily on 16- and 17-day toxicity and metabolism studies that took place from June through October 1993 and the *in vitro* metabolism and DNA-binding studies that took place from April 1993 through December 1996.

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The draft report on the toxicity and metabolism studies of chloral hydrate was evaluated by the reviewers listed below. These reviewers serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of these NTP studies are appropriate and ensure that the toxicity study report presents the experimental results and conclusions fully and clearly.

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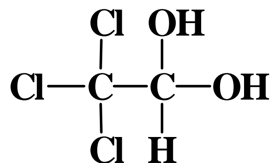
ABSTRACT



CHLORAL HYDRATE

CAS No. 302-17-0

Chemical Formula: $Cl_3CCH(OH)_2$ Molecular Weight: 165.40

Synonyms: 2,2,2-Trichloro-1,1-ethanediol; 1,1,1-trichloro-2,2-ethanediol; trichloroacetaldehyde monohydrate

Chloral hydrate is widely used as a sedative and a hypnotic in pediatric medicine. It is also a byproduct of water chlorination. Chloral hydrate has been shown to be genotoxic in numerous prokaryotic and eukaryotic assay systems including human lymphocytes *in vitro*. One of its metabolites, trichloroacetic acid, has demonstrated hepatocarcinogenic activity in mice. Trichloroethylene and perchloroethylene, both of which are metabolized to chloral hydrate, have been shown to be carcinogenic in rats and/or mice. Because of this evidence of carcinogenicity and because of the wide-spread use of chloral hydrate, 16- or 17-day range-finding toxicity studies and separate 16- or 17-day metabolism studies were performed in F344/N rats and $B6C3F_1$ mice in preparation for further long-term rodent studies. In addition, *in vitro* studies of the metabolism and DNA-binding capacity of chloral hydrate and its metabolites were performed. Genetic toxicity studies were conducted in *Salmonella typhimurium*, cultured Chinese hamster ovary cells, *Drosophila melanogaster*, and mouse bone marrow cells.

For the range-finding studies, groups of eight male and eight female F344/N Nctr BR rats and $B6C3F_1$/Nctr BR (C57BL/6N×C3H/HeN MTV^-) mice were administered 0, 50, 100, 200, 400, or 800 mg chloral hydrate per kg body weight in water by gavage 5 days per week for 17 days (rats) or 16 days (mice) for a total of 12 doses. One male rat receiving 800 mg/kg died after five doses. Two 800 mg/kg female rats died after dosing ended but before study termination. One male mouse in each group except the 400 mg/kg group died before the end of the study. Two 800 mg/kg female mice also died before the end of the study. The final mean body weight of 800 mg/kg male rats and the mean body weight gains of 400 and 800 mg/kg males were significantly less than those of the vehicle controls. The mean body weight gains of all groups of dosed male mice were

significantly greater than that of the vehicle control group. The only clinical finding in rats and mice attributed to chloral hydrate treatment was light sedation in the 400 mg/kg groups and heavy sedation in the 800 mg/kg groups; sedation subsided within 30 minutes or 3 hours, respectively. The liver weights of 400 mg/kg male mice and 800 mg/kg male and female mice were significantly greater than those of the vehicle control groups. No chemical-related lesions were observed in rats or mice.

Male and female rats and mice were administered a single dose of 50 or 200 mg chloral hydrate per kg body weight in water by gavage, or 12 doses of 50 or 200 mg/kg over 17 days (rats) or 16 days (mice). Plasma concentrations of chloral hydrate and its metabolites were determined 15 minutes, 1, 3, 6, and 24 hours, and 2, 4, 8, and 16 days after receiving 1 or 12 doses. Maximum concentrations of chloral hydrate were observed at the initial sampling point of 15 minutes. By 1 hour, the concentrations had dropped substantially, and by 3 hours, chloral hydrate could not be detected in rats or mice. Trichloroacetic acid was the major metabolite detected in the plasma. In rats, the concentrations rose slowly, with the peaks occurring between 1 and 6 hours after treatment. In mice, the peak concentrations were found 1 hour after dosing. The concentrations then slowly decreased such that by 2 days the metabolite could no longer be detected in rats or mice. Trichloroethanol was assayed both as the free alcohol and its glucuronide. In rats, the maximum concentrations of free trichloroethanol occurred at 15 minutes, while the peak concentrations of trichloroethanol glucuronide were found at 1 hour; by 3 hours, concentrations of both metabolites approached background levels. In mice, the maximum concentrations of both metabolites occurred at 15 minutes, and by 1 to 3 hours concentrations approached background levels. The plasma concentrations of chloral hydrate and its metabolites were dose dependent in rats and mice. In mice, plasma concentrations of trichloroacetic acid were significantly higher after a single dose than after 12 doses. None of the metabolic parameters appears to account for species differences that may exist in hepatocarcinogenicity.

The data from the study of metabolism and DNA adduct formation indicated that *in vitro* metabolism of 200 μM to 5 mM chloral hydrate by male B6C3F$_1$ mouse liver microsomes (control microsomes) generated free radical intermediates that resulted in endogenous lipid peroxidation, forming malondialdehyde, formaldehyde, acetaldehyde, acetone, and propionaldehyde. Similar concentrations of trichloroacetic acid and trichloroethanol, the primary metabolites of chloral hydrate, also generated free radicals and induced lipid peroxidation. Lipid peroxidation induced by trichloroacetic acid nearly equaled that induced by chloral hydrate, while that from trichloroethanol was three- to fourfold less.

Metabolism of 200 μM to 5 mM chloral hydrate, trichloroacetic acid, and trichloroethanol by liver microsomes of B6C3F$_1$ mice pretreated with pyrazole (pyrazole-induced microsomes) yielded lipid peroxidation products

at concentrations two- to threefold greater than those from liver microsomes of untreated mice. Additionally, chloral hydrate-induced lipid peroxidation catalyzed by control and pyrazole-induced microsomes was reduced significantly by 2,4-dichloro-6-phenylphenoxyethylamine, a general cytochrome P_{450} inhibitor. Human lymphoblastoid transgenic cells expressing cytochrome P_{450}2E1 metabolized 200 to 5,000 µg/mL chloral hydrate to reactants inducing mutations, whereas the parental cell line was inactive.

The malondialdehyde-modified DNA adduct, 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1,2α]purin-10(3H)-one (MDA-MG-1), formed from the metabolism of 1 mM chloral hydrate, trichloroacetic acid, and trichloroethanol by control B6C3F$_1$ mouse liver microsomes, mouse pyrazole-induced microsomes, male F344/N rat liver microsomes, and human liver microsomes in the presence and absence of calf thymus DNA was also determined. When incubated in the absence of calf thymus DNA, the amount of malondialdehyde formed from metabolism by pyrazole-induced mouse microsomes was twice that from rat or human liver microsomes. Amounts of chloral hydrate-induced and trichloroacetic acid-induced lipid peroxidation products formed from metabolism by rat and human liver microsomes were similar, and these quantities were about twice those formed from the metabolism of trichloroethanol. The quantity of MDA-MG-1 formed from the metabolism of chloral hydrate, trichloroacetic acid, and trichloroethanol by mouse, rat, and human liver microsomes exhibited a linear correlation with the quantity of malondialdehyde formed under incubation conditions in the absence of calf thymus DNA.

Chloral hydrate was shown to be mutagenic *in vitro* and *in vivo*. At doses from 1,000 to 10,000 µg/plate, it induced mutations in *S. typhimurium* strain TA100, with and without S9 activation; an equivocal response was obtained in *S. typhimurium* strain TA98 in the absence of S9, and no mutagenicity was detected with strain TA1535 or TA1537. Chloral hydrate at doses from 1,700 to 5,000 µg/mL induced sister chromatid exchanges; at doses from 1,000 to 3,000 µg/mL, chromosomal aberrations were induced in cultured Chinese hamster ovary cells, with and without S9. Results of a sex-linked recessive lethal test in *D. melanogaster* were unclear; administration of chloral hydrate by feeding produced an inconclusive increase in recessive lethal mutations, results of the injection experiment were negative. An *in vivo* mouse bone marrow micronucleus test with chloral hydrate at doses from 125 to 500 mg/kg gave a positive dose trend.

In summary, due to the absence of chloral hydrate-induced histopathologic lesions in rats and mice, no-observed-adverse-effect levels (NOAELs) were based on body weights of rats and liver weights of mice. The NOAELs for rats and mice were 200 mg/kg. Chloral hydrate was rapidly metabolized by rats and mice, with trichloroacetic acid occurring as the major metabolite. Peak concentrations of trichloroacetic acid occurred more quickly in mice. Plasma concentrations of chloral hydrate were dose dependent, but metabolic rates were

unaffected by dose or sex. Chloral hydrate was mutagenic *in vitro* and *in vivo*. Metabolism of chloral hydrate and its metabolites produced free radicals that resulted in lipid peroxidation in liver microsomes of mice, rats, and humans. Induction of cytochrome P_{450}2E1 by pyrazole increased the concentrations of lipid peroxidation products; inhibition of cytochrome P_{450}2E1 by 2,4-dinitrophenylhydrazine reduced these concentrations. Metabolism of chloral hydrate and its metabolites by mouse, rat, and human liver microsomes formed malondialdehyde, and in the presence of calf thymus DNA formed the DNA adduct MDA-MG-1.

INTRODUCTION

PHYSICAL PROPERTIES, USE, AND EXPOSURE

Chloral hydrate is produced by the addition of water to trichloroacetaldehyde (*Merck Index*, 1989). It is soluble in water, acetone, and methyl ethyl ketone and slightly soluble in turpentine, petroleum ether, carbon tetrachloride, benzene, and toluene. Chloral hydrate has an aromatic, penetrating and slightly acrid odor and a slightly bitter, caustic taste. Chloral hydrate is used medically as a sedative or hypnotic and as a rubefacient in topical preparations. It is often given to children as a sedative during dental and other medical procedures at doses of approximately 60 mg/kg body weight (Smith, 1990). In veterinary medicine it is used as a central nervous system depressant and sedative. It is used as a general anesthetic in cattle and horses (Rossoff, 1974). Chloral hydrate is used in the lumber industry for the bleaching of softwood pulp and is a byproduct of the chlorination of water. Concentrations in drinking water may reach 5 μg/L (USEPA, 1982).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

Studies of the metabolism of trichloroethylene in male $B6C3F_1$ mice have indicated that chloral hydrate is an early but rapidly metabolized intermediate in the metabolic scheme of trichloroethylene, with trichloroethanol and trichloroacetic acid being subsequent major metabolites (Green and Prout, 1985; Prout *et al.*, 1985). Metabolism of trichloroethanol is very similar in rats and mice; however, a higher concentration of trichloroacetic acid occurs in mice, suggesting a faster metabolism of chloral hydrate in mice compared to that in rats (Prout *et al.*, 1985). The direct study of chloral hydrate metabolism in male Swiss-Webster mice showed a 12-minute half-life ($t_{1/2}$) for the parent compound, with 56% converted to trichloroethanol ($t_{1/2}$=211 minutes), 11% converted to trichloroacetic acid, and 9.6% remaining as chloral hydrate (Cabana and Gessner, 1970); Abbas *et al.* (1996) demonstrated $t_{1/2}$s of 5 to 24 minutes in $B6C3F_1$ mice. In dogs, chloral hydrate is rapidly metabolized ($t_{1/2}$=4 minutes) to trichloroethanol and trichloroethanol-glucuronide (combined $t_{1/2}$=52 minutes) as well as trichloroacetic acid ($t_{1/2}$=5.5 days) (Breimer *et al.*, 1974).

Davidson and Beliles (1991) have reviewed literature showing that, *in vitro*, chloral hydrate may be reduced to trichloroethanol by cytosolic alcohol dehydrogenase, with NADH as the required coenzyme; however, Ikeda *et al.* (1980) demonstrated two NADPH-dependent enzymes in rat liver cytosol that also may catalyze this reduction. Similarly, human red blood cells reduce chloral hydrate to trichloroethanol (Davidson and Beliles,

1991). The oxidation of chloral hydrate to trichloroacetic acid may involve acetaldehyde dehydrogenase, but chloral hydrate reportedly is not a substrate for the human enzyme (Davidson and Beliles, 1991). A liver mitochondrial (NAD-dependent) aldehyde dehydrogenase may be the primary enzyme involved in trichloroacetic acid formation in rats (Ikeda *et al.*, 1980). Furthermore, carbonyl reductase from mouse or guinea pig lung has been shown to convert chloral hydrate to trichloroacetic acid and trichloroethanol in the presence of the reduced or oxidized cofactors, with trichloroacetic acid predominating in the NAD(P)+ linked reaction and equal amounts of trichloroethanol and trichloroacetic acid being produced in the NAD(P)H-linked reaction (Hara *et al.*, 1991).

Although the metabolism of trichloroethylene is qualitatively similar in rats and mice, male $B6C3F_1$ mice were found to metabolize inhaled trichloroethylene to a greater extent (262% more) than male Osborne-Mendel rats (Stott *et al.*, 1982). Furthermore, the metabolism of trichloroethylene administered orally in $B6C3F_1$ mice was linear over the range of 10 to 2,000 mg/kg, while in Osborne-Mendel rats, metabolism became constant and independent of dose at 1,000 mg/kg or greater (Prout *et al.*, 1985). When trichloroethylene was administered orally to female Wistar rats or NMRI mice at doses of 2, 20, or 200 mg/kg, there was no evidence of saturation of trichloroethylene metabolism in the mice, but saturation was apparent at 200 mg/kg in rats (Dekant *et al.*, 1986).

Humans

In humans, chloral hydrate is metabolized to trichloroethanol and trichloroacetic acid and is excreted in the urine as trichloroethanol, trichloroethanol-glucuronide, and trichloroacetic acid (Wade, 1977). When chloral hydrate is administered orally, a minimal amount of the parent compound is found in the blood, while the metabolites trichloroethanol, trichloroethanol-glucuronide, and trichloroacetic acid rise to peak concentrations within an hour of administration (Breimer *et al.*, 1974). The estimated $t_{1/2}$ of trichloroethanol and trichloroethanol glucuronide is about 7 hours, while the $t_{1/2}$ of trichloroacetic acid is 4 to 5 days. In a more recent study, the same metabolites were identified in the blood of an adult male and an infant male administered chloral hydrate orally (Gorecki *et al.*, 1990). In this study, trichloroacetic acid was the predominant metabolite in the adult, rising to peak concentrations 50 hours after administration. The predominant metabolite in the infant during the first 100 hours after administration was trichloroethanol. Chloral hydrate has also been shown to cross the placenta and to be secreted in breast milk (*MSDS*, 1991).

TOXICITY

Experimental Animals

The oral LD_{50} of chloral hydrate has been reported to range from 1,100 to 1,442 mg/kg in mice and 479 mg/kg in rats (Sanders *et al.*, 1982; *MSDS*, 1991). The intraperitoneal LD_{50} is 400 mg/kg in mice and 580 mg/kg in rats (*MSDS*, 1991).

In male Sprague-Dawley rats administered daily doses of 24, 48, 96, or 168 mg/kg in drinking water for 13 weeks, mean body weight, feed consumption, water consumption, and thymus weight were significantly decreased in the 168 mg/kg group (Daniel *et al.*, 1992a). Blood activities of lactate dehydrogenase and alanine aminotransferase were increased in all dosed groups, particularly in the 168 mg/kg group; aspartate aminotransferase activities were significantly increased in all dosed groups. Hepatocellular necrosis was observed in all but the 48 mg/kg group. No effects were observed in females receiving doses up to 288 mg/kg per day (Daniel *et al.*, 1992a).

In a 14-day gavage study in male Swiss (CD-1®) mice, increased liver weights, decreased spleen weights, and decreased blood lactate dehydrogenase levels were observed in mice administered daily doses of 144 mg/kg (Sanders *et al.*, 1982). In CD-1 mice given 0, 70, or 700 ppm (0, 17, or 170 mg/kg per day) in drinking water for 13 weeks, increases in body weight gain and relative liver weights were observed in males but not females (Sanders *et al.*, 1982). Body temperatures were somewhat depressed in males in each dosed group. Dose-related increases in the activities of liver microsomal enzymes cytochrome b-5, aniline hydroxylase, and aminopyrine-N-demethylase were observed in dosed males. An increased aniline hydroxylase activity was also observed in 700 ppm females. Serum lactate dehydrogenase and aspartate aminotransferase activities were also increased in males administered 700 ppm, while blood urea nitrogen concentrations decreased with increasing dose.

Trichloroethylene induced DNA strand breaks in rats and mice (Walles, 1986; Nelson and Bull, 1988). In one study, trichloroacetic acid, but not trichloroethanol, was shown to induce strand breaks in rat and mouse DNA (Nelson and Bull, 1988). In a subsequent study, trichloroacetic acid did not induce DNA strand breaks (Chang *et al.*, 1992). Although several studies have demonstrated covalent binding of trichloroethylene to DNA when incubated *in vitro* in the presence of microsomal fraction, covalent binding has not been convincingly demonstrated following *in vivo* administration of trichloroethylene (Uehleke and Poplawski-Tabarelli, 1977; Stott *et al.*, 1982; Bergman, 1983; Crebelli and Carere, 1989). Nevertheless, trichloroethylene has been shown to cause unscheduled DNA synthesis in isolated rat hepatocytes (Costa and Ivanetich, 1984) and in human lymphocytes (Perocco and Prodi, 1981).

Humans

In humans, chloral hydrate is corrosive to skin and mucous membranes. Therapeutic doses may cause gastritis with nausea and vomiting and, occasionally, allergic skin reactions. Chronic exposure may result in symptoms similar to chronic alcoholism (Wade, 1977). Hepatic damage with jaundice, renal damage with albuminuria, or heart damage may occur. The lethal human dose is estimated to be 5 to 10 grams (*MSDS*, 1991).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

Kallman *et al.* (1984) exposed CD-1 mice to 21 or 205 mg chloral hydrate/kg body weight per day in drinking water starting 3 weeks before gestation and continuing through pregnancy and lactation. A chemical-related increase in gestational weight gain was observed, but no effects on gestation length, litter size, pup weight, or pup mortality were noted. No gross malformations were noted in the offspring, nor were there any effects on development of neurobehavioral reflexes or motor control.

Studies in rodents on the effects of exposure to trichloroethanol during pregnancy have shown developmental delay with no clear signs of embryotoxicity or teratogenicity (Crebelli and Carere, 1989). In contrast, trichloroacetic acid caused soft tissue malformations in Long-Evans rats at incidences ranging from 9% at a dose of 330 mg/kg daily to 97% at 1,800 mg/kg daily. These malformations were primarily in the cardiovascular system. Dosing began on day 6 of pregnancy and continued for 10 days (Smith *et al.*, 1989).

Humans

No reproductive or developmental studies of chloral hydrate exposure in humans were found in a review of the literature.

IMMUNOTOXICITY

Experimental Animals

Chloral hydrate induced no alteration in humoral or cell-mediated immunity in male CD-1 mice administered 14.4 or 144 mg/kg by gavage for 14 days or in mice exposed to 0.07 or 0.7 mg/mL (70 or 700 ppm) in drinking water for 90 days; however, female mice exposed to the same doses for 13 weeks exhibited depressed humoral, but not cell-mediated, immune function (Kauffmann *et al.*, 1982). Immune function was evaluated by exposing spleen cells from treated females to sheep erythrocytes and assessing antibody production. The

number of antibody-forming cells per spleen was significantly reduced in each dosed group of females, but the number of antibody-forming cells per million cells was significantly reduced only in the 0.7 mg/mL group.

Both cell-mediated and humoral immunity were depressed by trichloroethylene in CD-1 mice, particularly in females (Davidson and Beliles, 1991).

Humans

In humans, sensitization may occur from repeated topical application of chloral hydrate (*MSDS*, 1991).

NEUROBEHAVIORAL TOXICITY

Experimental Animals

Male CD-1 mice administered chloral hydrate for 14 days by gavage or for 90 days in drinking water exhibited no behavioral responses besides those attributed to the acute effects of chemical exposure (Kallman *et al.,* 1984). Pregnant CD-1 mice were exposed to 205 mg/kg in drinking water through gestation and until the pups were weaned; the pups demonstrated a depressed retention of passive avoidance learning shortly after weaning (Kallman *et al.*, 1984). No effect was observed in mice exposed similarly to 21 mg/kg. Clinical findings of central nervous system toxicity, including ataxia, lethargy, convulsions, and hindlimb paralysis, have been reported in rats exposed chronically to trichloroethylene (NTP, 1988); mice show a period of excitement followed by a 15- to 30-minute subanesthetic state (Henschler *et al.*, 1984).

Humans

There is evidence suggesting neurotoxicity may result from chronic exposure of humans to trichloroethylene (Juntunen, 1986; Feldman *et al.*, 1988; Davidson and Beliles, 1991).

CARCINOGENICITY

Experimental Animals

In a skin paint study in "S" strain albino mice, the incidence of skin neoplasms was not increased significantly in animals treated with chloral hydrate followed by croton oil (Roe and Salaman, 1955). Rijhsinghani *et al.* (1986) reported that oral administration of a single dose of chloral hydrate (10 mg/kg) to eight newborn male $B6C3F_1$ mice caused a significant increase in the incidence of hepatic neoplasms in a 92-week study; three adenomas and three carcinomas were observed in dosed mice compared to two carcinomas in 19 control mice.

Daniel *et al.* (1992b) reported that chloral hydrate (1,000 ppm) was hepatocarcinogenic when administered to male B6C3F$_1$ mice in drinking water. The incidence of hepatocellular adenoma or carcinoma (combined) was 17/24 (71%) in treated mice versus 3/20 (15%) in control mice; the incidences of carcinoma were 2/20 (controls) and 11/24 (1,000 ppm group). In contrast, there was no increase in the incidence of hepatocellular carcinoma in male F344 rats administered up to 1,000 ppm (174 mg/kg per day) chloral hydrate in drinking water for 2 years (DeAngelo and George, 1995). A similar lack of tumorigenicity was reported for male and female Sprague-Dawley rats administered up to 135 mg/kg chloral hydrate per day in drinking water for 124 and 128 weeks, respectively (Leuschner and Beuscher, 1998).

Both trichloroethylene (NCI, 1976; NTP, 1990) and trichloroacetic acid (Herren-Freund *et al.*, 1987) have been shown to be hepatocellular carcinogens in B6C3F$_1$ mice. This has been shown in males and females with trichloroethylene but only in males with trichloroacetic acid. Forestomach papillomas and carcinomas were noted in Ha:ICR mice dosed with epoxide-stabilized trichloroethylene by gavage, and a significant increase in the incidence of malignant lymphoma was observed in female NMRI mice exposed to epoxide-free trichloroethylene by inhalation (Crebelli and Carere, 1989). In other inhalation studies in mice, the incidences of lung adenocarcinoma were increased in exposed male and female ICR and Swiss mice and in female B6C3F$_1$ mice, and hepatocellular carcinomas were induced in male Swiss mice and male and female B6C3F$_1$ mice. Trichloroethylene was not carcinogenic in Osborne-Mendel, Sprague-Dawley, or Wistar rats (Crebelli and Carere, 1989). A significantly increased incidence of renal adenocarcinoma was seen in male F344/N rats exposed to trichloroethylene; however, that study was considered inadequate due to high mortality (NTP, 1990). Increased incidences of renal adenocarcinoma and Leydig cell tumors were also observed in trichloroethylene-exposed Sprague-Dawley rats in another study (Crebelli and Carere, 1989). No studies of the carcinogenicity of trichloroethanol were found by Bruckner *et al.* (1989).

Elcombe *et al.* (1987) observed that trichloroethylene induced liver peroxisomes in mice but not in rats, while trichloroacetic acid induced peroxisomes in both species. More recently, DeAngelo *et al.* (1989) showed that trichloroacetic acid was a less effective peroxisome proliferator in rats than in mice. There is a more rapid conversion of trichloroethylene to trichloroacetic acid in mice than in rats (Prout *et al.*, 1985; Elcombe *et al.*, 1987); the difference in sensitivity between rats and mice to peroxisome induction by trichloroethylene appears to be due to this decreased rate of trichloroacetic acid formation and/or the resistance of rats to peroxisome induction by trichloroacetic acid. Perchloroethylene, a liver carcinogen in mice that is metabolized much like trichloroethylene, was found to induce peroxisomes more readily in mice than in rats. This result correlated with much higher levels of circulating trichloroacetic acid in mice than in rats (Odum *et al.*, 1988). These results have led to the suggestion that peroxisome induction by trichloroacetic acid has a causal role in liver carcinogenesis (Odum *et al.*, 1988).

Humans

No epidemiologic studies were found in the review of the literature.

GENETIC TOXICITY

Chloral hydrate has been tested for mutagenicity *in vitro* and *in vivo* in a variety of assays. Positive responses were obtained in many assays, particularly those which induced chromosomal damage in the form of aneuploidy.

Chloral hydrate gave positive results in the *Salmonella typhimurium* gene mutation assay in strain TA100, with and without S9 activation (Waskell, 1978; Bignami *et al.*, 1980; Haworth *et al.*, 1983); no mutagenic activity was detected with other strain/activation combinations. Purity of the chloral hydrate test samples in these studies ranged up to above 99%.

Positive results have also been seen with chloral hydrate in several genotoxicity assays in yeast. In *Saccharomyces cerevisiae*, chloral hydrate induced chromosomal malsegregation (Albertini, 1990), aneuploidy (Parry and James, 1988), disomy and diploidy (Sora and Agostini Carbone, 1987), and increased mitotic gene conversion in the presence of metabolic activation in the D7 diploid strain (Bronzetti *et al.*, 1984). In a diploid strain of *Aspergillus nidulans*, chloral hydrate exposure resulted in increased numbers of non-disjunction diploids and haploids, and hyperploidy was seen in the haploid strain 35 after treatment with chloral hydrate (Crebelli *et al.*, 1991). Aneuploidy was also observed in *A. nidulans* after chloral hydrate exposure (Crebelli and Carere, 1987).

In *Drosophila melanogaster*, chloral hydrate induced a small increase in the frequency of sex-linked recessive lethal mutations in germ cells of male flies administered the chemical by feeding (5,500 ppm in 5% sucrose); administration of chloral hydrate by abdominal injection (10,000 ppm in saline) did not induce germ cell mutations (Yoon *et al.*, 1985).

In mammalian cells treated with chloral hydrate *in vitro*, the observed genotoxic effects included aneuploidy in human lymphocytes (Vagnarelli *et al.*, 1990; Sbrana *et al.*, 1993) and Chinese hamster embryo cells (Furnus *et al.*, 1990; Natarajan *et al.*, 1993) and increased frequencies of kinetochore-positive micronucleated Cl-1 hamster cells (Degrassi and Tanzarella, 1988). However, no induction of DNA single-strand breaks was noted in rat or mouse hepatocytes or in human CCRF-CEM cells (Chang *et al.*, 1992) treated with chloral hydrate, and chloral hydrate failed to produce DNA-protein cross links when incubated with isolated rat liver nuclei (Keller and Heck, 1988).

In vivo, several studies have provided evidence of chloral hydrate-induced aneuploidy in spermatocytes of mice (Russo *et al.*, 1984; Liang and Pacchierotti, 1988; Miller and Adler, 1992), but not in oocytes (Mailhes *et al.*, 1988, 1993). Chloral hydrate exposure of premeiotic spermatocytes (Russo and Levis, 1992) and spermatogonial stem cells (Allen *et al.*, 1994; Nutley *et al.*, 1996) resulted in increased numbers of micronuclei in spermatids. In one experiment, however, kinetochore labeling of induced spermatid micronuclei did not indicate the presence of centromere-containing whole chromosomes, which would have been expected if aneuploidy had been induced (Allen *et al.*, 1994). In a study by Nutley *et al.* (1996), two methods of aneuploidy assessment were used (anti-kinetochore antibody staining and FISH with centromeric DNA probes), and both showed increased numbers of micronuclei with centromeric labels. Nutley *et al.* (1996) suggested that chloral hydrate induced structural chromosomal damage in treated spermatogonial stem cells. One study reported induction of single-strand breaks in hepatic cell DNA of rats and mice treated with chloral hydrate (Nelson and Bull, 1988), but a similar study failed to replicate these results (Chang *et al.*, 1992). Some somatic cell studies in rats and mice showed induction of micronuclei or chromosomal aberrations (Leopardi *et al.*, 1993), while others did not (Xu and Adler, 1990; Adler *et al.*, 1991).

STUDY RATIONALE AND DESIGN

The Food and Drug Administration nominated chloral hydrate for toxicity study based upon widespread human exposure, in addition to its potential hepatotoxicity and the toxicity of related chemicals. The NCTR conducted studies on chloral hydrate as part of an interagency agreement with the NIEHS to conduct comprehensive toxicological assessments of FDA priority chemicals nominated to the NTP. The *in vitro* metabolism and DNA binding studies described in this report were designed to facilitate the interpretation of the range-finding and metabolism study results, as well as an ongoing carcinogenicity bioassay. Data from all of the studies will be used to augment the regulatory decision process in terms of accurately assessing human health risk.

Sixteen- or seventeen-day gavage studies were performed in F344/N rats and B6C3F$_1$ mice. Endpoints included histopathology and organ weights. In separate studies, plasma metabolite concentrations were evaluated in F344/N rats and B6C3F$_1$ mice after 1 or 12 doses of 50 or 200 mg chloral hydrate/kg body weight. Chloral hydrate and its metabolites were also evaluated through *in vitro* metabolism and DNA binding studies using mouse, rat, and human microsomes.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF CHLORAL HYDRATE

Chloral hydrate was received in two batches from one lot (12HO289) purchased from Sigma Chemical Company (St. Louis, MO). Information on identity and purity was provided by the manufacturer.

Batch 1 of the chemical, a clear crystalline solid, was identified as chloral hydrate by nuclear magnetic resonance spectroscopy (NMR), which indicated unknown resonances at δ 5.76 and 5.70, representing molar percentages of 0.14% and 0.05%, respectively. For batch 2, NMR indicated the unknown resonances at δ 5.77 and 5.70, representing molar percentages of 0.18% and 0.42%, respectively. Purity was analyzed by gas chromatography with flame ionization detection, which indicated an impurity with a peak area of approximately 0.5% relative to the major peak for batch 1. Gas chromatography also revealed an impurity peak for batch 2 with the same retention time as that found in batch 1, but the peak area was too small to calculate. Mass spectral analysis of each batch indicated matches with chloral hydrate (99.5% for batch 1; 98.7% for batch 2) and with trichloroacetaldehyde (94.2% for batch 1; 99.1% for batch 2). Cumulative data indicated an overall purity of 99.5%.

Throughout the studies, bulk chloral hydrate was stored either in the original brown glass container, which was placed inside a plastic beaker inside a plastic canister (batch 1) or in a glass desiccator container (batch 2). Both batches were stored with silica gel, protected from light.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

Chloral hydrate crystals were weighed into a glass beaker and swirled with the appropriate amount of double-distilled water. Solutions were then filtered through 0.45-μm nylon filters. The concentration of the chloral hydrate dose formulations were verified using gas chromatography with flame ionization detection. Prior to dose administration, doses that were not within 10% of the target concentration were discarded. For the rat and mouse range-finding studies, 14 of 15 animal-room samples analyzed after dosing were within 10% of the target concentration; one 50 mg/mL formulation for rats was 118% of the target concentration, and one 100 mg/kg formulation for mice was 89% of the target concentration. For the rat and mouse metabolism studies, all animal-room samples analyzed after dosing were within 10% of the target concentration.

Stability studies of 6.5 and 16 mg/mL dose formulations of chloral hydrate were performed using gas chromatography. These studies indicated that both formulations were stable for at least 2 weeks when stored at room temperature exposed to ambient light and that the 16 mg/mL formulation was stable for up to 30 days when refrigerated at 1° to 4° C or at room temperature and protected from light. During the studies, dose formulations were stored in hypo-vials with Teflon-lined caps and aluminum seals at room temperature.

For rats, chloral hydrate was dissolved in distilled water at a concentration of 40 mg/mL for the 50, 100, and 200 mg/kg dose formulations and 160 mg/mL for the 400 and 800 mg/kg dose formulations. For mice, chloral hydrate was dissolved in distilled water at a concentration of 6.5 mg/mL for the 50 and 100 mg/kg dose formulations, 26 mg/mL for the 200 and 400 mg/kg dose formulations, and 52 mg/mL for the 800 mg/kg dose formulation. Final gavage solutions of chloral hydrate were dispensed by an automated dosing system that diluted the dose formulations by adding distilled water to give an appropriate dose based on the body weights of the dose group. The final dose volumes were no more than 1.0 mL for rats and no more than 0.5 mL for mice.

RANGE-FINDING STUDIES

Male and female F344/N rats and $B6C3F_1$ mice were provided by the NCTR breeding colony and were 4 weeks old at receipt. Rats and mice were 6 weeks old when the studies began. Groups of eight male and eight female rats and mice were administered 0, 50, 100, 200, 400, or 800 mg chloral hydrate per kg body weight in water by gavage 5 days per week for 17 (rats) or 16 (mice) days, including at least 2 consecutive days before necropsy, for a total of 12 doses. Rats were housed two per cage and mice were housed four per cage. Feed and water were available *ad libitum*. Clinical findings were recorded daily. Additional details concerning the study design and animal maintenance are provided in Table 1.

Complete necropsies were performed on all animals. Organs and tissues were examined grossly and the kidneys and liver were weighed. The kidney and liver as well as any tissues with grossly apparent lesions were fixed in 10% neutral buffered formalin, trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination.

METABOLISM STUDIES

Male and female F344/N Nctr BR rats and $B6C3F_1$/Nctr BR (C57BL/6N×C3H/HeN MTV^-) mice were provided by the NCTR breeding colony and were 4 weeks old at receipt. Rats and mice were 6 weeks old when

the studies began. Groups of 40 male and 40 female rats and mice were administered 50 or 200 mg chloral hydrate per kilogram body weight in water by gavage. Twenty males and 20 females per group were dosed once, and 20 males and 20 females were dosed 5 days per week for 17 (rats) or 16 (mice) days, including at least 2 consecutive days before the end of dosing, for a total of 12 doses. Rats were housed two per cage and mice were housed four per cage. Feed and water were available *ad libitum*. Clinical findings were recorded daily. Additional details concerning the study design and animal maintenance are provided in Table 1.

To analyze the plasma concentrations of chloral hydrate and its metabolites, blood samples were collected at 15 minutes, 1, 3, 6, and 24 hours, and 2, 4, 8, and 16 days after 1 or 12 doses of chloral hydrate. Animals were randomly assigned to blood collection groups such that all animals except those in the 24-hour groups were bled twice; animals in the 24-hour groups were bled only once. Thus, four male and four female rats and mice per group were bled at 15 minutes and 2 days, 1 hour and 4 days, 3 hours and 8 days, 6 hours and 16 days, or 24 hours. Animals were anesthetized with carbon dioxide; blood was collected from the retroorbital sinus into tubes containing ethylenediaminetetraacetic acid, centrifuged, and then stored at −60° C until analysis. Plasma concentrations of chloral hydrate and its metabolites were determined by electron-capture gas chromatography using a modification of the methods of Gorecki *et al.* (1990). Before centrifugation, plasma samples were added to 3 M sulfuric acid and extracted with methyl *t*-butyl ether (Baxter Healthcare Corp., Muskegon, MI) containing 1,1,1,2,-tetrachloroethane (Aldrich Chemical Co., Milwaukee, WI) as an internal standard. The samples were then centrifuged, and the organic phase was transferred to vials. Diazomethane solution was added to convert trichloroacetic acid to trichloroacetic acid methyl ester. The headspace was flushed with argon gas before storage and analysis. Trichloroethanol glucuronide was analyzed by the same methods, except plasma was treated with β-glucuronidase (Sigma Chemical Co., St. Louis, MO; EC 3.2.1.31, bacterial) at 37° C for 1 hour before extraction with methyl *t*-butyl ether.

TABLE 1
Experimental Designs and Materials and Methods in the 17-Day Studies of Chloral Hydrate

Range-Finding Studies	Metabolism Studies
Study Laboratory	
National Center for Toxicological Research (NCTR; Jefferson, AR)	National Center for Toxicological Research (NCTR; Jefferson, AR)
Strain and Species	
F344/N rats B6C3F$_1$ mice	F344/N rats B6C3F$_1$ mice
Animal Source	
NCTR breeding colony (Jefferson, AR)	NCTR breeding colony (Jefferson, AR)
Time Held Before Studies	
2 weeks	2 weeks
Average Age When Study Began	
6 weeks	6 weeks
Date of First Dose	
Rats: 16-17 August 1993 Mice: 14-15 June 1993	Rats: 4 October 1993 (single-dose groups) 4-6 October 1993 (12-dose groups) Mice: 7 July 1993 (single-dose groups) 19-21 July 1993 (12-dose groups)
Duration of Dose	
Rats: 12 doses over a 17-day period Mice: 12 doses over a 16-day period	Rats: One dose or 12 doses over a 17-day period Mice: One dose or 12 doses over a 16-day period
Date of Last Dose	
Rats: 1-2 September 1993 Mice: 30 June-1 July 1993	Rats: 20-22 October 1993 Mice: 3-5 August 1993
Date of Necropsy/Sacrifice	
Rats: 2-3 September 1993 Mice: 1-2 July 1993	Rats: 20-22 October 1993 Mice: 3-5 August 1993
Average Age at Necropsy/Sacrifice	
8 weeks	8 weeks
Size of Study Groups	
8 males and 8 females	40 males and 40 females
Method of Distribution	
Animals were distributed randomly into groups of approximately equal mean body weights.	Same as in range-finding studies
Animals Per Cage	
Rats: 2 Mice: 4	Rats: 2 Mice: 4
Method of Animal Identification	
Cage number and ear clip	Cage number and ear clip
Diet	
Autoclaved NIH-31 pellets (Purina Mills, Richmond, IN), available *ad libitum*	Same as in range-finding studies

Table 1
Experimental Designs and Materials and Methods in the 17-Day Studies of Chloral Hydrate (continued)

Range-Finding Studies	Metabolism Studies
Water	
Millipore-filtered water (Jefferson, municipal supply) via water bottle, available *ad libitum*	Same as in range-finding studies
Cages	
Polycarbonate (Lab Products, Seaford, DE)	Same as in range-finding studies
Bedding	
Hardwood chips (Northeastern Products, Warrensburg, NY)	Same as in range-finding studies
Animal Room Environment	
Temperature: 23° to 24° C (rats) 22° to 23° C (mice) Relative humidity: 40% to 60% (rats and mice) Fluorescent light: 12 hours/day Room air: 10 to 15 changes/hour	Temperature: 22° to 24° C (rats) 21° to 24° C (mice) Relative humidity: 38% to 62% (rats) 40% to 60% (mice) Fluorescent light: 12 hours/day Room air: 10 to 15 changes/hour
Doses	
0, 50, 100, 200, 400, or 800 mg/kg in water by gavage at a volume of up to 1 mL (rats) or 0.5 mL (mice)	50 or 200 mg/kg in water by gavage at a volume of up to 1 mL (rats) or 0.5 mL (mice)
Type and Frequency of Observation	
Animals were observed twice daily and were weighed on dose days. Clinical findings were recorded daily. The time necessary to recover from the sedating effect of chloral hydrate was recorded on days 1, 8, and 12.	Animals were observed twice daily and were weighed on dosing days (for determination of dose only). Clinical findings were recorded daily.
Method of Sacrifice	
CO_2 asphyxiation	CO_2 asphyxiation
Necropsy	
Necropsies were performed on all animals. The kidneys and livers were weighed.	None
Histopathology	
In addition to tissues with gross lesions, the kidneys and liver of all animals were examined microscopically.	None
Metabolite Analysis	
None	Blood was collected from four animals per group via the retroorbital sinus at 15 minutes, 1, 3, 6, and 24 hours, and 2, 4, 8, and 16 days after one treatment of chloral hydrate or after 12 treatments of chloral hydrate. Each animal was bled twice except animals evaluated at 24 hours, which were bled once.

Pharmacokinetic Analysis

Based upon the measured plasma concentrations of chloral hydrate its metabolites, a pharmacokinetic model was developed using an analog/digital hybrid computer system (Young *et al.*, 1981). The model, which is shown in Figure 1, along with the differential equations describing the model, assumed that chloral hydrate was metabolized to trichloroacetic acid and trichloroethanol, with the trichloroethanol being further metabolized to trichloroethanol glucuronide. It also assumed that all trichloroethanol was converted to trichloroethanol glucuronide and not excreted as the free alcohol (kEY = 0.0; [Y] = 0), and that 5% to 8% of the chloral hydrate was excreted directly in the urine (kCW) in mice (Cabana and Gessner, 1970). Absorption, metabolism, and elimination were considered to be first-order processes, and chloral hydrate, trichloroacetic acid, and trichloroethanol glucuronide were allowed to be eliminated from the plasma into the urine (or feces). The volumes of distribution of chloral hydrate and trichloroethanol were assigned values of 1 L/kg based upon the data of Cabana and Gessner (1970). The volumes of distribution of trichloroacetic acid and trichloroethanol glucuronide were variables. The ratio of the metabolism of chloral hydrate to trichloroacetic acid (kCA) and trichloroethanol (kCE) was fixed at a value of 5 based on the results of Cabana and Gessner (1970).

Statistical Methods

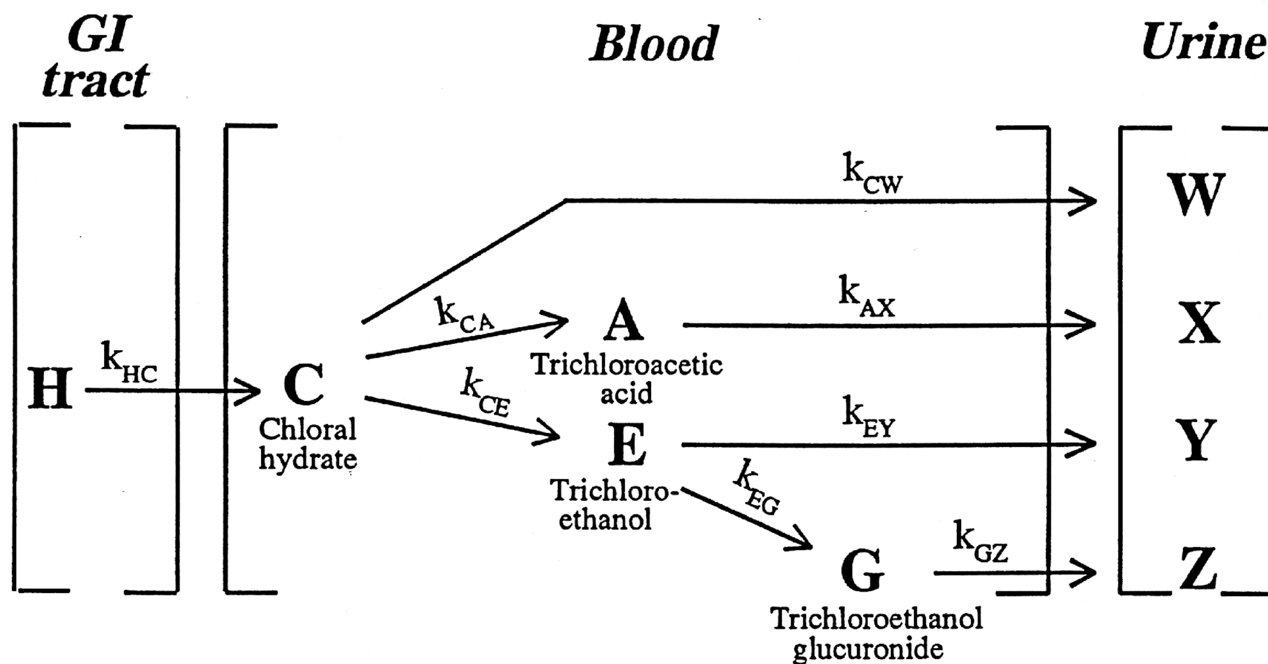
Calculation and Analysis of Lesion Incidence

The Fisher exact test, a procedure based on overall proportion of affected animals, was used to compare lesion incidences between dosed and control animals (Gart *et al.*, 1979). Dose-related trends were tested using the Cochran-Armitage trend (Armitage, 1971).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed using the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of dose-related trends and to determine whether a trend-sensitive test (Williams' test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-response (Dunnett's test). Trend-sensitive tests were used when Jonckheere's test was significant at $P<0.01$.

Peak plasma concentrations (C_{max}) of chloral hydrate and its metabolites were compared by a two-way analysis of variance (ANOVA), with sex and dose, or number of doses and dose, as fixed factors. Peak plasma



$$-dH/dt = k_{HC}[H]$$

$$dC/dt = k_{HC}[H] - k_{CA}[C] - k_{CE}[C] - k_{CW}[C]$$

$$dA/dt = k_{CA}[C] - k_{AX}[A]$$

$$dE/dt = k_{CE}[C] - k_{EG}[E] - k_{EY}[E]$$

$$dG/dt = k_{EG}[E] - k_{GZ}[G]$$

$$dW/dt = k_{CW}[C]$$

$$dX/dt = k_{AX}[A]$$

$$dY/dt = k_{EY}[E]$$

$$dZ/dt = k_{GZ}[G]$$

Figure 1
Pharmacokinetic Model for the Disposition of Chloral Hydrate in Mice

concentrations were compared between species with a one-way ANOVA. The area under the curve (AUC) was calculated using the trapezoid rule, and comparisons between dose, sex, number of doses, or species were made with an unpaired *t*-test. Half-lives were compared by one-way ANOVA, using gender, number of doses, or dose as fixed factors.

In Vitro Metabolism and DNA-Binding Studies

Procurement of Chemicals

Trichloroacetic acid, trichloroethanol, formaldehyde, malondialdehyde, acetaldehyde, acetone, propionaldehyde, N-*tert*-butyl-α-phenylnitrone, 2,4-dinitrophenylhydrazine, 1,1,1,2-tetrachloroethane, *tert*-butyl methyl ether, and 9-chloroanthracene were purchased from Aldrich Chemical Company (Milwaukee, WI). Chloral hydrate, arachidonic acid, hematin, horseradish peroxidase (type II), prostaglandin *H* synthase, pyrazole, α-tocopherol, menadione, chlorzoxazone, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, 3w′-dGMP, calf thymus DNA (sodium salt, type I), nuclease P1, micrococcal nuclease, and spleen phosphodiesterase were obtained from Sigma Chemical Company. T_4 polynucleotide kinase was purchased from U.S. Biochemical Corporation (Cleveland, OH). [1-^{14}C]Chloral hydrate (specific activity, 11.3 mCi/mmol) was synthesized at the study laboratory. Derivatives of formaldehyde, malondialdehyde, acetaldehyde, propionaldehyde, and acetone were prepared by reacting these compounds with 2,4-dinitrophenylhydrazine, as described by Shara *et al.* (1992), and were structurally confirmed by mass spectrometry and ultraviolet-visible absorption spectroscopy. The synthetic 3-(2-deoxy-β-D-erythro-pentafuranosyl) pyrimido[1,2-α]-purin-10(3H)-one (MDA-MG-1) standard was prepared by the procedure of Vaca *et al.* (1992). The malondialdehyde-modified 3′-dAMP adduct was prepared by reacting 3′-dAMP with malondialdehyde as described by Stone *et al.* (1990). This malondialdehyde-modified 3′-dAMP adduct was used as a marker for ^{32}P-postlabeling/thin-layer chromatography (TLC) analysis and was not characterized. All solvents used were high-performance liquid chromatography (HPLC) grade.

Microsome Preparation

Livers were obtained from male B6C3F$_1$ mice (17 to 20 g body weight) and male F344/N rats (120 to 150 g body weight) that were provided by the study laboratory's breeding colony. Twelve human liver samples, which were frozen in liquid nitrogen within 30 minutes of removal and stored at −80° C, were obtained from the U.S. Cooperative Human Tissue Network (Columbus, OH). Mouse, rat, and human liver microsomes were prepared following a the procedure of Fu *et al.* (1991). Mouse liver cytosol was obtained during the preparation of microsomes after centrifugation at 100,000 g for 1 hour and then stored at −80° C.

Pyrazole-induced mouse liver microsomes were prepared from mice injected intraperitoneally with a single dose of pyrazole (200 mg/kg body weight) 24 hours before sacrifice.

Protein concentrations were determined according to the procedure of Lowry *et al.* (1951). Cytochrome P_{450} content and P_{450}2E1 activity were determined as described by Omura and Sato (1964). The cytochrome P_{450} concentrations in the untreated mouse liver microsomes, pyrazole-induced mouse liver microsomes, rat liver microsomes, and human liver microsomes were 0.58 ± 0.04, 1.17 ± 0.23, 0.44 ± 0.11, and 0.58 ± 0.36 nmol/mg, respectively (mean $\pm$ standard deviation). The cytochrome P_{450}2E1 activities in these microsomes were 1.9 ± 0.3, 3.6 ± 0.5, 1.7 ± 0.3, and 1.9 ± 0.4 nmol/mg per minute, respectively (mean $\pm$ standard deviation).

Study Design

Metabolism of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Carbon Tetrachloride by Control and Pyrazole-Induced Mouse Liver Microsomes

A 2-mL incubation mixture was prepared with 200 mM Tris-hydrochloride buffer (pH 7.5) 3 mM magnesium chloride, 300 μM $NADP^+$, 5 mM glucose 6-phosphate, 0.2 IU glucose 6-phosphate dehydrogenase, 2 mg microsomal protein, and 5 mM substrate (chloral hydrate, trichloroacetic acid, trichloroethanol, or carbon tetrachloride).

Identification of chloral hydrate metabolites: The procedure of Gorecki *et al.* (1990) was followed to determine the yield of trichloroacetic acid and trichloroethanol from metabolism of chloral hydrate. After a 10-minute incubation at 37° C, the incubation mixture was combined with 3 M sulfuric acid and the internal standard 1,1,1,2-tetrachloroethane dissolved in *tert*-butyl methyl ether. After centrifugation at 1,500 g for 3 minutes, the organic layer was transferred to a vial and freshly prepared etheral diazomethane was added. The samples were stored under an argon headspace for gas chromatography analysis. The products from the metabolism of trichloroacetic acid and trichloroethanol were analyzed using a similar method.

Determination of free radicals formed by chloral hydrate, trichloroacetic acid, trichloroethanol, and carbon tetrachloride metabolism: To determine if free radicals were formed by the metabolism of chloral hydrate, 30 mM of a free radical trapping agent, N-*tert*-butyl-α-phenylnitrone, was added to the incubation mixture in a closed system, and the mixture was incubated for 10 minutes at 37° C with constant shaking. The incubation solution was transferred with a pressure-lock syringe (Precision Sampling Corporation, Baton Rouge, LA) to a centrifuge tube, mixed with chloroform:methanol (2:1), vortexed, and centrifuged at 3,000 g for 10 minutes. The chloroform layer was then removed for electron spin resonance spectral analysis.

Identification of lipid peroxidation products of chloral hydrate metabolism: To identify lipid peroxidation products of chloral hydrate metabolism, 200 μL of a freshly prepared 2,4-dinitrophenylhydrazine stock solution (30 mg 2,4-dinitrophenylhydrazine dissolved in 10 mL 2 M hydrochloric acid) was added to the incubation mixture remaining after free radical determination. After shaking in a closed system for 10 minutes, the products were extracted with ethyl acetate, the organic layer was collected, and the solvent was removed under reduced pressure. The residue was redissolved in acetonitrile for HPLC spectral analyses. Purified samples from HPLC were collected for subsequent structural confirmation by gas chromatography/mass spectrometry. Control experiments were performed using similar methods with either microsomes that were denatured by heating at 100° C for 1 minute or by omission of the NADPH-generating system.

Quantification of lipid peroxidation products of chloral hydrate, trichloroacetic acid, and trichloroethanol metabolism: In vitro metabolism of 5 mM chloral hydrate, trichloroacetic acid, and trichloroethanol by control microsomes and by pyrazole-induced microsomes was performed under conditions previously described for chloral hydrate metabolism. To compare the lipid peroxidation products formed in experiments using 0.1 and 1.0 mg/mL of enzyme-containing microsomal protein, the total lipid content was held constant by adding 0.9 mg/mL of heat-inactivated microsomes to the incubation mixtures containing 0.1 mg/mL microsomal protein. After incubation, 200 μL of a freshly prepared 2,4-dinitrophenylhydrazine solution was added to the mixture and incubation was continued with shaking in a closed system for an additional 10 minutes. Prior to solvent extraction, 26 nmol 9-chloroanthracene dissolved in 250 μL acetonitrile was added as an internal standard. The reaction mixture was then extracted with ethyl acetate, the organic layer was collected, and the solvent was removed under reduced pressure. The residue was redissolved in 500 μL acetonitrile for HPLC or in ethyl acetate for gas chromatography/mass spectrometry.

The ultraviolet-visible molar extinction coefficients ($M^{-1}cm^{-1}$) at 330 nm (ϵ 330) of the 2,4-dinitrophenylhydrazine hydrazone standards and 9-chloroanthracene in 45% acetonitrile in water were determined: malondialdehyde, 9.49×10^3; formaldehyde, 4.89×10^3; acetaldehyde, 1.25×10^4; acetone, 1.32×10^4; propionaldehyde, 1.51×10^4; chloral hydrate, 2.18×10^4; and 9-chloroanthracene, 3.82×10^3. To determine the recovery after extraction, a mixture of the six synthetic hydrazone standards was mixed with the microsomal reaction mixture. After incubation, 26 nmol 9-chloroanthracene was added, the mixture was treated as described previously, and the resulting products were separated and quantified by HPLC. The recovery of malondialdehyde, formaldehyde, acetaldehyde, acetone, propionaldehyde, and chloral hydrate as hydrazones and 9-chloroanthracene was 76.1 ± 9.3, 78.3 ± 7.7, 85.5 ± 9.1, 91.6 ± 10.7, 91.2 ± 7.9, 93.7 ± 11.9, and 95.4 ± 10.6, respectively (mean ± standard deviation). After normalization, the quantities of the lipid peroxidation products formed from the experiments described previously were calculated. The

chloral hydrate-induced lipid peroxidation products were further confirmed by gas chromatography/mass spectrometry.

Inhibitory Effect of 2,4-Dichloro-6-phenylphenoxyethylamine on Chloral Hydrate-Induced Lipid Peroxidation in Mouse Liver Microsomal Metabolism

The metabolism of 200 μM chloral hydrate by control or pyrazole-induced microsomes was performed as previously described in the absence or presence of 100 or 500 μM 2,4-dichloro-6-phenylphenoxyethylamine, a cytochrome P_{450} inhibitor.

Metabolism of Chlorzoxazone by Mouse Liver Microsomes in the Presence and Absence of 2,4-Dichloro-6-phenylphenoxyethylamine

The metabolism of 2 μM chlorzoxazone by both control and pyrazole-induced microsomes in the presence and absence of 100 or 500 μM 2,4-dichloro-6-phenylphenoxyethylamine was conducted to determine whether control and pyrazole-induced microsomes contain cytochrome P_{450}2E1. A 200-μM chlorzoxazone stock solution was freshly prepared in 50 mM potassium hydroxide. The incubation mixture was preincubated at 37° C for 3 minutes prior to the addition of 10 μL of a chlorzoxazone stock solution. The mixture was then incubated at 37° C for 15 minutes. To isolate and quantify the chlorzoxazone metabolite, 6-hydroxychlorzoxazone, 1 μg 5-fluoro-2(3H)-benzoxazolone in 50 μL 40% phosphoric acid was added as an internal standard after incubation and before solvent extraction. The incubation mixture was then extracted twice with methylene chloride. The combined organic phases were dried under nitrogen gas, redissolved in acetonitrile:water (1:1) for HPLC analysis, and eluted isocratically at 1 mL/minute with acetonitrile:0.5% phosphoric acid (2:3).

Metabolism of Chloral Hydrate with Mouse Liver Microsomes in the Presence of NADH

The metabolism of 200 μM chloral hydrate by control and pyrazole-induced microsomes was performed as previously described except that 300 μM NADH, instead of NADPH, was added to the incubation mixture.

Effect of Xanthine Oxidase, Allopurinol, and NADPH on the Metabolism of Chloral Hydrate by Mouse Liver Microsomes

To test whether xanthine oxidase catalyzes free radical reactions of chloral hydrate, the metabolism of 200 μM chloral hydrate by control and pyrazole-induced microsomes was performed as previously described with the addition of 200 μM hypoxanthine (dissolved in 2 μL dimethyl sulfoxide) or 100 or 500 μM allopurinol (dissolved in 1 or 5 μL dimethyl sulfoxide).

Metabolism of Chloral Hydrate and Trichloroacetic Acid by Control and Pyrazole-Induced Mouse Liver Microsomes in the Presence and Absence of Antioxidants

The metabolism of chloral hydrate and trichloroacetic acid was conducted by following the procedure described previously. The 2-mL incubation mixture contained 100 mM phosphate buffer (pH 7.5), 3 mM magnesium chloride, 300 μM $NADP^+$, 5 mM glucose 6-phosphate, 1 IU glucose 6-phosphate dehydrogenase, 2 mg microsomal protein (untreated or pyrazole-induced), and 1 mM chloral hydrate or trichloroacetic acid. To test the inhibitory effect of antioxidants, 0 to 200 μM concentrations of α-tocopherol, menadione, β-carotene, ascorbic acid, or a combination of ascorbic acid and α-tocopherol, were also added. After the mixture was shaken at 37° C for 15 minutes, 1 mL of a freshly prepared stock solution of 2,4-dinitrophenylhydrazine (30 mg dissolved in 10 mL 2 M hydrochloric acid) was added, and the mixture was shaken in a closed system for an additional 10 minutes. Following the addition of 20 nmol 9-chloroanthracene, an internal standard, the mixture was extracted with ethyl acetate. The organic layer was collected and the solvent was removed under reduced pressure. The residue was redissolved in 500 μL acetonitrile for HPLC analyses. Lipid peroxidation products were quantified as previously described.

Metabolism of Chloral Hydrate by Male B6C3F$_1$ Mouse Liver Cytosol

The ability of mouse liver cytosolic enzymes to metabolize chloral hydrate was also investigated. The incubations were conducted in 50 mM potassium phosphate buffer (pH 7.2). Heat-denatured (100° C for 1 minute) microsomes were added to the cytosolic incubations at a concentration of 1 mg/mL to serve as the source of lipid. The incubation mixtures contained one of the following: (a) buffer only; (b) 5 mM chloral hydrate; (c) 5 mM chloral hydrate and 200 μM NADH; (d) 5 mM chloral hydrate and 200 μM NADPH; (e) 5 mM chloral hydrate and 200 μM $NADP^+$; (f) microsomal protein; (g) 5 mM chloral hydrate and heat-denatured microsomal protein; (h) 5 mM chloral hydrate, heat-denatured microsomal protein, and 200 μM NADH; (i) 5 mM chloral hydrate, heat-denatured microsomal protein, and 200 μM NADPH; or (j) 5 mM chloral hydrate, heat-denatured microsomal protein, and 200 μM $NADP^+$. After 400 μL of a freshly prepared stock solution of 2,4-dinitrophenylhydrazine (30 mg dissolved in 10 mL 2 M hydrochloric acid) was added, the incubation mixture was shaken in a closed system for 10 minutes. The products were then extracted with ethyl acetate, the organic layer was collected, and the solvent was removed under reduced pressure. The residue was redissolved in 500 μL acetonitrile for HPLC analyses. For quantification, the 9-chloroanthracene internal standard was added prior to solvent extraction as previously described.

Enzymatic Reactions of Chloral Hydrate Catalyzed by Horseradish Peroxidase and Prostaglandin H Synthase

To test whether horseradish peroxidase and prostaglandin H synthase catalyze free radical reactions of chloral hydrate, a solution of 50 μM [1-^{14}C]chloral hydrate (specific activity, 11.3 mCi/mmol) dissolved in 10 mL

50 mM sodium phosphate buffer (pH 7.0) was incubated with 0.1 mg/mL horseradish peroxidase in the presence of 100 μM hydrogen peroxide at 37° C for 20 minutes in a closed system connected to a trap containing 5 mL of a saturated barium hydroxide aqueous solution. After incubation, the precipitated barium carbonate was collected and radioactivity was measured.

In the same reaction vessel, a solution of 50 μM [1-^{14}C]chloral hydrate in 50 mM sodium phosphate buffer (pH 6.0) was incubated aerobically with 1,000 IU prostaglandin H synthase, 10 mM arachidonic acid, and 500 μM hematin at 37° C for 2 minutes. After incubation, the precipitated barium carbonate was collected and radioactivity was measured.

Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Rat and Human Liver Microsomes

The metabolism of 1 mM chloral hydrate, trichloroacetic acid, and trichloroethanol was performed as previously described. Lipid peroxidation products were quantified as previously described.

Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Mouse, Rat, and Human Liver Microsomes in the Presence of Calf Thymus DNA

Chloral hydrate, trichloroacetic acid, and trichloroethanol (1 mM) were incubated with untreated mouse liver microsomes, pyrazole-induced mouse liver microsomes, rat liver microsomes, or human liver microsomes in the presence of 1 mg/mL calf thymus DNA for 1 hour to produce DNA adducts. After incubation, the mixture was cooled and extracted twice with buffer-saturated phenol, twice with phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated by the addition of 5 M sodium chloride and ice-cold ethanol.

The 3′,5′-bisphosphate deoxynucleotides (DNA adducts) were then analyzed following the ^{32}P-postlabeling/TLC procedure of Vaca *et al.* (1995) with the exception that for TLC separation, 20 × 20 PEI TLC plates were used and developed employing different solvent systems. The DNA from each of the incubations was enzymatically digested with micrococcal nuclease and spleen phosphodiesterase in 20 mM sodium succinate (pH 6.0) and 10 mM calcium chloride at 37° C for 3 hours. The resulting 3′-monophosphate DNA adducts were incubated with nuclease P1 (Reddy and Randerath, 1986) and then phosphorylated with T_4 polynucleotide kinase and 200 μCi [γ-^{32}P]ATP at 37° C for 1 hour. For chromatography, the incubation mixture was applied to a 20 × 20 cm PEI cellulose plate (Macherey-Nagel, River Vale, NJ). The solvents used were D1, 500 mM ammonium formate (pH 3.5) (a 5-cm wick was attached to the plate and the solvent was developed to about 3 cm of the wick); D2, 3.5 M ammonium formate (pH 3.5); and D3, 1.2 M lithium chloride, 0.5 M tris-hydrochloride, and 6.0 M urea (pH 8.0). Autoradiography was performed with DuPont Cronex films. The

radioactivity of the resulting spots on the PEI plates was quantified by Cerekov counting. The relative adduct levels (RALs) were calculated following the procedure of Reddy and Randerath (1986). To characterize further the adducts after D3 separation, the radioactive spots with the R_f value identical to that of the synthesized malondialdehyde-3′-dGMP standards were extracted with 3.5 M ammonium formate (pH 3.5). After centrifugation at 3,000 g for 30 minutes, the supernatant was injected onto an Ultrasphere ODS column (Beckman) eluted with 100 mM sodium phosphate buffer (pH 3.0) at 1.0 mL/minute in line with a Radiomatic FLO-ONE\Beta A-515 system (Packard Instrument Co., Downers Grove, IL).

Inhibition by Antioxidants of Malondialdehyde-Modified DNA Adducts Formed from the Metabolism of Chloral Hydrate by Mouse Pyrazole-Induced Liver Microsomes in the Presence of Calf Thymus DNA

Four-hour incubations of chloral hydrate with pyrazole-induced mouse liver microsomes were conducted as described above in the presence of 1 mg/mL calf thymus DNA and 0 to 200 μM concentrations of α-tocopherol, menadione, β-carotene, ascorbic acid, or a combination of ascorbic acid and α-tocopherol. After incubation, the mixture was cooled with ice and extracted twice with buffer-saturated phenol, twice with phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated by the addition of 5 M sodium chloride and 2 mL ice-cold ethanol. The DNA adducts were then analyzed by the ^{32}P-postlabeling/TLC procedure as described previously.

Cytotoxicity Assays

H2EI V2 human lymphoblastoid cells expressing cytochrome P_{450}2E1 were obtained from Gentest Corporation (Woburn, MA). The cells were cultured in RPMI 1640 with 2 mM histidinol (Gentest) supplemented with 10% iron-supplemented fetal calf serum (FCS, Hyclone, Logan, UT), 100 IU penicillin/mL, 100 μg/mL streptomycin (Gibco, Gaithersburg, MD), and 2 mM L-glutamine (Gibco). The cultures were incubated at 37° C in 5% CO_2. The cell concentrations were determined every 48 to 72 hours using a hemacytometer. The cells were maintained at a density of 3.5×10^5 to 5×10^5 cells/mL. To reduce background mutants, cells were cultured in RPMI 1640, 2 mM L-glutamine, 2×10^{-5} cells/mL hypoxanthine, 8×10^{-7} cells/mL aminopterin, and 3.2×10^{-5} cells/mL thymidine (HAT supplement, Gibco) for 72 hours. The cells were then cultured in antibiotic-free RPMI 1640 medium with 10% FCS, 2 mM L-glutamine, 2×10^{-4} cells/mL hypoxanthine, and 3.2×10^{-5} cells/mL thymidine for 24 hours. The cells were then allowed to proliferate in complete medium for a minimum of 72 hours before chemical treatment.

For exposure to chloral hydrate, the cells were centrifuged, the supernatant was aspirated, and the number of cells was determined using a hemacytometer. Cells were reseeded at 5×10^5 cells/mL in RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Chloral hydrate

was dissolved in distilled water at 50 mg/mL; calculated volumes were added to achieve doses of 0 to 5,000 μg/mL, and the cells were exposed for 28 hours with no serum present. The cells were collected by centrifugation, counted, and seeded at 5×10^5 cells/mL in complete medium. Cells were maintained in 5% carbon dioxide at 37° C. Cytotoxicity was determined by comparing growth in the exposed population to growth in the control population and expressed as percent survival.

Mutagenicity Assays

Mutation frequency at the tk and hprt loci: Mutation frequency at the *tk* locus was determined by plating cells from the cytotoxicity assay 3 days after exposure ended and at the *hprt* locus 6 or 7 days postexposure. Mutant frequency at the *hprt* locus was measured as described by Penman and Crespi (1987) and at the *tk* locus as described by Crespi *et al*. (1985). Triplicate cultures were used to determine the mutation frequency at each loci. Positive colonies were counted using a microscope, and mutation frequency was calculated using the Poisson formula by Furth *et al*. (1981).

Mutagenicity in Salmonella typhimurium: Reversion to the *his*$^+$ phenotype of *S. typhimurium* histidine auxotrophic strain TA104 was used according to the procedures outlined by Maron and Ames (1983). The post-mitochondrial supernatant fraction (S9, 0.5 mL/plate) was prepared from a liver homogenate of Aroclor 1254-pretreated male Sprague-Dawley rats. The effect of 0 to 6 mg chloral hydrate, trichloroacetic acid, or trichloroethanol per plate was tested under three different conditions: (a) without S9, (b) with S9, and (c) preincubated with S9 for 30 minutes at 37° C in a shaking water bath before the addition of the tester strains. Once the optimum concentrations of chloral hydrate (4 mg/plate), trichloroacetic acid (2 mg/plate), and trichloroethanol (4 mg/plate) were determined, the effects of various antioxidants (range from 0 to 90 μM) were assayed at the optimum concentration of each test compound under the S9 preincubation conditions described above.

Inhibition of chloral hydrate- and trichloroacetic acid-induced S. typhimurium mutagenicity by antioxidants: The synergistic effect of α-tocopherol and ascorbic acid was tested by using a sub-optimal concentration of α-tocopherol (20 μM) with varying ascorbic acid concentrations. The effects of antioxidants alone were also determined as a control.

GENETIC TOXICOLOGY

S. typhimurium Mutagenicity Test Protocol

Testing was performed as reported by Haworth *et al.* (1983). Chloral hydrate was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *S. typhimurium* tester strains TA98, TA100, TA1535, and TA1537 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of chloral hydrate. The high dose was limited by experimental design to 10,000 μg/plate. All positive assays were repeated under the conditions that elicited the positive response.

A positive response in the *S. typhimurium* assay is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is of insufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

Chinese Hamster Ovary Cell Cytogenetics Assays

Testing was performed as reported by Galloway *et al.* (1987). Chloral hydrate was sent to the laboratory as a coded aliquot by Radian Corporation. It was tested in cultured Chinese hamster ovary (CHO) cells for induction of sister chromatid exchanges (SCEs) and chromosomal aberrations (Abs), both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of bromodeoxyuridine-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least three doses of chloral hydrate; the high dose was limited by toxicity. A single flask per dose was used, and tests yielding equivocal or positive results were repeated.

Sister Chromatid Exchange Test: In the SCE test without S9, CHO cells were incubated for 26 hours with chloral hydrate in supplemented McCoy's 5A medium. Bromodeoxyuridine (BrdU) was added 2 hours after culture initiation. After 26 hours, the medium containing chloral hydrate was removed and replaced with fresh medium plus BrdU and Colcemid, and incubation was continued for 2 hours. Cells were then harvested by

mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. In the SCE test with S9, cells were incubated with chloral hydrate, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing serum and BrdU and no chloral hydrate, and incubation proceeded for an additional 26 hours, with Colcemid present for the final 2 hours. Harvesting and staining were the same as for cells treated without S9. All slides were scored blind and those from a single test were read by the same person. Fifty second-division metaphase cells were scored for frequency of SCEs/cell from each dose level. Because significant chemical-induced cell cycle delay was seen at some of the higher doses tested, incubation time for these cultures was lengthened to ensure a sufficient number of scorable (second-division metaphase) cells.

For the SCE data, statistical analyses were conducted on the slopes of the dose-response curves and the individual dose points (Galloway *et al.*, 1987). An SCE frequency 20% above the concurrent solvent control value was chosen as a statistically conservative positive response. The probability of this level of difference occurring by chance at one dose point is less than 0.01; the probability for such a chance occurrence at two dose points is less than 0.001. An increase of 20% or greater at any single dose was considered weak evidence of activity; increases at two or more doses indicated that the trial was positive. The response was determined to be equivocal when a statistically significant trend ($P<0.005$) in the absence of any responses reaching 20% above background was observed.

Chromosomal Aberrations Test: In the Abs test without S9, cells were incubated in McCoy's 5A medium with chloral hydrate for 18.5 hours; Colcemid was added and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with chloral hydrate and S9 for 2 hours, after which the treatment medium was removed and the cells incubated for 10.5 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9. The harvest time for the Abs test was based on the cell cycle information obtained in the SCE test: because cell cycle delay was anticipated in the absence of S9, the incubation period for these cultures was extended.

Cells were selected for scoring on the basis of good morphology and completeness of karyotype (21 ± 2 chromosomes). All slides were scored blind and those from a single test were read by the same person. One hundred first-division metaphase cells were scored at most dose levels; occasionally, when a high percentage of aberrant cells was present in the culture, fewer cells were scored. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations).

Chromosomal aberration data are presented as the percentage of cells with aberrations. To arrive at a statistical decision, analyses were conducted on both the dose-response curve and individual dose points. For a single trial, a statistically significant ($P \leq 0.05$) difference for one dose point and a significant trend ($P \leq 0.015$) was considered weak evidence for a positive response; significant differences for two or more doses indicated the trial was positive. A positive trend, in the absence of a statistically significant increase at any one dose resulted in an equivocal response (Galloway *et al.,* 1987). Ultimately, the trial responses were based on a consideration of the statistical analyses as well as the biological information available to the reviewers.

Drosophila melanogaster Test Protocol

The assays for induction of sex-linked recessive lethal (SLRL) mutations were performed with adult flies as described in Yoon *et al.* (1985). Chloral hydrate was supplied as a coded aliquot from Radian Corporation. It was assayed in the SLRL test by feeding for 3 days to adult Canton-S wild-type males no more than 24 hours old at the beginning of treatment. Because a clearly positive response was not obtained, chloral hydrate was retested by injection into adult males.

To administer chloral hydrate by injection, a glass Pasteur pipette was drawn out in a flame to a microfine filament, and the tip was broken off to allow delivery of the test solution. Injection was performed either manually, by attaching a rubber bulb to the other end of the pipette and forcing through sufficient solution (0.2 to 0.3 μL) to slightly distend the abdomen of the fly, or by attaching the pipette to a microinjector that automatically delivered a calibrated volume. Flies were anaesthetized with ether and immobilized on a strip of tape. Injection into the thorax, under the wing, was performed with the aid of a dissecting microscope.

Toxicity tests were performed to set concentrations of chloral hydrate at a level that would induce 30% mortality after 72 hours of feeding or 24 hours after injection, while keeping induced sterility at an acceptable level. Canton-S males were allowed to feed for 72 hours on an aqueous solution of chloral hydrate in 5% sucrose. In the injection experiments, 24- to 72-hour-old Canton-S males were treated with a solution of chloral hydrate dissolved in saline and allowed to recover for 24 hours. A concurrent saline control group was also included. Treated males were mated to three *Basc* females for 3 days and were given fresh females at 2-day intervals to produce three matings of 3, 2, and 2 days (in each case, sample sperm from successive matings were treated at successively earlier post-meiotic stages). F_1 heterozygous females were mated with their siblings and then placed in individual vials. F_1 daughters from the same parental male were kept together to identify clusters. (A cluster occurs when a number of mutants from a given male result from a single spontaneous premeiotic mutation event and is identified when the number of mutants from that male exceeds the number predicted by a Poisson distribution.) If a cluster was identified, all data from the male in question were discarded.

Presumptive lethal mutations were identified as vials containing fewer than 5% of the expected number of wild-type males after 17 days; these were retested to confirm the response.

SLRL data were analyzed by simultaneous comparison with the concurrent and historical controls using a normal approximation to the binomial test (Margolin *et al.*, 1983). A test result was considered positive if the P value was less than 0.01 and the mutation frequency in the tested group was greater than 0.10% or if the P value was less than 0.05 and the frequency in the treatment group was greater than 0.15%. A test was considered to be inconclusive if the P value was between 0.05 and 0.01 but the frequency in the treatment group was between 0.10% and 0.15% or if the P value was between 0.10 and 0.05 but the frequency in the treatment group was greater than 0.10%. A test was considered negative if the P value was greater than 0.10 or if the frequency in the treatment group was less than 0.10%.

Mouse Bone Marrow Micronucleus Test Protocol

Preliminary range-finding studies were performed. Factors affecting dose selection included chemical solubility and toxicity and the extent of cell cycle delay induced by chloral hydrate exposure. The standard three-exposure protocol is described in detail by Shelby *et al.* (1993). Male mice were injected intraperitoneally three times at 24-hour intervals with chloral hydrate dissolved in phosphate-buffered saline; the total dosing volume was 0.4 mL. Solvent control animals were injected with 0.4 mL of phosphate-buffered saline only. The positive control mice received injections of cyclophosphamide. The mice were killed 24 hours after the final injection, and blood smears were prepared from bone marrow cells obtained from the femurs. Air-dried smears were fixed and stained; 2,000 polychromatic erythrocytes (PCEs) were scored for the frequency of micronucleated cells in each of four or five animals per dose group.

The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among PCEs was analyzed by a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dose group is less than or equal to 0.025 divided by the number of dose groups. A final determination of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final decision is made by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitude of those effects.

Evaluation Protocol

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and differing results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The results presented in the Abstract of this Toxicity Study Report represent a scientific judgement of the overall evidence for activity of the chemical in an assay.

RESULTS

RATS

17-DAY RANGE-FINDING STUDY

One male receiving 800 mg/kg died after receiving five doses. All other animals survived to receive 12 doses, though two 800 mg/kg females died before the end of the study. The final mean body weight of 800 mg/kg males was significantly less than that of the vehicle control group. In addition, the mean body weight gains of 400 and 800 mg/kg males were significantly less than that of the vehicle controls (Table 2). Final mean body weights and body weight gains of all female dosed groups were similar to those of the vehicle control group. The only clinical finding attributed to chloral hydrate treatment was light sedation in the 400 mg/kg groups and heavy sedation in the 800 mg/kg dose groups; sedation subsided within 30 minutes or 3 hours, respectively.

Differences in organ weights generally reflected reduced mean body weights. Mild to moderate kidney mineralization occurred in all dosed and control females; the severity of this lesion was not dose related (Table A2). No treatment-related gross or microscopic lesions were observed in males or females.

TABLE 2
Survival and Body Weights of Rats in the 17-Day Gavage Study of Chloral Hydrate

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g) Initial	Final	Change	Final Weight Relative to Controls (%)
Male					
0	8/8	121 ± 4	190 ± 7	69 ± 3	
50	8/8	118 ± 2	185 ± 3	67 ± 2	97
100	8/8	120 ± 3	189 ± 4	69 ± 2	99
200	8/8	120 ± 3	185 ± 6	65 ± 3	97
400	8/8	119 ± 4	179 ± 7	60 ± 3*	94
800	7/8[c]	114 ± 4	168 ± 7*	54 ± 4**	88
Female					
0	8/8	102 ± 3	133 ± 3	31 ± 1	
50	8/8	103 ± 2	137 ± 2	33 ± 1	102
100	8/8	100 ± 2	136 ± 2	36 ± 2	102
200	8/8	104 ± 4	136 ± 3	32 ± 1	102
400	8/8	102 ± 2	135 ± 3	33 ± 1	101
800	6/8[d]	100 ± 2	126 ± 4	26 ± 3	95

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** Significantly different ($P \leq 0.01$) from the vehicle control group by Williams' test
[a] Number of animals surviving/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Died after the fifth dose; body weight excluded from body weight means
[d] Died after dosing ended but before terminal sacrifice; body weights included in means

17-Day Metabolism Study

Metabolite Analysis

No treatment-related findings of clinical toxicity were observed. Initially, plasma concentrations of chloral hydrate and its metabolites were determined 15 minutes, 1, 3, 6, and 24 hours, and 2, 4, 8, and 16 days after receiving one or the last of 12 doses. Because the metabolite concentrations fell to the limits of detection (1 μg/mL) within approximately 24 hours after dosing, subsequent analyses were restricted to samples obtained within 2 days after the last dose (Table C1 and Figures C1 through C4).

After a single dose of chloral hydrate, the maximum plasma concentrations of chloral hydrate in males and females were observed at the initial sampling time of 15 minutes (Figures C1 and C3). By 1 hour, plasma concentrations of chloral hydrate had dropped substantially, and by 3 hours, chloral hydrate could not be detected. Concentrations of chloral hydrate in plasma decreased by approximately 50% during storage at −80° C; thus, peak concentrations may have been underestimated by a factor of two. Following a single treatment, the peak concentration of chloral hydrate did not differ significantly between males and females; however, there was a dose-related difference ($P=0.003$).

After 12 treatments, the maximum concentrations of chloral hydrate also occurred at the initial sampling time of 15 minutes (Figures C2 and C4). As had been observed following the single dose, the concentrations of chloral hydrate decreased rapidly, and no chloral hydrate was detected 3 hours after the final dose. The peak concentrations of chloral hydrate after 12 doses did not differ significantly between males and females. There was a dose-related difference ($P=0.005$) in males and females. The peak concentrations of chloral hydrate did not differ significantly between 1 and 12 doses. The area under the curve for chloral hydrate was greater for the 200 mg/kg groups than for the 50 mg/kg groups, tended to remain constant with multiple doses, and did not differ between males and females (Table C2).

Trichloroacetic acid was the major metabolite detected in the plasma. After a single dose of 200 mg/kg, the concentration rose slowly and peaked approximately 6 hours after dosing in males (Figure C1) and approximately 3 hours after dosing in females (Figure C3). The concentrations then decreased, and after 2 days the metabolite could no longer be detected. The maximum concentration of trichloroacetic acid from the 50 mg/kg dose in males and females occurred at 1 hour. A slow increase in the plasma concentration of trichloroacetic acid also occurred after 12 chloral hydrate treatments (Figures C2 and C4). The area under the curve for trichloroacetic acid showed significant ($P<0.05$) differences between the 50 and 200 mg/kg doses (Table C2).

Trichloroethanol was assayed both as the free alcohol and its glucuronide conjugate. After 1 or 12 doses, the maximum concentration of free trichloroethanol occurred at 15 minutes, and by 3 hours it approached background concentrations (Figures C1 through C4). The peak concentrations of trichloroethanol did not vary significantly between groups receiving one dose and those receiving 12 doses; however, there was a significant difference ($P<0.001$) between doses. A significant sex difference was observed in the peak plasma concentration of free trichloroethanol; the concentration was 1.3- to 2.0-fold greater ($P<0.017$) in females than in males (Figures C1 through C4). The peak concentrations of trichloroethanol glucuronide tended to occur 1 hour after dosing, especially in the 200 mg/kg groups. A significant ($P<0.001$) dose response was observed for trichloroethanol glucuronide.

The area under the curve for free trichloroethanol showed significant ($P<0.05$) differences between doses, but not between males and females (except for males treated one time with 200 mg/kg) or between the number of doses (Table C2). Similar results were found with the area under the curve for trichloroethanol glucuronide.

Pharmacokinetic Analysis

A pharmacokinetic analysis was conducted to model the metabolite data. Initially, the model was developed to fit plasma concentrations of metabolites for 24 hours after the final dose of chloral hydrate. Because the plasma concentrations for all metabolites, with the exception of trichloroacetic acid, were very low by 6 hours after the last treatment, the final model was limited only to the data collected up to 6 hours after the final dose. Simulations were extended to 8 hours for the purpose of presentation.

Representative metabolic profiles generated by a hybrid computer model are shown in Figure C5, and the data are summarized in Table C3. None of the rate constants differed significantly between male and female rats. Likewise, the rate constants did not differ significantly between the groups receiving one dose and those receiving 12 doses. There was a significant difference in the conversion of trichloroethanol to trichloroethanol glucuronide between the 50 and 200 mg/kg doses, with the rate being 1.7-fold faster at the 50 mg/kg dose.

MICE

16-DAY RANGE-FINDING STUDY

One male in each group except the 400 mg/kg group died before the end of the study (Table 3). Two 800 mg/kg female mice also died before the end of the study. The mean body weight gains of all groups of dosed males were significantly greater than that of the vehicle control group. The final mean body weights of dosed males and females and the mean body weight gains of dosed females were similar to those of the vehicle control groups. The only clinical finding attributed to chloral hydrate treatment was light sedation in the 400 mg/kg groups and heavy sedation in the 800 mg/kg dose groups; sedation subsided within 30 minutes or 3 hours, respectively.

TABLE 3
Survival and Body Weights of Mice in the 16-Day Gavage Study of Chloral Hydrate

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g) Initial	Final	Change	Final Weight Relative to Controls (%)
Male					
0	7/8[c]	22.7 ± 0.5	25.3 ± 0.4	2.5 ± 0.4	
50	7/8[c]	22.5 ± 0.5	26.2 ± 0.6	3.7 ± 0.3*	103
100	7/8[c]	23.0 ± 0.3	26.7 ± 0.3	3.7 ± 0.4*	105
200	7/8[c]	23.3 ± 0.6	27.1 ± 0.7	4.1 ± 0.2**	107
400	8/8	22.5 ± 0.5	26.4 ± 0.5	4.0 ± 0.1**	104
800	7/8[c]	22.1 ± 0.5	25.7 ± 0.5	3.7 ± 0.2*	101
Female					
0	8/8	19.0 ± 0.4	20.1 ± 0.3	1.1 ± 0.2	
50	8/8	18.8 ± 0.2	20.4 ± 0.4	1.6 ± 0.2	101
100	8/8	18.5 ± 0.4	19.6 ± 0.5	1.1 ± 0.2	98
200	8/8	19.2 ± 0.3	20.7 ± 0.4	1.5 ± 0.5	103
400	8/8	18.9 ± 0.3	20.8 ± 0.4	1.9 ± 0.4	104
800	6/8[c]	19.2 ± 0.3	20.9 ± 0.5	1.5 ± 0.5	104

* Significantly different (P≤0.05) from the vehicle control group by Dunnett's test
** P≤0.01
[a] Number of animals surviving/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Died on or before the fifth dose day

The liver weights of 400 mg/kg males and 800 mg/kg males and females were significantly greater than those of the vehicle control groups (Table B2). Foci of subacute inflammation of the liver were observed in all exposed and vehicle control groups of male and female mice (Tables A3 and A4). No lesions were observed that could be attributed to treatment with chloral hydrate.

16-Day Metabolism Study

Metabolite Analysis

No treatment-related findings of clinical toxicity were observed. Initially, plasma concentrations of chloral hydrate and its metabolites were planned to be determined 15 minutes, 1, 3, 6, and 24 hours, and 2, 4, 8, and 16 days after receiving 1 or the last of 12 doses. Because the metabolite concentrations reached the limits of detection (1 µg/mL) within approximately 24 hours after dosing, subsequent analyses were restricted to samples obtained within 2 days after the last dose (Table C4 and Figures C6 through C9).

After a single dose of chloral hydrate, the maximum plasma concentration of chloral hydrate was observed at the initial sampling time of 15 minutes (Figures C6 and C8). By 1 hour, the concentrations had dropped substantially, and by 3 hours, chloral hydrate could not be detected. Following a single treatment, the peak concentrations of chloral hydrate did not differ significantly between males and females; however, there was a significant difference ($P=0.016$) between doses.

When assayed after 12 doses, the maximum concentrations of chloral hydrate also occurred at the initial sampling time of 15 minutes (Figures C7 and C9). As was observed following the single treatment, the concentrations of chloral hydrate decreased rapidly, and no chloral hydrate was detected 3 hours after the final dose. Concentrations of chloral hydrate in plasma decreased by approximately 50% during storage at −80° C; thus, peak concentrations may have been underestimated by a factor of two. The peak concentrations of chloral hydrate after 12 doses did not differ significantly between males and females. There was a significant ($P=0.007$) difference between doses.

The area under the curve for chloral hydrate was greater for the 200 mg/kg groups than the 50 mg/kg groups, tended to increase with multiple doses, and did not differ between males and females (Table C5).

Trichloroacetic acid was the major metabolite detected in the plasma. After a single dose, the peak concentrations occurred 1 hour after dosing (Figures C6 and C8). The concentrations then slowly decreased such that by 2 days, the metabolite could no longer be detected. As with chloral hydrate, the peak concentrations of trichloroacetic acid did not differ between males and females; however, there was a significant difference ($P<0.001$) between doses.

The results observed with trichloroacetic acid after 12 doses were similar to those observed after a single dose: the concentrations tended to peak 1 hour after dosing, the metabolite could not be detected 2 days after the last dose, similar concentrations were found in males and females, and there was a significant difference ($P<0.001$)

between doses (Figures C7 and C9). In addition, the peak concentrations of trichloroacetic acid were significantly ($P<0.002$) greater after a single dose of chloral hydrate than after 12 doses.

The area under the curve for trichloroacetic acid reflected the trends observed with the peak concentrations, with there being significant differences between doses ($P<0.05$), but not between males and females (Table C5). Further, the area-under-the-curve values were significantly ($P<0.05$) greater after one dose than after 12 doses.

Trichloroethanol was assayed both as the free alcohol and its glucuronide conjugate. After 1 or 12 doses, the maximum concentration of free trichloroethanol occurred at 15 minutes, and by 1 hour it approached background concentrations (Figures C6 through C9). The peak concentrations of trichloroethanol did not differ between groups receiving one dose and those receiving 12 doses of chloral hydrate. A difference was observed between males and females dosed once, with the concentrations of trichloroethanol being higher in females ($P=0.018$) than males. This difference was not observed between males and females receiving 12 doses. There was also a significant difference ($P<0.001$) between doses (Figures C6 through C9). A significant ($P<0.03$) difference between males and females was observed in trichloroethanol glucuronide concentrations, with the peak concentrations being approximately two-fold greater in male mice than in female mice after 1 or 12 doses of chloral hydrate. A significant ($P<0.007$) dose response was also observed.

Results of area-under-the-curve analyses for free trichloroethanol and trichloroethanol glucuronide were similar to the peak concentration analyses: with free trichloroethanol there were significant ($P<0.05$) differences between doses, but not between males or females or between the number of treatments (Table C5). The difference between males and females in the peak concentrations of trichloroethanol glucuronide was not observed with the area under the curve. This appeared to be due to a longer half-life of the glucuronide in female mice than in male mice.

Pharmacokinetic Analysis

A pharmacokinetic analysis was conducted to model the metabolite data. Initially, the model was developed to fit plasma concentrations of metabolites for 24 hours after the final dose of chloral hydrate. Because the plasma concentrations for all metabolites, with the exception of trichloroacetic acid, were very low by 6 hours after the last treatment, the final model was limited to the data from 0 to 6 hours.

Representative metabolic profiles generated by a hybrid computer model are shown in Figure C5, and the data are summarized in Table C3. None of the rate constants differed significantly between male and female mice. Likewise, the rate constants did not differ significantly between the 50 and 200 mg/kg doses. There was a

significant difference in the excretion of trichloroacetic acid between 1 and 12 doses, with the rate of elimination being 2.1-fold greater after 12 doses of chloral hydrate.

In vitro Metabolism and DNA-Binding Studies

Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Control and Pyrazole-Induced Mouse Liver Microsomes

Identification of chloral hydrate metabolites: Chloral hydrate metabolites formed by incubation with control microsomes were assayed by gas chromatography. Through comparison to synthetic standards, the chromatographic peaks eluting at 2.96, 5.2, and 6.0 minutes were identified as the recovered chloral hydrate, trichloroacetic acid, and trichloroethanol, respectively (Figure D1). Incubation of chloral hydrate with pyrazole-induced microsomes gave similar results, with the exception that the yields of trichloroacetic acid and trichloroethanol were approximately twice those produced from the incubation of chloral hydrate with control microsomes.

Determination of free radicals formed by chloral hydrate, trichloroacetic acid, and trichloroethanol metabolism: Electron spin resonance spectral measurements indicated that the incubation mixture from metabolism of chloral hydrate by control microsomes formed free radical species. This electron spin resonance spectral pattern (Figure D2) was similar to that formed from the metabolism of carbon tetrachloride (Albano *et al.*, 1982). Both electron spin resonance spectra showed strong triplets of doublets with hyperfine splitting constants α_N 1.4 mT and α_H 0.175 mT in a 50-gauss scan, which is characteristic of carbon free radicals. No electron spin resonance spectral signals were detected from incubation products of the two control experiments, one using boiled microsomes and the other in the absence of an NADPH generating system.

Similar to those of chloral hydrate and carbon tetrachloride, the electron spin resonance spectra of trichloroacetic acid and trichloroethanol (Figure D2) showed strong triplets of doublets with hyperfine splitting constants α_N 1.4 mT and α_H 0.175 mT in a 50-gauss scan. Although all the electron spin resonance spectra in Figure D2 exhibited a similar pattern, the relative intensities of these spectra were different, with carbon tetrachloride $\geq$ chloral hydrate $\geq$ trichloroacetic acid $>>$ trichloroethanol.

Identification of lipid peroxidation products of chloral hydrate metabolism: The HPLC profiles of 2,4-dinitrophenylhydrazine-derived lipid peroxidation products formed from the metabolism of chloral hydrate by control microsomes and by pyrazole-induced microsomes are shown in Figure D3. The HPLC retention times of the identified 2,4-dinitrophenylhydrazine derivatives of malondialdehyde, formaldehyde, acetaldehyde,

acetone, propionaldehyde, and chloral hydrate were 3.8, 4.4, 5.7, 7.3, 8.2, and 11.1 minutes, respectively. 2,4-Dinitrophenylhydrazine eluted at 2.7 minutes.

Analysis by gas chromatography/mass spectrometry of the 2,4-dinitrophenylhydrazine-derived lipid peroxidation products yielded retention times and mass spectra identical to those of the 2,4-dinitrophenylhydrazine derivatives of malondialdehyde, formaldehyde, acetaldehyde, acetone, and propionaldehyde standards. The mass spectra were similar to those reported in the literature (Ekström *et al.*, 1988; Shara *et al.*, 1992). The following gas chromatography retention times were determined: malondialdehyde, 10.4 minutes; formaldehyde, 10.5 minutes; acetaldehyde, 10.2 minutes and 11.3 minutes; propionaldehyde, 11.7 minutes and 11.9 minutes; and acetone, 11.8 minutes. The M+1 isotope ions in the mass spectra of the dinitrophenylhydrazones were elevated due to a space-charging effect in the ion trap. Identical mass spectra were observed for the *cis* and *trans* isomers of acetaldehyde dinitrophenylhydrazones. Similarly, the isomeric dinitrophenylhydrazones of propionaldehyde were resolved into two gas chromatograph peaks with identical mass spectra.

Quantification of lipid peroxidation products of chloral hydrate, trichloroacetic acid, and trichloroethanol: The quantities of lipid peroxidation products formed from the metabolism of chloral hydrate for 10 and 15 minutes with 0.1 or 1.0 mg/mL microsomal protein (control and pyrazole-induced microsomes) are summarized in Table D1. These results indicate that lipid peroxidation from the metabolism of chloral hydrate by control microsomes was about 8- to 10-fold greater than that from incubation in the absence of chloral hydrate ($P<0.02$). The amount of lipid peroxidation from metabolism with pyrazole-induced microsomes was about 20-fold greater than background ($P<0.03$). In all cases, microsomes from pyrazole-induced mice enhanced chloral hydrate-induced lipid peroxidation by twofold that from control microsomes, a value that was marginally ($P<0.07$) significant (Table D1). Among the chloral hydrate-induced lipid peroxidation products, acetone, malondialdehyde, acetaldehyde, and formaldehyde were formed in similar amounts and to a greater extent than propionaldehyde ($P<0.03$; Table D1 and Figures D4 and D5).

The quantities of lipid peroxidation products formed from the metabolism of trichloroacetic acid and trichloroethanol for 10 and 15 minutes with 0.1 or 1.0 mg/mL microsomal protein are also summarized in Table D1. Similar to chloral hydrate, the total amount of trichloroacetic acid- and trichloroethanol-induced lipid peroxidation catalyzed by pyrazole-induced microsomes was about twofold greater than that from control microsomes, a difference that was marginally significant ($P<0.07$). While the level of trichloroacetic acid-induced lipid peroxidation was very similar to that induced by chloral hydrate, trichloroethanol induced three- to fourfold less lipid peroxidation ($P<0.05$; Table D1).

Inhibitory Effect of 2,4-Dichloro-6-phenylphenoxyethylamine on Chloral Hydrate-Induced Lipid Peroxidation in Mouse Liver Microsomal Metabolism

No significant decrease in chloral hydrate-induced lipid peroxidation resulted from 100 μM 2,4-dichloro-6-phenylphenoxyethylamine. When 500 μM of 2,4-dichloro-6-phenylphenoxyethylamine was used, the inhibition of total chloral hydrate-induced lipid peroxidation from control microsomes was 67% ($P<0.02$), while with pyrazole-induced microsomes, the inhibition was 58% ($P<0.07$; Table D2).

Metabolism of Chlorzoxazone by Mouse Liver Microsomes in the Presence and Absence of 2,4-Dichloro-6-phenylphenoxyethylamine

The concentrations of the metabolite 6-hydroxychlorzoxazone formed from incubation with control and pyrazole-induced microsomes were 1.9 ± 0.3 and 4.2 ± 0.7 nmol/mg per minute, respectively (mean ± standard deviation). With the incorporation of 100 μM 2,4-dichloro-6-phenylphenoxyethylamine in the incubation with control or pyrazole-induced microsomes, the yield of 6-hydroxychlorzoxazone was not decreased significantly (Table D3). Increasing the concentration to 500 μM 2,4-dichloro-6-phenylphenoxyethylamine resulted in a 78.9% inhibition ($P<0.03$) with control microsomes and a 71.4% inhibition ($P<0.03$) with pyrazole-induced microsomes (Table D3).

Metabolism of Chloral Hydrate with Mouse Liver Microsomes in the Presence of NADH

The yield of lipid peroxidation catalyzed by NADH was 10- to 12-fold less than that by NADPH ($P<0.04$; Table D4).

Effect of Xanthine Oxidase, Allopurinol, and NADPH on the Metabolism of Chloral Hydrate by Mouse Liver Microsomes

In the absence of NADPH, the chloral hydrate-induced lipid peroxidation was essentially at the background level, while the chloral hydrate-induced lipid peroxidation was about 10-fold greater in the presence of NADPH ($P<0.02$; Table D5). The incorporation of 200 μM hypoxanthine or 100 or 500 μM allopurinol did not affect chloral hydrate-induced lipid peroxidation significantly (Table D5).

Metabolism of Chloral Hydrate and Trichloroacetic Acid by Control and Pyrazole-Induced Mouse Liver Microsomes in the Presence and Absence of Antioxidants

α-Tocopherol, β-carotene, menadione, and a combination of ascorbic acid and α-tocopherol exhibited significant inhibitory effects on chloral hydrate-induced lipid peroxidation ($P<0.05$; Figure D6 and Table D6). All of the antioxidants showed a dose-response relationship from 10 to 100 μM. Beyond 100 μM there was no further effect. Ascorbic acid did not exhibit an inhibitory effect. Among these antioxidants, a combination of ascorbic

acid and α-tocopherol showed the strongest inhibitory effect, followed by α-tocopherol, menadione, and β-carotene. At 200 μM, a combination of α-tocopherol and ascorbic acid reduced chloral hydrate-induced mutagenicity to near the background level. When tested at a dose of 25 μM or greater, a combination of α-tocopherol and ascorbic acid exhibited a higher inhibitory effect than α-tocopherol alone ($P<0.05$).

Similar to inhibition of chloral hydrate-induced lipid peroxidation, α-tocopherol effectively inhibited trichloroacetic acid-induced lipid peroxidation ($P<0.05$), while menadione was less effective (Figure D7). The inhibition of each of the lipid peroxidation products, malondialdehyde, formaldehyde, acetaldehyde, acetone, and propionaldehyde, by α-tocopherol and menadione was analyzed (Table D7). In general, each of the lipid peroxidation products was inhibited proportionally.

Metabolism of Chloral Hydrate by Male B6C3F$_1$ Mouse Liver Cytosol

After incubation, HPLC analysis of the reaction residues indicated that no chloral hydrate-induced lipid peroxidation products were formed from any of the assays.

Enzymatic Reactions of Chloral Hydrate Catalyzed by Horseradish Peroxidase and Prostaglandin H Synthase

In two assays with horseradish peroxidase, an average 3.04% ± 0.77% of the chloral hydrate (mean ± standard deviation) was converted into carbon dioxide. The carbon dioxide formed in two assays with prostaglandin H synthase was 5.21% ± 1.19%.

Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Rat and Human Liver Microsomes

The HPLC profiles of 2,4-dinitrophenylhydrazine-derived lipid peroxidation products formed from metabolism of chloral hydrate by rat liver microsomes and human liver microsomes are shown in Figure D8. The total quantities of the chloral hydrate-, trichloroacetic acid-, and trichloroethanol-induced lipid peroxidation products formed from metabolism by rat and human liver microsomes were similar (Table D8). As was observed with mouse liver microsomes (Table D1), the total quantity of chlorohydrate- and trichloracetic acid-induced lipid peroxidation products was approximately twofold greater than that observed with trichloroethanol-induced microsomes, a value that was marginally significant ($P<0.07$; Table D8).

Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Mouse, Rat, and Human Liver Microsomes in the Presence of Calf Thymus DNA

In all cases, the only DNA adduct detected was the malondialdehyde-modified DNA adduct 3-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimido[1,2α]purin-10(3H)-one (MDA-MG-1), the structure of which is shown in Figure 2. The quantities of this adduct from each incubation are summarized in Tables D9 and D10. For comparison, the quantities of malondialdehyde formed in incubations in the absence of calf thymus DNA are also given. The amounts of the MDA-MG-1 adduct formed from metabolism by rat and human liver microsomes were similar, and these were significantly less than that observed with pyrazole-induced mouse microsomes ($P<0.05$). The total quantities of trichloroethanol-induced MDA-MG-1 adduct from each of the microsomes were significantly less that the quantities found from incubations conducted with chloral hydrate and trichloroacetic acid ($P<0.05$).

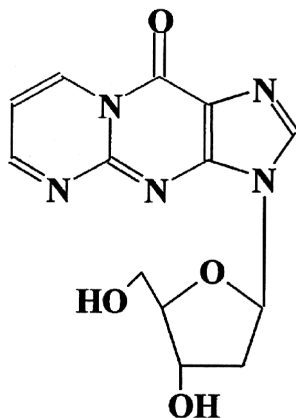


FIGURE 2
3-(2-Deoxy-ß-D-erythro-pentofuranosyl)pyrimido[1,2α]purin-10(3H)-one (MDA-MG-1)

Inhibition by Antioxidants of Malondialdehyde-Modified DNA Adducts Formed from the Metabolism of Chloral Hydrate by Mouse Pyrazole-Induced Liver Microsomes in the Presence of Calf Thymus DNA

The results are summarized in Table D11 and Figure D9. For comparison, the yields of malondialdehyde formed from incubation of chloral hydrate with mouse pyrazole-induced liver microsomes in the absence of calf thymus DNA are also given. Ascorbic acid was did not inhibit the formation of either malondialdehyde or MDA-MG-1. α-Tocopherol demonstrated a significant inhibition ($P<0.05$) at all doses tested. A combination of α-tocopherol and ascorbic acid exhibited the strongest inhibitory effect on MDA-MG-1 formation. The inhibitory effect of a combination of α-tocopherol and ascorbic acid at 100 or 200 μM was significantly greater than that of α-tocopherol alone ($P<0.01$). The quantity of chloral hydrate-induced MDA-MG-1 exhibited a linear correlation with the quantity of malondialdehyde in the microsomes ($P<0.0001$; Figure D10).

Cytotoxicity Assay

Cytotoxicity data are shown in Figure D11. Cell survival was approximately 60% at the lowest chloral hydrate dose tested. At the highest dose tested, survival was approximately 45%.

Mutagenicity Assays

Mutation at the tk and hprt loci: In transgenic cells containing cytochrome P_{450}2E1, chloral hydrate induced a dose-dependent increase in mutant fractions at both the *hprt* and *tk* loci. However, at the highest dose tested (5,000 μg/mL), approximately fivefold more mutants were recovered at the *tk* locus than at the *hprt* locus (Figure D11). This differential sensitivity occurred at doses in which 50% of the cells survived and indicated that the DNA damage caused by chloral hydrate primarily results in large deletions (DeMarini *et al.*, 1989). When the parent cell line containing the vector without the P_{450} cDNA was treated with the same concentrations of chloral hydrate, mutations were not detected at either locus (data not shown). These results indicated that cytochrome P_{450}2E1 is responsible for metabolism of chloral hydrate to a DNA-damaging species.

Mutagenicity in Salmonella typhimurium: Chloral hydrate, trichloroacetic acid, and trichloroethanol all exhibited the greatest mutagenicity when the test was conducted after preincubation with S9 for 3 minutes. Chloral hydrate was the most mutagenic compound and exhibited a linear dose-response relationship (Figure D12). Trichloroacetic acid was less mutagenic and was cytotoxic at 4 mg/plate. Trichloroethanol was the least mutagenic of the three compounds.

Inhibition of chloral hydrate- and trichloroacetic acid-induced S. typhimurium mutagenicity by antioxidants: Chloral hydrate was assayed at 4 mg/plate; due to cytotoxicity, trichloroacetic acid was assayed at 2 mg/plate.

Because of the low mutagenicity of trichloroethanol, the effect of vitamins on trichloroethanol-induced mutations was omitted. Both α-tocopherol and menadione inhibited chloral hydrate-induced mutation significantly ($P<0.05$) and exhibited a positive dose-response relationship (Figure D13). However, α-tocopherol and menadione inhibited trichloroacetic acid-induced mutagenicity only at doses of 50 µM or greater. When tested at 87 µM, α-tocopherol and menadione inhibited 97% and 78% of chloral hydrate-induced mutagenicity, respectively.

Inhibition of trichloroacetic acid-induced mutagenicity by ascorbic acid and by a combination of ascorbic acid and α-tocopherol is shown in Figure D14. Ascorbic acid exhibited a significant inhibitory effect only at the 80 µM dose. All dose combinations of α-tocopherol and ascorbic acid exhibited significant inhibition ($P<0.05$).

GENETIC TOXICOLOGY

Chloral hydrate gave positive responses in both *in vitro* and *in vivo* mutagenicity assays. It induced mutations in *S. typhimurium* strain TA100, with and without liver S9 activation enzymes; an equivocal response was obtained in *S. typhimurium* strain TA98 in the absence of S9; and no mutagenicity was detected with strain TA1535 or TA1537, with or without S9 (Table E1; Haworth *et al.*, 1983). In addition to gene mutations in bacterial cells, chloral hydrate was shown to produce chromosomal damage in mammalian cells. It induced significant increases in sister chromatid exchanges (Table E2) and chromosomal aberrations (Table E3) in cultured Chinese hamster ovary cells, with and without S9. Results of sex-linked recessive lethal tests in *Drosophila melanogaster* were inconclusive (Table E4; Yoon *et al.*, 1985). Chloral hydrate, administered by feeding in 5% sucrose, produced only a small increase in sex-linked recessive lethal mutations in the germ cells of male flies; this result was considered inconclusive. A second sex-linked recessive lethal assay that used injection as the route of administration gave negative results.

An *in vivo* mammalian mutagenicity study, a mouse bone marrow micronucleus test, was performed with chloral hydrate (Table E5). In this test, male mice injected with 125 to 500 mg/kg showed a significant dose-related trend in the frequency of micronucleated erythrocytes in bone marrow sampled 24 hours after treatment. Thus, chloral hydrate gave positive responses in both *in vivo* and *in vitro* assays for chromosomal damage.

DISCUSSION

Chloral hydrate, a widely used sedative and hypnotic, was nominated for study by the Food and Drug Administration based on widespread human exposure, the potential hepatotoxicity of chloral hydrate, and the toxicity of related chemicals. For rats and mice, metabolic profiles of chloral hydrate were developed at doses known to induce hepatic neoplasms in mice.

Chloral hydrate is hepatocarcinogenic to male B6C3F$_1$ mice when administered in drinking water at a concentration of 1,000 ppm, equivalent to 166 mg/kg body weight per day (Daniel *et al.*, 1992a). Male B6C3F$_1$ mice administered 800 or 1,400 ppm chloral hydrate in drinking water (equivalent to 87 or 165 mg chloral hydrate/kg body weight per day, respectively) had increased incidences of multiple hepatic neoplasms compared to the controls (DeAngelo and George, 1995). In contrast, only limited toxicity has been observed in male and female Sprague-Dawley rats administered up to 2,400 ppm (288 mg/kg per day) chloral hydrate for 90 days (Daniel *et al.*, 1992b), and no increases in the incidences of hepatocellular carcinomas occurred in male F344 rats administered up to 1,000 ppm (174 mg/kg per day) chloral hydrate in drinking water for 2 years (DeAngelo and George, 1995). Likewise, there was not an increase in tumorigenicity in male and female Sprague-Dawley rats administered up to 135 mg/kg chloral hydrate per day in drinking water for 124 and 128 weeks, respectively (Leuschner and Beuscher, 1998).

In the range-finding studies, chloral hydrate induced dose-related decreases in mean body weight gains of male rats; there were no changes in organ weights. In rats, sedation times were constant throughout the study, indicating no induction of microsomal enzymes; this finding is consistent with the absence of effects on liver weights. Dosed male mice had significantly increased mean body weight gains; increases in liver weights occurred in male mice in the 400 and 800 mg/kg groups and female mice in the 800 mg/kg group. Increases in liver weight in mice administered chloral hydrate have been observed in other studies (Sanders *et al.*, 1982; Daniel *et al.*, 1992b) and were presumably due to an increase in the synthesis of microsomal enzymes, which could, in turn, explain the decreased sedation observed at later time points in mice in the 800 mg/kg groups in the current studies. No treatment-related gross or microscopic lesions were observed in rats or mice.

In the metabolism studies, peak plasma concentrations of chloral hydrate in male and female rats and mice were detected at the initial sampling time of 15 minutes. The peak concentrations did not differ between males and females or between the number of doses but were dose proportional. Peak concentrations of chloral hydrate

were also significantly (P=0.03) greater in rats than in mice. One hour after administration, the concentrations of chloral hydrate were substantially reduced, and 3 hours after treatment, no chloral hydrate could be detected. A pharmacokinetic model based upon these data indicated a plasma half-life ($t_{½}$) of 8.4 minutes in rats and 7.2 minutes in mice. These values are faster than the whole-body $t_{½}$ determined in male Swiss Webster mice after intraperitoneal administration (12 minutes; Cabana and Gessner, 1970) or the $t_{½}$ obtained following intravenous administration to male $B6C3F_1$ mice (5-24 minutes; Abbas *et al.*, 1996). These results suggest that the absorption and systemic distribution of chloral hydrate following an oral dose are not limiting factors in its metabolism. The values for $t_{½}$ are also substantially faster than those observed in infants and children (1-10 hours; Mayers *et al.*, 1991).

The metabolic reduction of chloral hydrate leads to the formation of trichloroethanol, which can be conjugated to give trichloroethanol glucuronide. Both of these metabolites were detected in the plasma of rats and mice administered chloral hydrate, and the assumption was made that the trichloroethanol was converted to the glucuronide before being excreted. This assumption seemed valid because free trichloroethanol appears to be a very minor urinary metabolite compared to trichloroethanol glucuronide (Gorecki *et al.*, 1990; Abbas *et al.*, 1996). The peak concentration of free trichloroethanol occurred at 0.25 hour, and the $t_{½}$ of 4.5 minutes in mice was similar to that of chloral hydrate. This $t_{½}$ is considerably faster than that of 211 minutes given by Cabana and Gessner (1970) for Swiss Webster mice administered chloral hydrate intraperitoneally or to those of 15.6 to 21.6 minutes reported by Abbas *et al.* (1996) in male $B6C3F_1$ mice treated intravenously. The reason for this difference is not known but may reflect differences in the route of administration, the amount given, or the mouse strain. The $t_{½}$ of trichloroethanol in rats (8.0 minutes) was also significantly (P=0.001) greater than that observed in mice.

Trichloroethanol is considered to be the metabolite responsible for the sedating activity of chloral hydrate (AHFS, 1999). Upon repeated dosing with higher doses of chloral hydrate (i.e, 400 and 800 mg/kg), mice showed a decreased sedation, suggesting a decreased formation of trichloroethanol or a faster rate of metabolism. Although the peak concentrations and the area under the curve for trichloroethanol were dose dependent, the values did not differ significantly between 1 and 12 doses; thus, there must be another reason (e.g., central nervous system tolerance) for the decrease in sedation that was observed upon repeated administration of chloral hydrate. Upon repeated dosing of rats with chloral hydrate, there was no change in the sedating effect, which suggested that there might be similar concentrations of trichloroethanol. The interpretation was consistent with the observation that the peak concentrations or the area under the curve of trichloroethanol did not differ between 1 and 12 doses.

In mice, trichloroethanol glucuronide had a $t_{1/2}$ of 7.3 minutes, which is significantly ($P<0.001$) shorter than the 24 minutes observed in rats and the 52-minute $t_{1/2}$ reported for dogs (Breimer *et al.*, 1974). It is also much shorter than the 7 to 40 hours observed in humans; these values decrease with increasing age (Breimer *et al.*, 1974; Mayers *et al.*, 1991).

Trichloroacetic acid was the most persistent metabolite detected in mice, with a $t_{1/2}$ of 8.5 hours. Fisher *et al.* (1991) have reported a pronounced sex difference in the elimination of trichloroacetic acid from B6C3F_1 mice administered trichloroethylene, with the $t_{1/2}$ in males (5.6 hours) being twice that of females (2.2 hours). In a subsequent study (Fisher and Allen, 1993), values of 24.8 and 11.2 hours were obtained for males and females, respectively. No such sex difference was observed in the current studies, but the concentrations of trichloroacetic acid were found to be significantly greater after a single dose then after 12 doses. This appeared to be a consequence of a 1.8-fold increase ($P=0.006$) in the rate of elimination of trichloroacetic acid after 12 doses of chloral hydrate. The $t_{1/2}$ of trichloroacetic acid in mice (8.5 hours) did not differ significantly from the $t_{1/2}$ of 11.2 hours found in rats; however, it was considerably shorter than the 4 to 5 days reported for humans (Breimer *et al.*, 1974).

There is considerable uncertainty concerning the mechanism by which chloral hydrate induces liver neoplasms in male B6C3F_1 mice. Given the short $t_{1/2}$ of chloral hydrate, attention has focused on its metabolites, in particular trichloroacetic acid. Trichloroacetic acid is known to be hepatocarcinogenic in B6C3F_1 mice (Herren-Freund *et al.*, 1987; Bull *et al.*, 1990; DeAngelo and Daniel, 1990) but does not induce hepatic neoplasms in rats (Bull *et al.*, 1993). Fisher *et al.* (1991) suggested that the greater sensitivity of mice as compared to rats to the carcinogenic effects of trichloroethylene is due to a greater body burden of trichloroacetic acid in mice. This could be particularly important for human risk assessment (Fisher and Allen, 1993) given the very long $t_{1/2}$ of trichloroacetic acid in humans (Breimer *et al.*, 1974). Nevertheless, while the increased body burden of trichloroacetic acid could explain the difference in carcinogenicity of trichloroethylene between mice and rats, this mechanism may not be applicable to chloral hydrate because the area under the curve of trichloroacetic acid following multiple doses with chloral hydrate was 1.5-fold greater in rats than in mice.

Dichloroacetic acid is another metabolite considered to be important in the carcinogenicity of trichloroethylene. Dichloroacetic acid is carcinogenic in B6C3F_1 mice (Herren-Freund *et al.*, 1987; Bull *et al.*, 1990; DeAngelo *et al.*, 1991), and Bull *et al.* (1993) found substantial concentrations of dichloroacetic acid in the blood of mice administered trichloroethylene, while none was found in the blood of rats treated with identical doses of trichloroethylene. Dichloroacetic acid was also found at concentrations approaching those of trichloroacetic acid in male B6C3F_1 mice treated intravenously with chloral hydrate (Abbas *et al.*, 1996). More recently,

Henderson *et al.* (1997) reported the presence of dichloroacetic acid in the plasma of children treated orally with chloral hydrate. In contrast, dichloroacetic acid was not detected (<1 μg per mL) in the plasma of rats or mice during the present studies. While this does not preclude the rapid excretion of any dichloroacetic acid that may have been formed, this seems unlikely given the persistence noted by Abbas *et al.* (1996). Nonetheless, the failure to detect dichloroacetic acid suggests that it may not play a role in the carcinogenicity of chloral hydrate following oral exposure. In accord with this conclusion, Merdink *et al.* (1998) were not able to detect dichloroacetic acid in B6C3F$_1$ mice treated with chloral hydrate and indicated that previous reports of this metabolite may be the result of an experimental artifact.

Results of metabolism and DNA adduct formation studies indicated that *in vitro* metabolism of chloral hydrate by male B6C3F$_1$ mouse liver microsomes generated free radical intermediates that resulted in endogenous lipid peroxidation, forming malondialdehyde, formaldehyde, acetaldehyde, acetone, and propionaldehyde. Induction of endogenous lipid peroxidation by xenobiotics through generation of free radical species results in alterations of cellular function, genotoxic damage, and tumor initiation (Comporti, 1985; Vaca *et al.*, 1988). Malondialdehyde, formaldehyde, and acetaldehyde are known carcinogens, and the DNA adduct of malondialdehyde has been detected in humans (Kerns *et al.*, 1983; Sellakumar *et al.*, 1985; Woutersen *et al.*, 1986; Woutersen and Feron, 1987; Basu *et al.*, 1988; Feron *et al.*, 1991; Chaudhary *et al.*, 1994) and is mutagenic (Benamira *et al.*, 1995). Trichloroacetic acid and trichloroethanol, the primary metabolites of chloral hydrate, also generated free radicals and induced lipid peroxidation. Lipid peroxidation induced by trichloroacetic acid nearly equaled that induced by chloral hydrate, while that from trichloroethanol was three- to fourfold less, suggesting that oxidative metabolism of chloral hydrate to trichloroacetic acid is a predominant pathway and reductive metabolism to trichloroethanol is a relatively minor route.

Pyrazole is a specific inducer of cytochrome P$_{450}$2E1. To determine if this cytochrome P$_{450}$ isoform is responsible for the metabolism of chloral hydrate, trichloroacetic acid, and trichloroethanol, incubations were conducted with liver microsomes of B6C3F$_1$ mice pretreated with pyrazole. The pyrazole-induced microsomes yielded lipid peroxidation products at concentrations two- to threefold greater than those from liver microsomes of untreated mice. Chlorzoxazone is a substrate for cytochrome P$_{450}$2E1 and is metabolized to 6-hydroxychlorzoxazone (Peter *et al.*, 1990). To confirm the involvement of cytochrome P$_{450}$2E1, the metabolism of chlorzoxazone by both control and pyrazole-induced microsomes was examined; both types of microsomes generated 6-hydroxychlorzoxazone, with the greater amount being generated by the pyrazole-induced microsomes. Additional support for the involvement of cytochrome P$_{450}$ in the metabolism of chloral hydrate was obtained by demonstrating a decrease in lipid peroxidation when conducting microsomal incubations in the presence of 2,4-dichloro-6-phenylphenoxyethylamine, a general cytochrome P$_{450}$ inhibitor. These findings

suggest that cytochrome P_{450} is the enzyme system responsible for metabolic activation of chloral hydrate leading to lipid peroxidation and that cytochrome P_{450}2E1 is the major isozyme. Human cytochrome P_{450}2E1 has been reported to be the major cytochrome isozyme involved in catalyzing the metabolism of halogen-containing chemicals of low molecular weight, including carbon tetrachloride, chloroform, methylene chloride, chloromethane, α,α,α-trichloroethane, 1,2-dichloropropane, ethylene dichloride, vinyl chloride, and trichloroethylene (Guengerich *et al.*, 1981). The current study results are consistent with these findings and suggest that cytochrome P_{450}2E1 could be the major isozyme involved in the metabolic activation of chloral hydrate and trichloroacetic acid in humans.

The metabolism of chloral hydrate, trichloroacetic acid, and trichloroethanol by pyrazole-induced mouse liver microsomes, untreated mouse liver microsomes, rat liver microsomes, and human liver microsomes in the presence of calf thymus DNA resulted in the formation of the malondialdehyde-modified DNA adduct, MDA-MG-1, an adduct that has been detected in human liver and breast cells and leukocytes (Chaudhary *et al.*, 1994; Vaca *et al.*, 1995) and represents a mutagenic lesion in *Escherichia coli* (Benamira *et al.*, 1995).

The amount of malondialdehyde formed from metabolism by pyrazole-induced mouse microsomes was twice that from rat or human liver microsomes. The amount of MDA-MG-1 formed from the metabolism of chloral hydrate, trichloroacetic acid, and trichloroethanol by mouse, rat, and human liver microsomes exhibited a linear correlation with the quantity of malondialdehyde formed under incubation conditions in the absence of calf thymus DNA. The quantities of malondialdehyde and MDA-MG-1 induced by trichloroacetic acid in the microsomes were nearly equal to those induced by chloral hydrate, while the quantities induced by trichloroethanol were two- to threefold less than those induced by chloral hydrate or trichloroacetic acid. These results suggest that metabolism of chloral hydrate to trichloroacetic acid is a principal activation pathway and that reductive metabolism to trichloroethanol produces less lipid peroxidation and subsequent MDA-MG-1 formation. MDA-MG-1 is persistent in the mouse liver, having a $t_{½}$ of 12.5 days (Kautiainen *et al.*, 1993); therefore, it could serve as a biomarker for the study of xenobiotic-induced and naturally formed lipid peroxidation and endogenous DNA adduct formation.

The mutagenicity, lipid peroxidation, and MDA-MG-1 adduct formation induced by chloral hydrate and trichloroacetic acid were inhibited by the vitamins α-tocopherol, menadione, β-carotene, and a combination of ascorbic acid and α-tocopherol. The inhibitory effect was dose related; the combination of ascorbic acid and α-tocopherol was the most effective inhibitor. This inhibitory effect may be due to the ability of antioxidants to quench the free radicals formed by chloral hydrate and its metabolites. Among the reactive oxyradicals generated by lipid peroxidation, peroxy radicals are the most abundant, and their reaction with another fatty acid

is the slowest step in the lipid peroxidation free radical process. The free radical chain-breaking antioxidant, α-tocopherol, efficiently quenches peroxy radicals (Comporti, 1985; Vaca *et al.*, 1988; Liebler, 1993); the effectiveness of the combination of ascorbic acid and α-tocopherol is partly due to the recycling of α-tocopherol by ascorbic acid. This synergistic effect is in good agreement with many *in vitro* results reported in the literature (Aust *et al.*, 1993).

In summary, due to the absence of chloral hydrate-induced lesions in rats and mice, no-observed-adverse-effect levels (NOAELs) were based on body weights of rats and liver weights of mice. The NOAELs for rats and mice were 200 mg/kg. Chloral hydrate was rapidly metabolized by rats and mice, with trichloroacetic acid occurring as the major metabolite. Peak concentrations of trichloroacetic acid occurred more quickly in mice. Plasma concentrations of chloral hydrate were dose dependent, but metabolic rates were unaffected by dose or gender. Chloral hydrate was mutagenic *in vitro* and *in vivo*. Metabolism of chloral hydrate and its metabolites produced free radicals that resulted in lipid peroxidation in liver microsomes of mice, rats, and humans. Induction of cytochrome P_{450}2E1 by pyrazole increased the concentrations of lipid peroxidation products; inhibition of cytochrome P_{450}2E1 by 2,4-dinitrophenylhydrazine reduced these concentrations. Metabolism of chloral hydrate and its metabolites by mouse, rat, and human liver microsomes formed malondialdehyde, and in the presence of calf thymus DNA formed the DNA adduct MDA-MG-1. Antioxidants reduced the amount of lipid peroxidation products and MDA-MG-1 formation by liver microsomes.

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APPENDIX A
SUMMARY OF NONNEOPLASTIC LESIONS IN RATS AND MICE

TABLE A1 Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 17-Day Range-Finding Gavage Study of Chloral Hydrate A-2

TABLE A2 Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 17-Day Range-Finding Gavage Study of Chloral Hydrate A-4

TABLE A3 Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 16-Day Range-Finding Gavage Study of Chloral Hydrate A-6

TABLE A4 Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 16-Day Range-Finding Gavage Study of Chloral Hydrate A-8

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 17-Day Range-Finding Gavage Study of Chloral Hydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Disposition Summary						
Animals initially in study	8	8	8	8	8	8
Early deaths						
Natural death						1
Survivors						
Terminal sacrifice	8	8	8	8	8	7
Animals examined microscopically	8	8	8	8	8	8
Alimentary System						
None						
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
Testes	(1)				(1)	
Degeneration, marked					1 (100%)	
Mineralization, marked					1 (100%)	
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 17-Day Range-Finding Gavage Study of Chloral Hydrate

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Special Senses System						
None						
Urinary System						
Kidney	(8)	(8)	(8)	(8)	(8)	(8)
Autolysis, minimal						2 (25%)
Degeneration, minimal, renal tubule			1 (13%)			
Dilatation, minimal, renal tubule					1 (13%)	2 (25%)
Inflammation, chronic, focal, minimal						1 (13%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

Table A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 17-Day Range-Finding Gavage Study of Chloral Hydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Disposition Summary						
Animals initially in study	8	8	8	8	8	8
Survivors						
Died after dose day 12						1
Terminal sacrifice	8	8	8	8	8	7
Animals examined microscopically	8	8	8	8	8	8
Alimentary System						
Liver	(8)	(8)	(8)	(8)	(8)	(8)
Developmental malformation	2 (25%)					
Focal cell change, mild			1 (13%)			
Focal cell change, minimal	1 (13%)					
Hematopoietic cell proliferation, minimal			3 (38%)	1 (13%)		
Infiltration, cellular, mast cell, minimal		1 (13%)				
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
None						
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 17-Day Range-Finding Gavage Study of Chloral Hydrate

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Respiratory System						
None						
Special Senses System						
Eye	(1)			(1)	(1)	
Developmental malformation, left, optic nerve					1 (100%)	
Hypoplasia, left, retina					1 (100%)	
Urinary System						
Kidney	(8)	(8)	(8)	(8)	(8)	(8)
Autolysis, minimal						1 (13%)
Degeneration, minimal, renal tubule				1 (13%)		2 (25%)
Dilatation, mild, renal tubule						1 (13%)
Dilatation, minimal, renal tubule	1 (13%)	2 (25%)				1 (13%)
Infarction, mild		1 (13%)				
Mineralization, mild	5 (63%)	7 (88%)	5 (63%)	6 (75%)	8 (100%)	4 (50%)
Mineralization, minimal	3 (38%)	1 (13%)	2 (25%)	2 (25%)		3 (38%)
Mineralization, moderate			1 (13%)			1 (13%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 16-Day Range-Finding Gavage Study of Chloral Hydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Disposition Summary						
Animals initially in study	8	8	8	8	8	8
Early deaths						
Natural deaths	1	1	1	1		1
Survivors						
Terminal sacrifice	7	7	7	7	8	7
Animals examined microscopically	8	8	8	8	8	8
Alimentary System						
Liver	(8)	(8)	(8)	(8)	(8)	(8)
Hematopoietic cell proliferation, marked			1 (13%)			
Hematopoietic cell proliferation, mild				1 (13%)		
Inflammation, subacute, mild	1 (13%)					
Inflammation, subacute, minimal	2 (25%)	1 (13%)	2 (25%)	2 (25%)	2 (25%)	5 (63%)
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
None						
Hematopoietic System						
Spleen	(2)		(1)			
Hematopoietic cell proliferation, marked			1 (100%)			
Hyperplasia, lymphoid, moderate	1 (50%)					
Integumentary System						
Skin	(1)		(1)			
Abscess, marked			1 (100%)			
Musculoskeletal System						
Bone	(2)					
Developmental malformation, sternum	1 (50%)					
Skeletal muscle	(2)					
Inflammation, chronic, marked, diaphragm	1 (50%)					

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 16-Day Range-Finding Gavage Study of Chloral Hydrate

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Nervous System						
None						
Respiratory System						
Lung	(2)					
Abscess, marked	1 (50%)					
Special Senses System						
None						
Urinary System						
Kidney	(8)	(8)	(8)	(8)	(8)	(8)
Inflammation, chronic, minimal	1 (13%)					

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 16-Day Range-Finding Gavage Study of Chloral Hydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Disposition Summary						
Animals initially in study	8	8	8	8	8	8
Early deaths						
Natural deaths						2
Survivors						
Terminal sacrifice	8	8	8	8	8	6
Animals examined microscopically	8	8	8	8	8	8
Alimentary System						
Liver	(8)	(8)	(8)	(8)	(8)	(8)
Inflammation, subacute, minimal	7 (88%)	8 (100%)	6 (75%)	8 (100%)	8 (100%)	5 (63%)
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
None						
Hematopoietic System						
None						
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 16-Day Range-Finding Gavage Study of Chloral Hydrate

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Special Senses System						
None						
Urinary System						
None						

[a] Number of animals examined microscopically at the site and the number of animals with lesion

APPENDIX B
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE B1 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 17-Day Range-Finding Gavage Study of Chloral Hydrate B-2
TABLE B2 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Range Finding Gavage Study of Chloral Hydrate B-3

TABLE B1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 17-Day Range-Finding Gavage Study of Chloral Hydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Male						
n	8	8	8	8	7	7
Necropsy body wt	176 ± 6	170 ± 3	173 ± 4	170 ± 6	165 ± 7	153 ± 6**
R. Kidney						
Absolute	0.773 ± 0.029	0.787 ± 0.017	0.814 ± 0.024	0.779 ± 0.024	0.801 ± 0.042	0.769 ± 0.032
Relative	4.39 ± 0.08	4.63 ± 0.06*	4.71 ± 0.04**	4.59 ± 0.07**	4.85 ± 0.05**	5.05 ± 0.09**
L. Kidney						
Absolute	0.790 ± 0.030	0.785 ± 0.018	0.833 ± 0.033	0.801 ± 0.021	0.795 ± 0.031	0.779 ± 0.037
Relative	4.48 ± 0.08	4.62 ± 0.07	4.81 ± 0.09*	4.72 ± 0.08*	4.83 ± 0.06**	5.11 ± 0.11**
Liver						
Absolute	8.291 ± 0.356	8.273 ± 0.176	8.472 ± 0.224	8.363 ± 0.322	8.077 ± 0.371	8.276 ± 0.346
Relative	46.96 ± 1.06	48.62 ± 0.49	49.05 ± 0.56	49.19 ± 0.84	49.03 ± 0.34	54.38 ± 1.50**
Female						
n	8	8	8	8	8	7
Necropsy body wt	120 ± 2	126 ± 2	124 ± 3	123 ± 3	123 ± 3	116 ± 3
R. Kidney						
Absolute	0.598 ± 0.011	0.629 ± 0.011	0.613 ± 0.016	0.628 ± 0.021	0.623 ± 0.017	0.599 ± 0.015
Relative	5.00 ± 0.12	5.02 ± 0.07	4.96 ± 0.07	5.09 ± 0.08	5.06 ± 0.11	5.19 ± 0.08
L. Kidney						
Absolute	0.586 ± 0.012	0.608 ± 0.014	0.610 ± 0.018	0.648 ± 0.017*	0.626 ± 0.014	0.602 ± 0.018
Relative	4.89 ± 0.10	4.85 ± 0.09	4.93 ± 0.07	5.26 ± 0.08*	5.08 ± 0.08*	5.22 ± 0.11*
Liver						
Absolute	5.650 ± 0.175	5.632 ± 0.104	5.526 ± 0.173	5.805 ± 0.141	5.766 ± 0.149	5.498 ± 0.129
Relative	47.21 ± 1.59	44.86 ± 0.36	44.62 ± 0.61	47.18 ± 0.79	46.79 ± 0.74	47.63 ± 0.90

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** Significantly different ($P \leq 0.01$) from the vehicle control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE B2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Range-Finding Gavage Study of Chloral Hydrate[a]

	Vehicle Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg	800 mg/kg
Male						
n	7	7	7	7	8	7
Necropsy body wt	22.0 ± 0.4	22.2 ± 0.6	22.9 ± 0.2	23.3 ± 0.4	23.2 ± 0.4	22.7 ± 0.5
R. Kidney						
Absolute	0.190 ± 0.004	0.191 ± 0.008	0.194 ± 0.006	0.201 ± 0.005	0.197 ± 0.005	0.195 ± 0.004
Relative	8.63 ± 0.15	8.61 ± 0.25	8.47 ± 0.26	8.63 ± 0.20	8.51 ± 0.15	8.58 ± 0.22
L. Kidney						
Absolute	0.172 ± 0.004	0.177 ± 0.005	0.185 ± 0.005	0.185 ± 0.004	0.186 ± 0.004	0.182 ± 0.005
Relative	7.79 ± 0.15	7.99 ± 0.22	8.08 ± 0.19	7.93 ± 0.10	8.02 ± 0.09	8.00 ± 0.16
Liver						
Absolute	1.107 ± 0.038	1.096 ± 0.050	1.111 ± 0.038	1.178 ± 0.036	1.289 ± 0.024**	1.251 ± 0.039**
Relative	50.40 ± 2.35	49.25 ± 1.34	48.57 ± 1.56	50.49 ± 0.77	55.71 ± 1.26*	55.05 ± 0.93*
Female						
n	8	8	8	8	8	6
Necropsy body wt	16.9 ± 0.4	17.3 ± 0.2	16.8 ± 0.4	17.9 ± 0.1*	18.1 ± 0.3*	18.5 ± 0.5**
R. Kidney						
Absolute	0.140 ± 0.004	0.140 ± 0.003	0.134 ± 0.004	0.141 ± 0.004	0.147 ± 0.004	0.149 ± 0.004
Relative	8.23 ± 0.14	8.08 ± 0.13	7.98 ± 0.21	7.90 ± 0.20	8.14 ± 0.17	8.06 ± 0.15
L. Kidney						
Absolute	0.132 ± 0.004	0.128 ± 0.002	0.126 ± 0.003	0.133 ± 0.005	0.135 ± 0.007	0.139 ± 0.005
Relative	7.78 ± 0.17	7.41 ± 0.10	7.49 ± 0.20	7.43 ± 0.22	7.44 ± 0.30	7.49 ± 0.09
Liver						
Absolute	0.800 ± 0.025	0.754 ± 0.018	0.755 ± 0.021	0.808 ± 0.016	0.854 ± 0.023	0.928 ± 0.021**
Relative	47.19 ± 0.82	43.57 ± 0.56	44.95 ± 0.72	45.19 ± 0.75	47.24 ± 0.63	50.16 ± 0.83**

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' test
** $P \leq 0.01$
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX C
METABOLISM ANALYSIS RESULTS

TABLE C1	Plasma Concentrations of Chloral Hydrate and Its Metabolites in Male and Female Rats in the 17-Day Metabolism Study of Chloral Hydrate	C-2
FIGURE C1	Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male Rats after a Single Gavage Dose of 50 or 200 mg/kg Chloral Hydrate	C-4
FIGURE C2	Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male Rats after 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate	C-5
FIGURE C3	Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Female Rats after a Single Gavage Dose of 50 or 200 mg/kg Chloral Hydrate	C-6
FIGURE C4	Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Female Rats after 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate	C-7
TABLE C2	Maximum Plasma Concentrations and Areas Under the Curve of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male and Female Rats after 1 or 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate	C-8
FIGURE C5	Representative Metabolic Profile Based on the Pharmacokinetic Model for the Metabolism of Chloral Hydrate in Rats	C-9
TABLE C3	Half-Life Values of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide for Male and Female Rats in the 17-Day Metabolism Study of Chloral Hydrate	C-10
TABLE C4	Plasma Concentrations of Chloral Hydrate and Its Metabolites in Male and Female Mice in the 16-Day Metabolism Study of Chloral Hydrate	C-11
FIGURE C6	Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male Mice after a Single Gavage Dose of 50 or 200 mg/kg Chloral Hydrate	C-13
FIGURE C7	Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male Mice after 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate	C-14
FIGURE C8	Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Female Mice after a Single Gavage Dose of 50 or 200 mg/kg Chloral Hydrate	C-15
FIGURE C9	Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Female Mice after 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate	C-16
TABLE C5	Maximum Plasma Concentrations and Areas Under the Curve of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male and Female Mice after 1 or 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate	C-17
FIGURE C10	Representative Metabolic Profile Based on the Pharmacokinetic Model for the Metabolism of Chloral Hydrate in Mice	C-18
TABLE C6	Half-Life Values of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide for Male and Female Mice in the 16-Day Metabolism Study of Chloral Hydrate	C-19

TABLE C1
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Male and Female Rats in the 17-Day Metabolism Study of Chloral Hydrate[a]

	15 minutes	1 hour	3 hours	6 hours	1 day	2 days
Male						
50 mg/kg (1 dose)						
Chloral hydrate	41.7 ± 20.9	—[b]	—	—	—	—
Trichloroacetic acid	96.1 ± 16.2	97.3 ± 8.5	96.1 ± 8.1	75.3 ± 5.0	9.8 ± 1.4	—
Trichloroethanol	40.8 ± 3.7	11.4 ± 5.0	—	—	—	—
Trichloroethanol glucuronide	121.8 ± 5.8	61.6 ± 6.2	9.4 ± 4.3	—	—	—
200 mg/kg (1 dose)						
Chloral hydrate	83.4 ± 9.8	13.3 ± 2.1	—	—	—	—
Trichloroacetic acid	161.0 ± 11.0	288.2 ± 8.5	331.1 ± 26.8	356.8 ± 18.0	34.3 ± 5.0	—
Trichloroethanol	165.3 ± 16.2	145.9 ± 10.0	9.4 ± 2.7	6.7 ± 0.8	—	—
Trichloroethanol glucuronide	157.3 ± 22.4	353.4 ± 4.6	48.2 ± 3.9	4.7 ± 1.2	—	—
50 mg/kg (12 doses)						
Chloral hydrate	27.2 ± 6.3	—	—	—	—	—
Trichloroacetic acid	53.2 ± 6.7	114.4 ± 17.3	134.6 ± 31.8	90.0 ± 3.9	13.5 ± 2.1	—
Trichloroethanol	26.1 ± 2.7	16.1 ± 2.7	10.0 ± 4.3	8.7 ± 3.1	9.4 ± 4.3	—
Trichloroethanol glucuronide	68.3 ± 12.4	97.7 ± 25.1	14.1 ± 6.2	—	—	—
200 mg/kg (12 doses)						
Chloral hydrate	125.1 ± 27.2	37.5 ± 6.4	—	—	—	—
Trichloroacetic acid	200.7 ± 5.2	301.1 ± 30.9	392.3 ± 25.5	401.5 ± 67.8	51.4 ± 2.8	12.2 ± 1.4
Trichloroethanol	131.8 ± 25.5	66.3 ± 12.0	16.1 ± 2.7	—	—	—
Trichloroethanol glucuronide	134.5 ± 15.8	307.9 ± 67.3	76.3 ± 9.6	12.7 ± 6.9	—	—

TABLE C1
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Male and Female Rats in the 17-Day Metabolism Study of Chloral Hydrate

	15 minutes	1 hour	3 hours	6 hours	1 day	2 days
Female						
50 mg/kg (1 dose)						
Chloral hydrate	13.9 ± 6.1	—	—	—	—	—
Trichloroacetic acid	93.0 ± 8.3	109.5 ± 5.0	91.8 ± 5.7	80.2 ± 4.2	4.3 ± 0.3	—
Trichloroethanol	38.8 ± 5.4	7.4 ± 1.9	—	—	—	—
Trichloroethanol glucuronide	123.1 ± 17.4	41.5 ± 6.6	—	—	—	—
200 mg/kg (1 dose)						
Chloral hydrate	201.9 ± 90.1	33.9 ± 13.9	—	—	—	—
Trichloroacetic acid	238.1 ± 7.7	325.6 ± 16.2	359.9 ± 32.5	307.2 ± 42.4	23.9 ± 3.2	—
Trichloroethanol	226.2 ± 13.4	145.2 ± 30.8	14.1 ± 0.4	—	—	—
Trichloroethanol glucuronide	202.8 ± 24.4	436.4 ± 117.5	30.1 ± 6.2	4.7 ± 1.2	—	—
50 mg/kg (12 doses)						
Chloral hydrate	9.7 ± 4.6	—	—	—	—	—
Trichloroacetic acid	115.1 ± 11.3	163.4 ± 6.1	146.9 ± 8.5	127.9 ± 9.9	9.8 ± 2.1	—
Trichloroethanol	44.2 ± 3.0	16.1 ± 4.0	—	—	—	—
Trichloroethanol glucuronide	103.1 ± 6.7	75.6 ± 9.4	5.4 ± 0.8	—	—	—
200 mg/kg (12 doses)						
Chloral hydrate	79.8 ± 34.5	13.3 ± 3.1	—	—	—	—
Trichloroacetic acid	375.8 ± 46.5	419.2 ± 12.4	514.1 ± 38.2	555.7 ± 11.0	53.9 ± 8.8	10.4 ± 0.7
Trichloroethanol	303.2 ± 59.9	93.7 ± 26.3	14.7 ± 1.2	8.7 ± 2.3	6.7 ± 1.9	—
Trichloroethanol glucuronide	191.4 ± 16.6	226.9 ± 12.8	58.9 ± 8.5	12.0 ± 4.3	—	—

[a] Data are given in μM of metabolite (mean ± standard error for at least three rats).
[b] Not detected

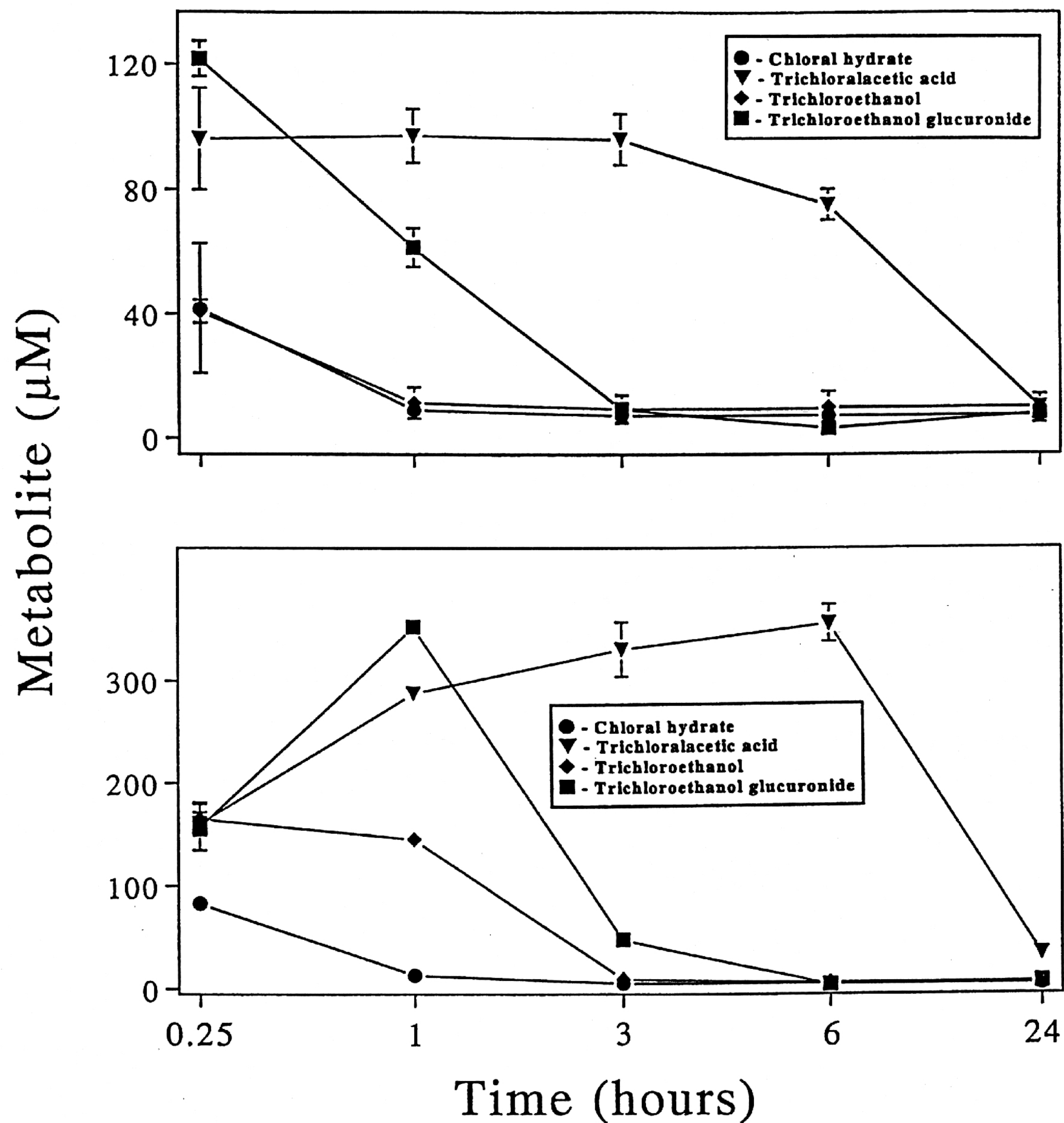

FIGURE C1
Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male Rats after a Single Gavage Dose of 50 or 200 mg/kg Chloral Hydrate
(Data are presented as the mean ± standard error for at least three rats.)

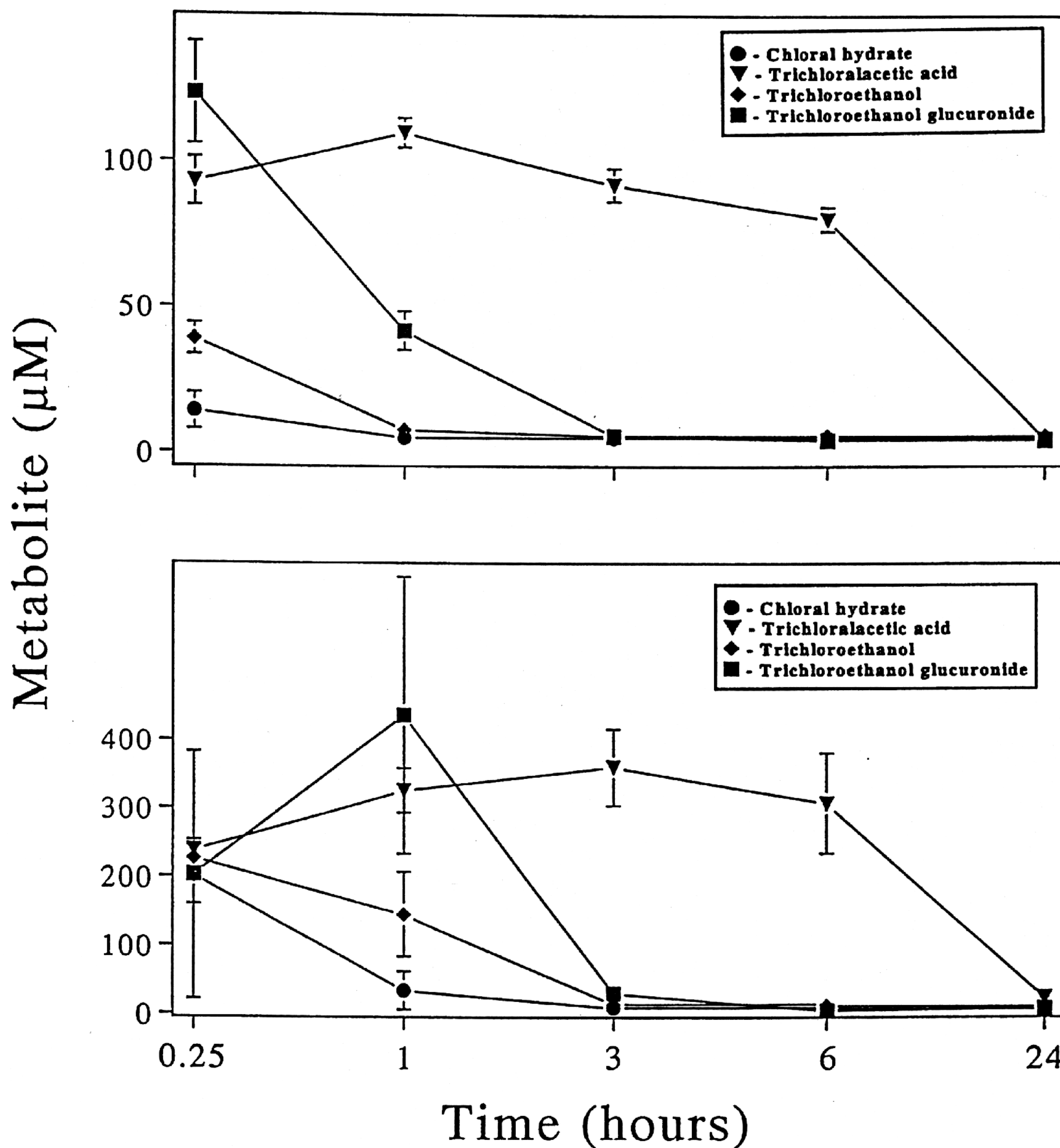

FIGURE C2
Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male Rats after 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate
(Data are presented as the mean ± standard error for at least three rats.)

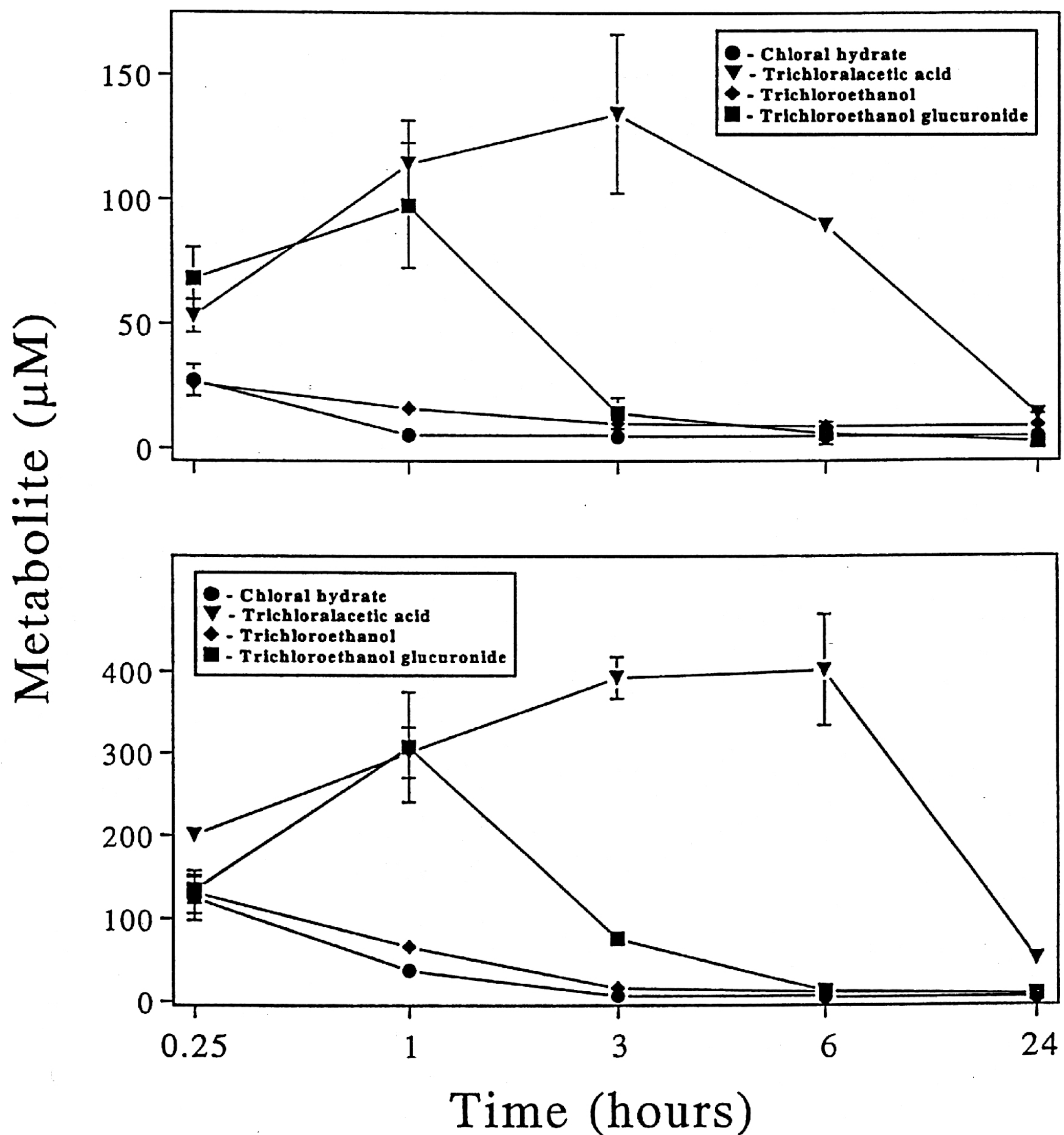

FIGURE C3
Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Female Rats after a Single Gavage Dose of 50 or 200 mg/kg Chloral Hydrate
(Data are presented as the mean ± standard error for at least three rats.)

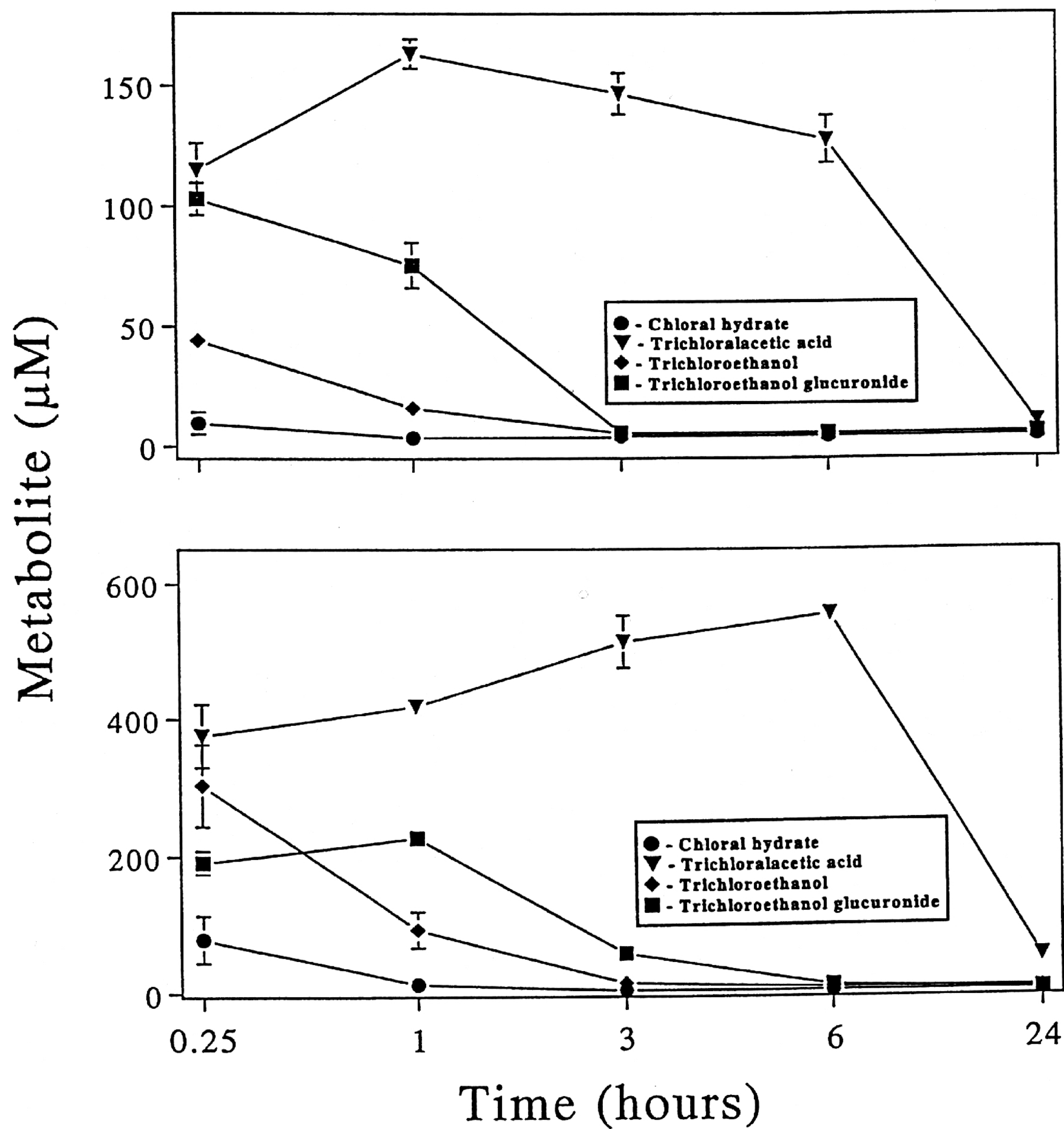

FIGURE C4
Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Female Rats after 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate
(Data are presented as the mean ± standard error for at least three rats.)

TABLE C2
Maximum Plasma Concentrations and Areas Under the Curve of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male and Female Rats after 1 or 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate[a]

	C_{max} (µM)				AUC (µmol • hour)			
	Chloral hydrate	TCA	TCE	TCE glucuronide	Chloral hydrate	TCA	TCE	TCE glucuronide
Male								
50 mg/kg (1 dose)	41.7 ± 20.9	97.3 ± 8.5	40.8 ± 3.7	121.8 ± 5.8	57 ± 13	1,289 ± 59	69 ± 15	159 ± 14
200 mg/kg (1 dose)	83.4 ± 9.8	356.8 ± 18.0	165.3 ± 16.2	353.4 ± 4.6	70 ± 6	5,339 ± 206	296 ± 17	672 ± 14
50 mg/kg (12 doses)	27.2 ± 6.3	134.6 ± 31.8	26.1 ± 2.7	97.7 ± 25.1	37 ± 4	1,580 ± 94	70 ± 12	204 ± 39
200 mg/kg (12 doses)	125.1 ± 27.2	392.3 ± 25.5	131.8 ± 25.5	307.9 ± 67.3	122 ± 16	6,148 ± 717	198 ± 21	684 ± 96
Female								
50 mg/kg (1 dose)	13.9 ± 6.1	109.5 ± 5.0	38.8 ± 5.4	123.1 ± 17.4	31 ± 4	1,296 ± 47	46 ± 5	123 ± 12
200 mg/kg (1 dose)	201.9 ± 90.1	359.9 ± 32.5	226.2 ± 13.4	436.4 ± 117.5	156 ± 46	4,877 ± 454	337 ± 49	758 ± 163
50 mg/kg (12 doses)	9.7 ± 4.6	163.4 ± 6.1	44.2 ± 3.0	103.1 ± 6.7	25 ± 4	2,066 ± 108	60 ± 7	164 ± 15
200 mg/kg (12 doses)	79.8 ± 34.5	555.7 ± 11.0	303.2 ± 59.9	226.9 ± 12.8	65 ± 16	8,323 ± 171	292 ± 45	549 ± 29

[a] Mean ± standard error. Plasma concentrations of chloral hydrate and its metabolites were determined 0.25, 1, 3, 6, and 24 hours and 2, 4, 8, and 16 days after receiving 1 or 12 doses. C_{max}=maximum plasma concentration; TCA=trichloroacetic acid; TCE=trichloroethanol

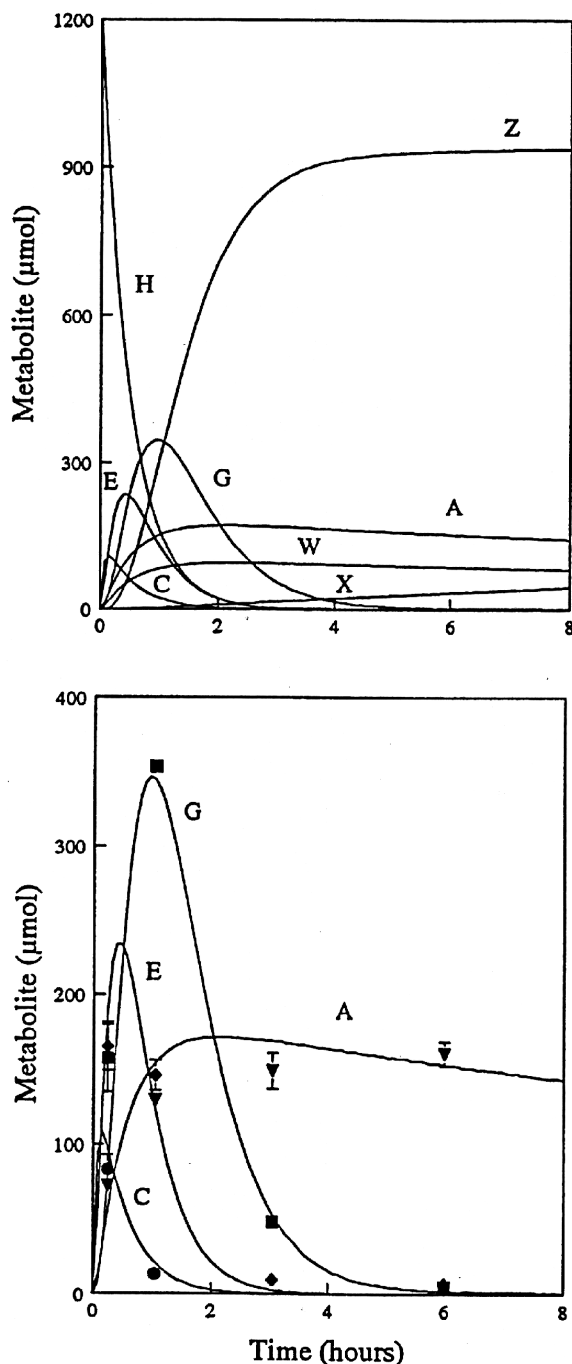

FIGURE C5
Representative Metabolic Profile Based on the Pharmacokinetic Model for the Metabolism of Chloral Hydrate in Male and Female Rats [results for males treated with a single gavage dose of 200 mg/kg. The following abbreviations are used: H, chloral hydrate in gastrointestinal tract; C, chloral hydrate in plasma; A, trichloroacetic acid in plasma; E, trichloroethanol in plasma; G, trichloroethanol glucuronide in plasma; and W, X, Y, and Z, urinary (or fecal) excretion of chloral hydrate, trichloroacetic acid, trichloroethanol, and trichloroethanol glucuronide, respectively. The lower panel compares the pharmacokinetic model to the experimentally determined plasma concentrations (mean ± standard error) of chloral hydrate (●), trichloroacetic acid (▼), trichloroethanol (◆), and trichloroethanol glucuronide (■). Note that the ordinate is presented as amount (µmol) and not concentration (µM) as in Figures C1-C4 and that the data for trichloroacetic acid reflect the volume of distribution (V_{dA}) being <1 L/kg (see Table C3)]

TABLE C3
Half-Life Values of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide for Male and Female Rats in the 17-Day Metabolism Study of Chloral Hydrate[a]

	Half-Life (hours)									
	V_{dA}	k_{HC}	k_{CW}	k_{CE}	k_{CA}	k_{AX}	k_{EY}	k_{EG}	k_{GZ}	Σk_{Ci}[b]
Male										
50 mg/kg (1 dose)	0.350	0.213	1.233	0.139	0.694	6.930	0.0	0.099	0.292	0.106
200 mg/kg (1 dose)	0.450	0.347	0.504	0.055	0.277	18.730	0.0	0.185	0.462	0.042
50 mg/kg (12 doses)	0.350	0.308	0.616	0.068	0.347	11.177	0.0	0.089	0.554	0.052
200 mg/kg (12 doses)	0.350	0.252	0.740	0.079	0.396	7.966	0.0	0.111	0.554	0.061
Female										
50 mg/kg (1 dose)	0.400	0.252	0.462	0.050	0.252	9.240	0.0	0.089	0.222	0.039
200 mg/kg (1 dose)	0.400	0.173	1.386	0.150	0.749	9.240	0.0	0.185	0.462	0.115
50 mg/kg (12 doses)	0.270	0.277	0.462	0.050	0.252	12.375	0.0	0.121	0.370	0.039
200 mg/kg (12 doses)	0.350	0.163	0.616	0.068	0.347	13.860	0.0	0.185	0.252	0.052
Average half-life	0.365	0.248	0.752	0.082	0.414	11.190	0.0	0.133	0.396	0.063

[a] V_{dA}=volume of distribution of trichloroacetic acid. The V_d for chloral hydrate, trichloroethanol, and trichloroethanol glucuronide was 1 L/kg. k_{HC}=rate of chloral hydrate absorption from gastrointestinal tract; k_{CW}=rate of chloral hydrate excretion; k_{CE}=rate of chloral hydrate conversion to trichloroethanol; k_{CA}=rate of chloral hydrate conversion to trichloroacetic acid; k_{AX}=rate of trichloroacetic acid excretion; k_{EY}=rate of trichloroethanol excretion; k_{EG}=rate of trichloroethanol conversion to trichloroethanol glucuronide; k_{GZ}=rate of trichloroethanol glucuronide excretion

[b] $\Sigma k_{Ci} = k_{CW} + k_{CE} + k_{CA}$

TABLE C4
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Male and Female Mice in the 16-Day Metabolism Study of Chloral Hydrate[a]

	15 minutes	1 hour	3 hours	6 hours	1 day	2 days
Male						
50 mg/kg (1 dose)						
Chloral hydrate	8.5 ± 3.5	—[b]	—	—	—	—
Trichloroacetic acid	140.1 ± 24.2	180.5 ± 22.1	148.7 ± 28.2	112.6 ± 6.9	9.2 ± 1.2	—
Trichloroethanol	20.7 ± 3.1	—	—	—	—	—
Trichloroethanol glucuronide	137.9 ± 10.0	6.0 ± 1.6	—	—	—	—
200 mg/kg (1 dose)						
Chloral hydrate	47.8 ± 13.3	5.4 ± 0.9	—	—	—	—
Trichloroacetic acid	362.9 ± 57.2	588.1 ± 24.8	447.4 ± 34.2	369.6 ± 21.5	34.3 ± 14.5	—
Trichloroethanol	151.3 ± 44.1	11.4 ± 1.7	—	—	—	—
Trichloroethanol glucuronide	273.1 ± 34.0	97.0 ± 11.2	10.0 ± 6.2	—	—	—
50 mg/kg (12 doses)						
Chloral hydrate	12.7 ± 4.6	—	—	—	—	—
Trichloroacetic acid	126.7 ± 19.9	99.1 ± 14.8	90.0 ± 31.1	52.0 ± 29.3	9.8 ± 2.1	—
Trichloroethanol	16.7 ± 4.4	—	—	—	—	—
Trichloroethanol glucuronide	113.1 ± 7.7	2.0 ± 0.8	10.0 ± 2.7	—	—	—
200 mg/kg (12 doses)						
Chloral hydrate	98.5 ± 49.3	—	—	—	—	—
Trichloroacetic acid	432.7 ± 31.8	555.7 ± 38.9	410.0 ± 34.3	270.5 ± 19.8	30.0 ± 5.3	7.3 ± 0.7
Trichloroethanol	182.0 ± 20.8	12.0 ± 0.8	—	—	—	—
Trichloroethanol glucuronide	320.6 ± 71.6	45.5 ± 8.9	—	—	—	—

TABLE C4
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Male and Female Mice in the 16-Day Metabolism Study of Chloral Hydrate

	15 minutes	1 hour	3 hours	6 hours	1 day	2 days
Female						
50 mg/kg (1 dose)						
Chloral hydrate	12.1 ± 5.3	—	—	—	—	—
Trichloroacetic acid	116.9 ± 20.5	200.7 ± 4.2	172.6 ± 13.8	127.9 ± 1.0	—	—
Trichloroethanol	48.9 ± 5.8	—	—	—	—	—
Trichloroethanol glucuronide	64.2 ± 16.5	11.4 ± 2.3	—	—	—	—
200 mg/kg (1 dose)						
Chloral hydrate	30.8 ± 11.5	4.8 ± 2.4	—	—	—	—
Trichloroacetic acid	324.4 ± 37.1	515.9 ± 25.5	462.1 ± 17.0	412.5 ± 36.4	16.5 ± 2.1	4.9 ± 1.4
Trichloroethanol	167.3 ± 22.1	37.5 ± 3.5	—	—	—	—
Trichloroethanol glucuronide	133.9 ± 12.0	137.2 ± 19.3	10.0 ± 1.6	12.0 ± 2.7	—	—
50 mg/kg (12 doses)						
Chloral hydrate	12.1 ± 7.6	—	—	—	—	—
Trichloroacetic acid	121.8 ± 28.2	153.6 ± 6.0	105.9 ± 6.0	65.5 ± 3.2	3.7 ± 1.4	—
Trichloroethanol	24.1 ± 9.7	—	—	—	—	—
Trichloroethanol glucuronide	57.6 ± 20.4	—	—	—	—	—
200 mg/kg (12 doses)						
Chloral hydrate	54.4 ± 30.6	—	—	—	—	—
Trichloroacetic acid	368.4 ± 30.9	393.5 ± 33.5	298.0 ± 34.6	204.4 ± 18.0	8.0 ± 0.7	—
Trichloroethanol	213.5 ± 16.8	14.1 ± 3.5	—	—	—	—
Trichloroethanol glucuronide	163.3 ± 32.4	47.5 ± 7.7	—	—	—	—

[a] Data are given in μM of metabolite (mean ± standard error for at least three rats).
[b] Not detected

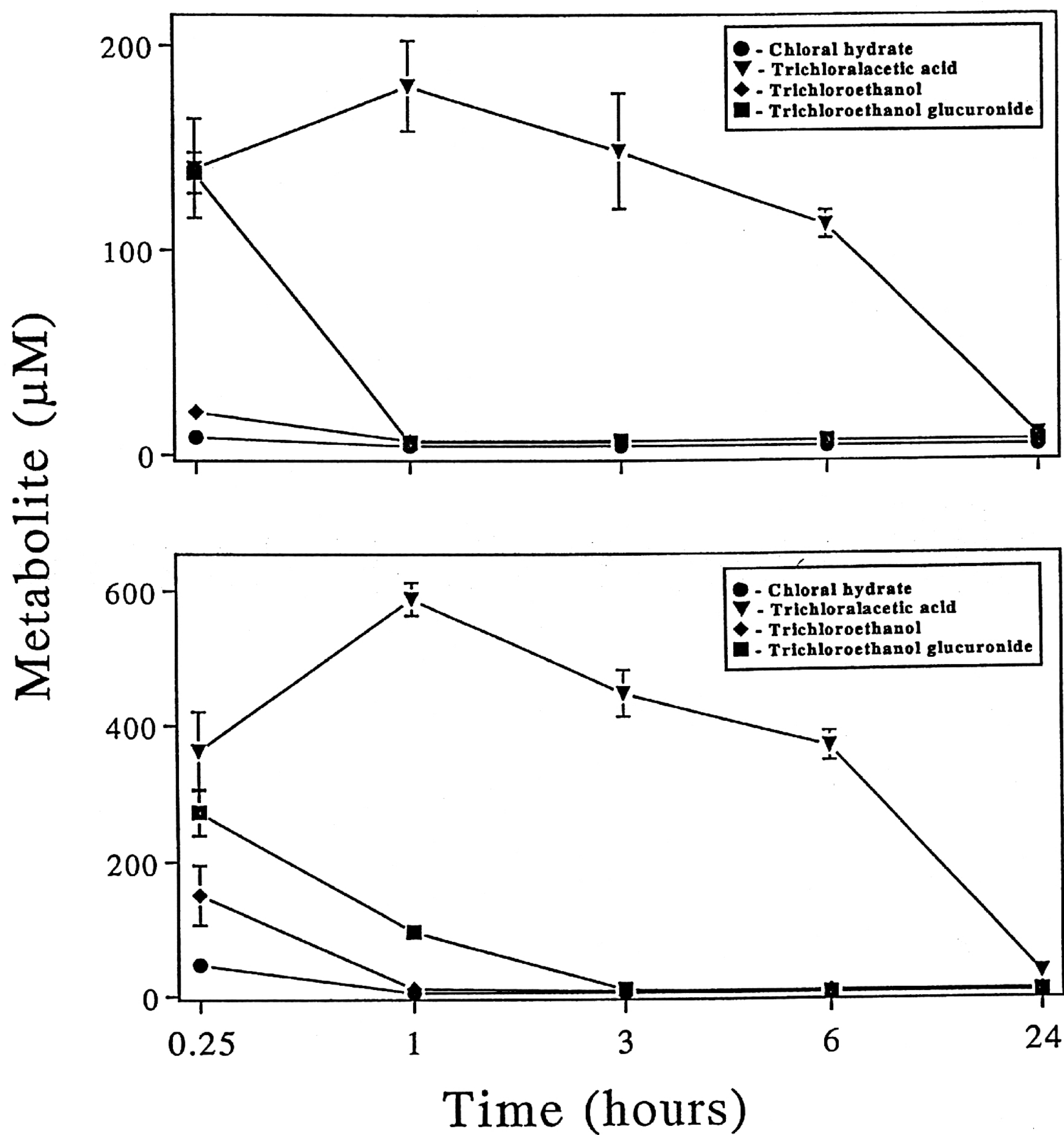

FIGURE C6
Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male Mice after a Single Gavage Dose of 50 or 200 mg/kg Chloral Hydrate
(Data are presented as the mean ± standard error for at least three mice.)

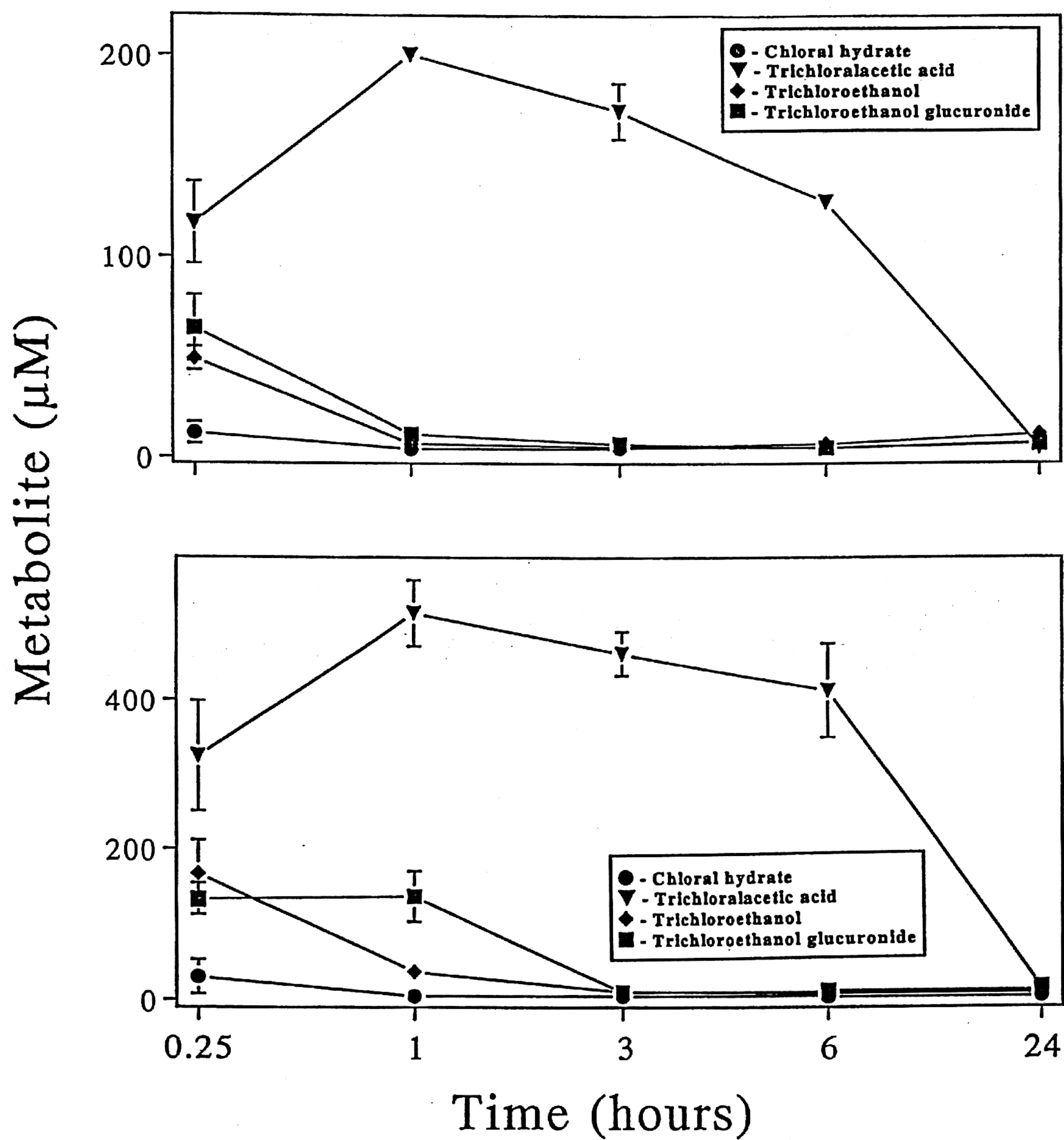

Figure C7
Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male Mice after 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate
(Data are presented as the mean ± standard error for at least three mice.)

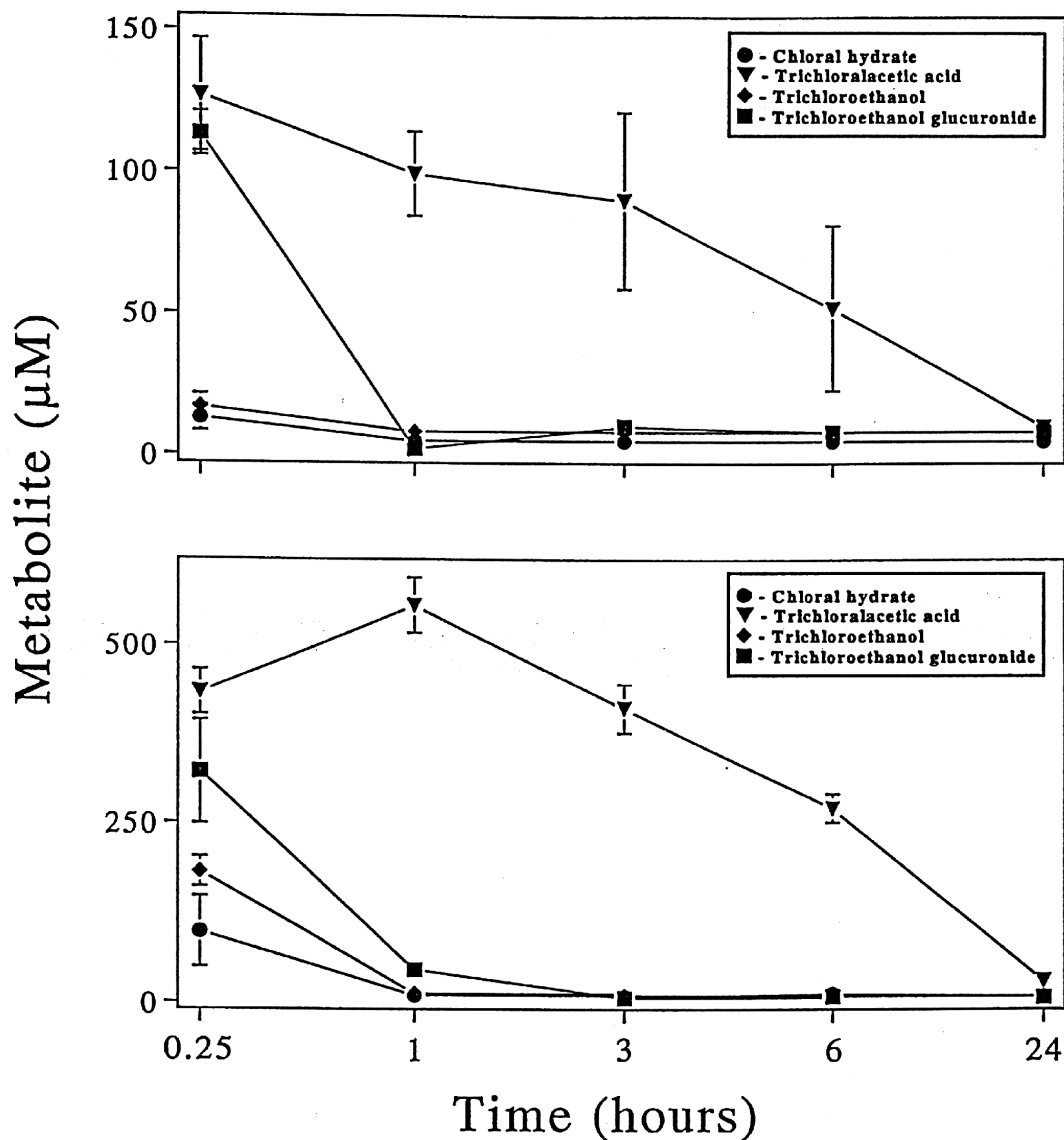

FIGURE C8
Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Female Mice after a Single Gavage Dose of 50 or 200 mg/kg Chloral Hydrate
(Data are presented as the mean ± standard error for at least three mice.)

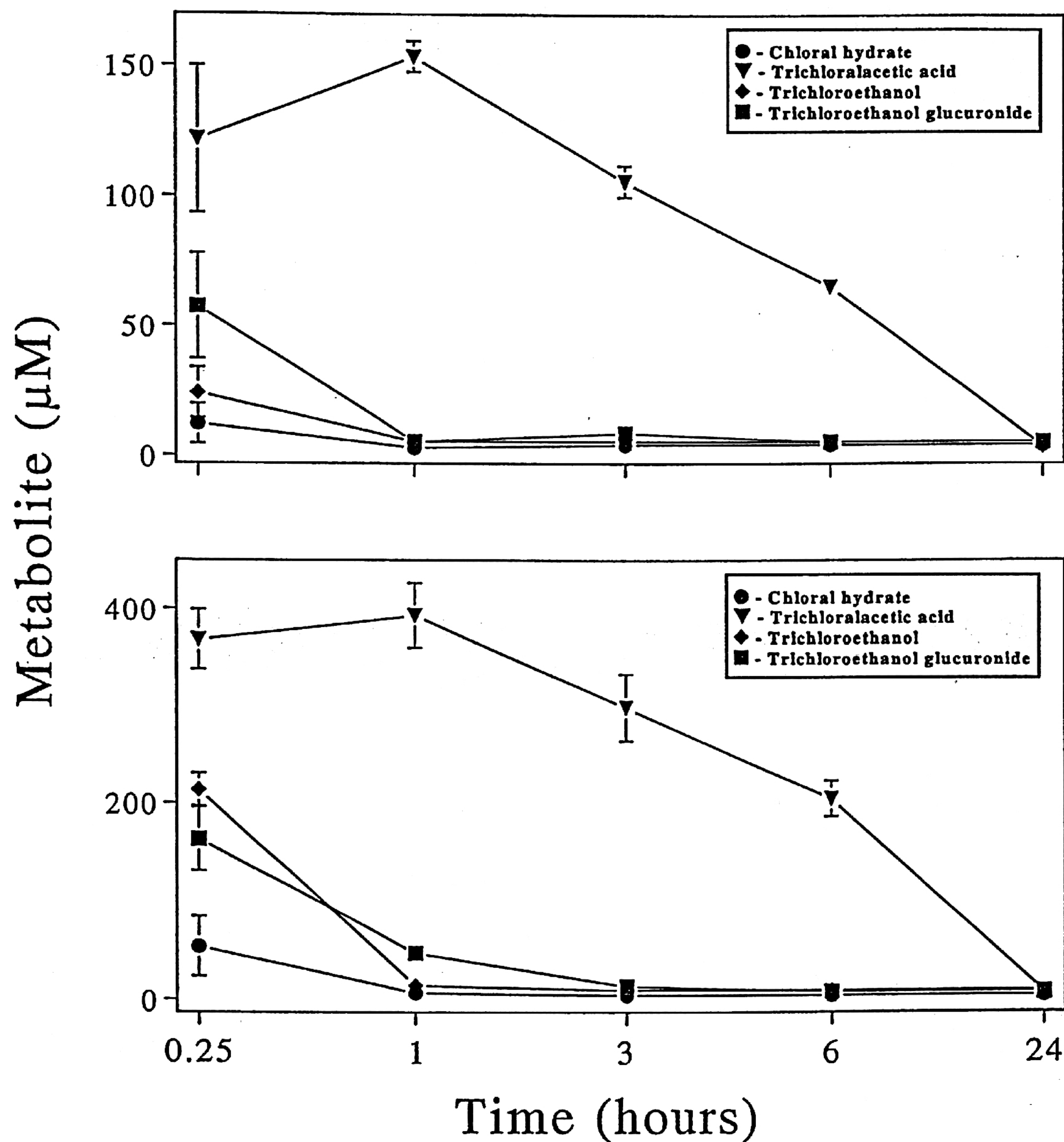

FIGURE C9
Plasma Concentrations of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Female Mice after 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate
(Data are presented as the mean ± standard error for at least three mice.)

TABLE C5
Maximum Plasma Concentrations and Areas Under the Curve of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide in Male and Female Mice after 1 or 12 Gavage Doses of 50 or 200 mg/kg Chloral Hydrate[a]

	C_{max} (µM)				AUC (µmol • hour)			
	Chloral hydrate	TCA	TCE	TCE glucuronide	Chloral hydrate	TCA	TCE	TCE glucuronide
Male								
50 mg/kg (1 dose)	8.5 ± 3.5	180.5 ± 22.1	20.7 ± 3.1	137.9 ± 10.0	26 ± 4	1,938 ± 108	44 ± 11	87 ± 11
200 mg/kg (1 dose)	47.8 ± 13.3	588.1 ± 24.8	151.3 ± 44.1	273.1 ± 34.0	46 ± 6	6,263 ± 278	105 ± 19	269 ± 26
50 mg/kg (12 doses)	12.7 ± 4.6	126.7 ± 19.9	16.7 ± 4.4	113.1 ± 7.7	31 ± 4	1,043 ± 319	49 ± 10	82 ± 9
200 mg/kg (12 doses)	98.5 ± 49.3	555.7 ± 38.9	182.0 ± 20.8	320.6 ± 71.6	86 ± 24	5,062 ± 236	120 ± 13	210 ± 34
Female								
50 mg/kg (1 dose)	12.1 ± 5.3	200.7 ± 4.2	48.9 ± 5.8	64.2 ± 16.5	30 ± 4	2,138 ± 39	51 ± 4	64 ± 7
200 mg/kg (1 dose)	30.8 ± 11.5	515.9 ± 25.5	167.3 ± 22.1	137.2 ± 19.3	36 ± 7	6,466 ± 387	153 ± 14	282 ± 28
50 mg/kg (12 doses)	12.1 ± 7.6	153.6 ± 6.0	24.1 ± 9.7	57.6 ± 20.4	25 ± 4	1,243 ± 41	38 ± 6	59 ± 13
200 mg/kg (12 doses)	54.4 ± 30.6	393.5 ± 33.5	213.5 ± 16.8	163.3 ± 32.4	46 ± 14	3,642 ± 214	137 ± 10	172 ± 19

[a] Mean ± standard error. Plasma concentrations of chloral hydrate and its metabolites were determined 0.25, 1, 3, 6, and 24 hours and 2, 4, 8, and 16 days after receiving 1 or 12 doses. C_{max}=maximum plasma concentration; TCA=trichloroacetic acid; TCE=trichloroethanol

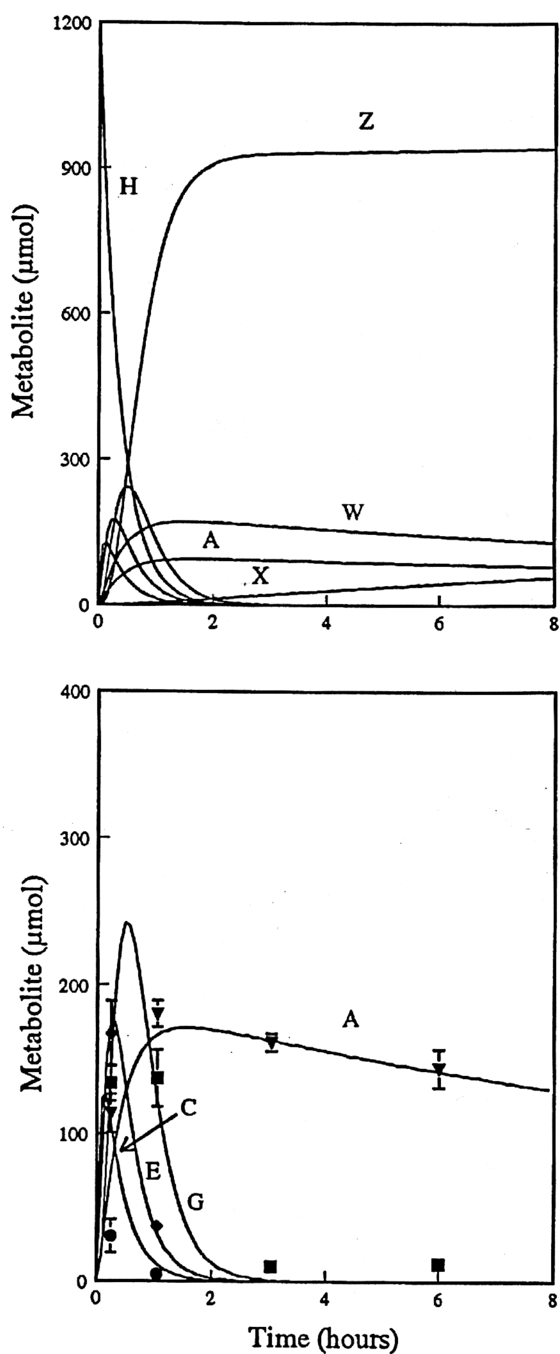

FIGURE C9

Representative Metabolic Profile Based on the Pharmacokinetic Model for the Metabolism of Chloral Hydrate in Mice [Results shown are for females treated with a single gavage dose of 200 mg/kg. The following abbreviations are used: H, chloral hydrate in gastrointestinal tract; C, chloral hydrate in plasma; A, trichloroacetic acid in plasma; E, trichloroethanol in plasma; G, trichloroethanol glucuronide in plasma; and W, X, Y, and Z, urinary (or fecal) excretion of chloral hydrate, trichloroacetic acid, trichloroethanol, and trichloroethanol glucuronide, respectively. The lower panel compares the pharmacokinetic model to the experimentally determined plasma concentrations (mean ± standard error) of chloral hydrate (●), trichloroacetic acid (▼), trichloroethanol (◆), and trichloroethanol glucuronide (■). Note that the ordinate is presented as amount (µmol) and not concentration (µM) as in Figures C6-C9 and that the data for trichloroacetic acid reflect the volume of distribution (V_{dA}) being <1 L/kg (see Table C6)]

TABLE C6
Half-Life Values of Chloral Hydrate, Trichloroacetic Acid, Trichloroethanol, and Trichloroethanol Glucuronide for Male and Female Mice in the 16-Day Metabolism Study of Chloral Hydrate[a]

	Half-Life (hours)									
	V_{dA}	k_{HC}	k_{CW}	k_{CE}	k_{CA}	k_{AX}	k_{EY}	k_{EG}	k_{GZ}	Σk_{Ci}[b]
Male										
50 mg/kg (1 dose)	0.280	0.347	0.504	0.055	0.277	11.177	0.0	0.055	0.079	0.042
200 mg/kg (1 dose)	0.320	0.213	0.462	0.050	0.252	9.240	0.0	0.069	0.168	0.039
50 mg/kg (12 doses)	0.350	0.347	0.504	0.055	0.277	6.187	0.0	0.069	0.069	0.042
200 mg/kg (12 doses)	0.320	0.213	0.616	0.068	0.347	6.930	0.0	0.084	0.101	0.052
Female										
50 mg/kg (1 dose)	0.230	0.198	0.462	0.050	0.252	9.240	0.0	0.089	0.111	0.039
200 mg/kg (1 dose)	0.350	0.252	0.462	0.050	0.252	13.860	0.0	0.089	0.168	0.039
50 mg/kg (12 doses)	0.320	0.231	0.616	0.068	0.347	5.544	0.0	0.055	0.168	0.052
200 mg/kg (12 doses)	0.400	0.213	0.616	0.068	0.347	5.544	0.0	0.089	0.101	0.052
Average half-life	0.321	0.252	0.530	0.058	0.294	8.465	0.0	0.075	0.121	0.045

[a] V_{dA}=volume of distribution of trichloroacetic acid. The V_d for chloral hydrate, trichloroethanol, and trichloroethanol glucuronide was 1 L/kg. k_{HC}=rate of chloral hydrate absorption from gastrointestinal tract; k_{CW}=rate of chloral hydrate excretion; k_{CE}=rate of chloral hydrate conversion to trichloroethanol; k_{CA}=rate of chloral hydrate conversion to trichloroacetic acid; k_{AX}=rate of trichloroacetic acid excretion; k_{EY}=rate of trichloroethanol excretion; k_{EG}=rate of trichloroethanol conversion to trichloroethanol glucuronide; k_{GZ}=rate of trichloroethanol glucuronide excretion

[b] $\Sigma k_{Ci}=k_{CW}+k_{CE}+k_{CA}$

APPENDIX D
IN VITRO METABOLISM AND DNA-BINDING RESULTS

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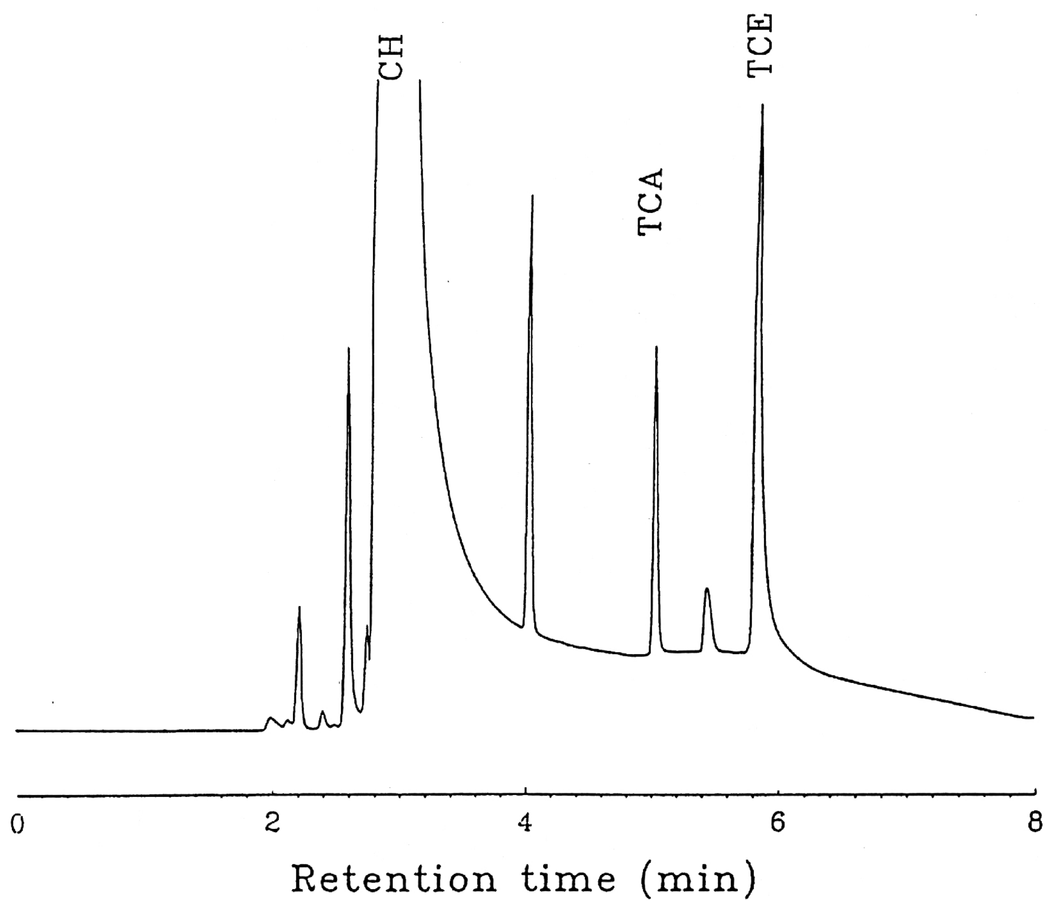

FIGURE D1
Gas Chromatogram of Metabolites Formed from the Metabolism of Chloral Hydrate by Male B6C3F$_1$ Mouse Liver Microsomes
(CH = chloral hydrate, TCA = trichloracetic acid, TCE - trichloroethanol

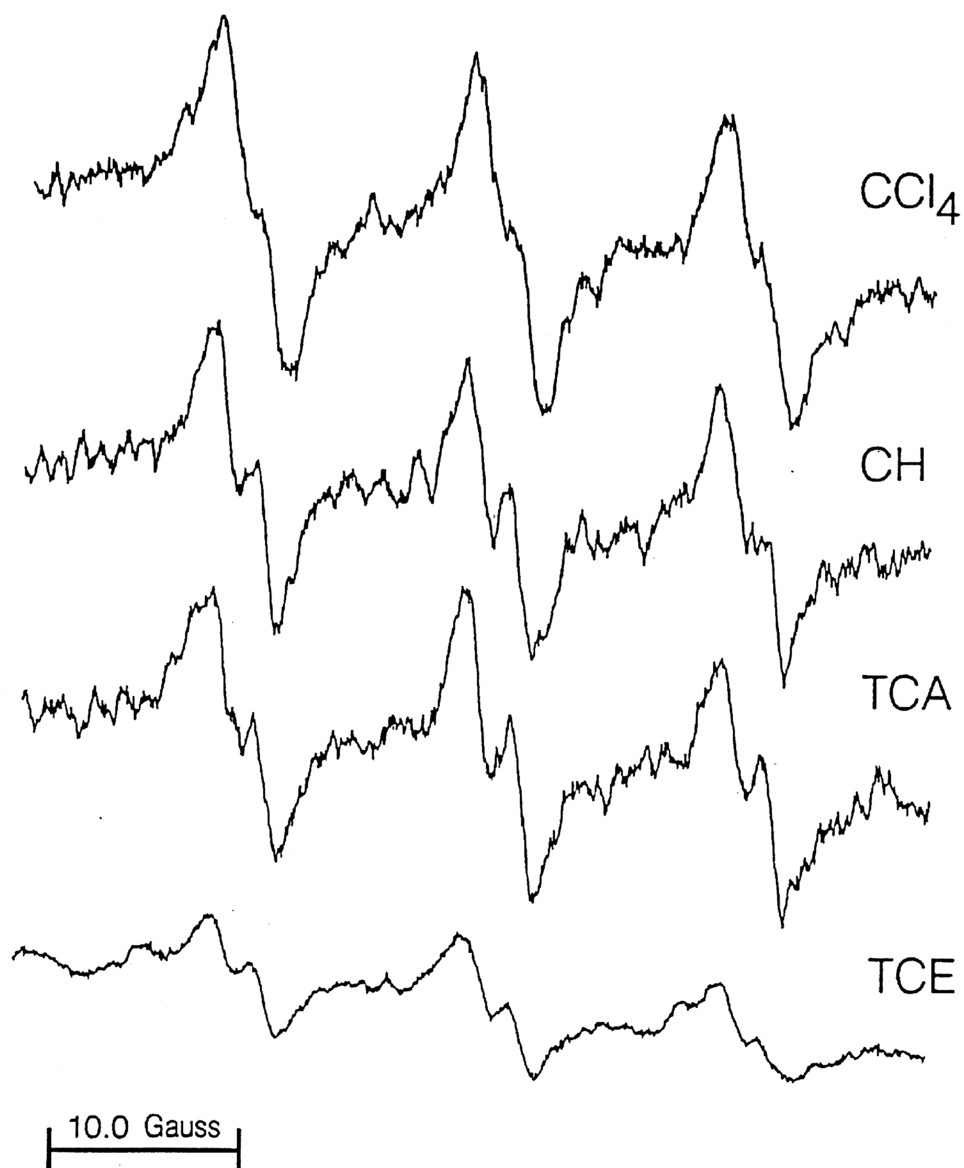

FIGURE D2
Spin-Trapped Electron Spin Resonance Spectra Formed from Metabolism of Carbon Tetrachloride (CCl_4), Chloral Hydrate (CH), Trichloroacetic Acid (TCA), and Trichloroethanol (TCE) by Male B6C3F$_1$ Mouse Liver Microsomes

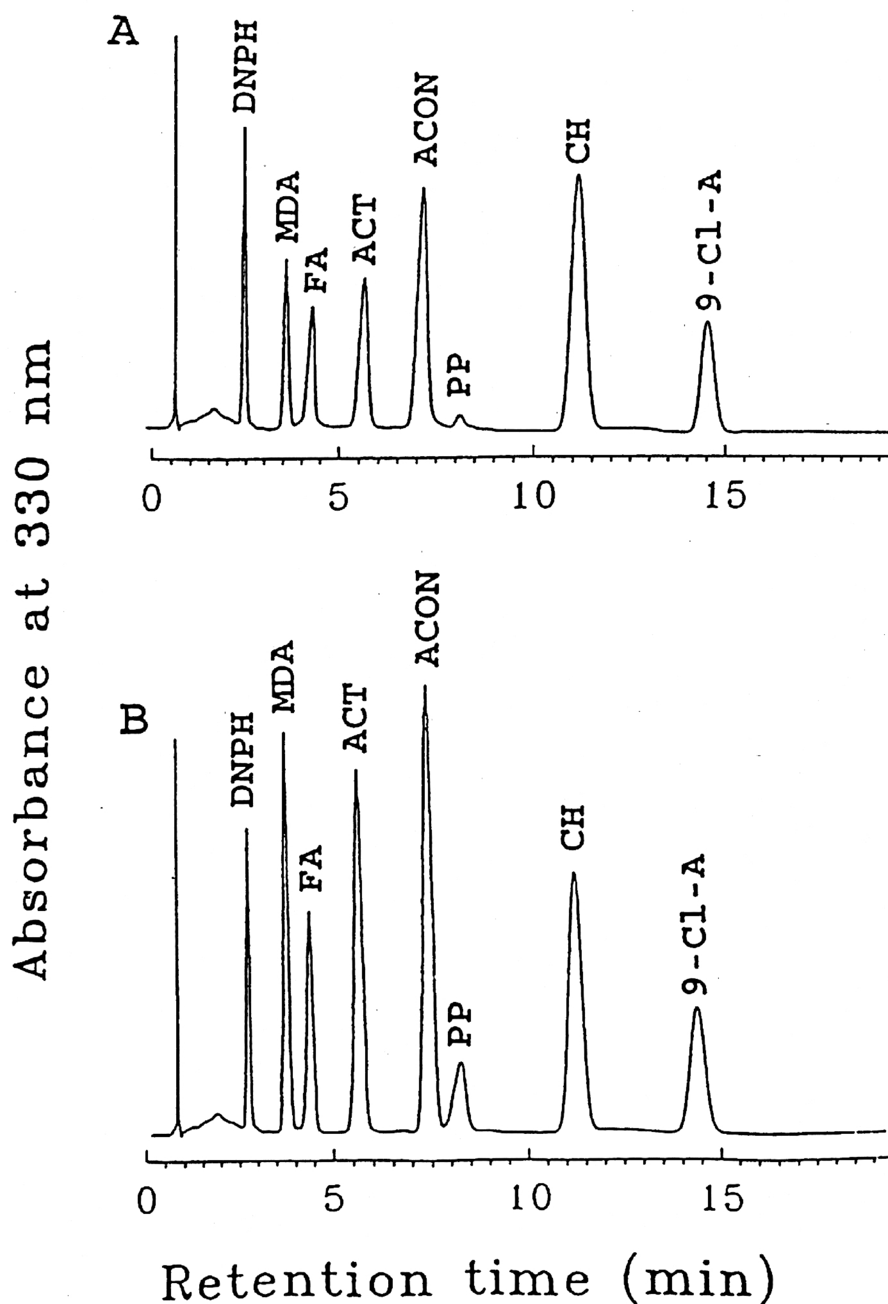

FIGURE D3
High-Performance Liquid Chromatography Profiles of 2,4-Dinitrophenylhydrazine-Derived Lipid Peroxidation Products Formed from the incubation of Chloral Hydrate with (A) Mouse Control Microsomes or (B) Mouse Pyrazole-Induced Microsomes (MDA = malondialdehyde, FA= formaldehyde, ACT = acetaldehyde, ACON = acetone, PP = propionaldehyde, CH = chloral hydrate, and 9-Cl-A = 9-chloroanthracene)

TABLE D1
Comparison of Lipid Peroxidation Products Formed from Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Control or Pyrazole-Induced Male B6C3F$_1$ Mouse Liver Microsomes[a]

Lipid Peroxidation Product	10-Minute Incubation				15-Minute Incubation			
	Water	Chloral Hydrate	TCA	TCE	Water	Chloral Hydrate	TCA	TCE
0.1 mg/mL microsomal protein								
Malondialdehyde								
Pyrazole-induced	2.1 ± 0.4	105.2 ± 19.7	90.7 ± 14.4	18.3 ± 4.1	2.5 ± 0.4	138.9 ± 23.8	127.9 ± 20.7	23.6 ± 4.4
Control	2.5 ± 0.5	49.9 ± 8.1	53.3 ± 9.8	8.8 ± 2.0	3.0 ± 0.5	66.3 ± 8.9	55.3 ± 8.3	10.2 ± 1.6
Formaldehyde								
Pyrazole-induced	3.4 ± 0.6	66.7 ± 11.2	107.1 ± 17.6	23.7 ± 4.9	4.3 ± 0.7	107.1 ± 20.8	147.7 ± 22.8	28.7 ± 4.3
Control	3.1 ± 0.5	31.3 ± 5.6	45.9 ± 7.3	11.0 ± 1.6	3.5 ± 0.5	51.7 ± 7.3	68.8 ± 7.9	13.6 ± 2.1
Acetaldehyde								
Pyrazole-induced	4.6 ± 0.9	87.0 ± 15.8	112.8 ± 19.2	31.4 ± 6.0	6.1 ± 1.2	114.2 ± 20.4	167.5 ± 34.2	57.8 ± 9.3
Control	4.3 ± 0.9	44.6 ± 7.1	66.5 ± 10.8	12.5 ± 2.1	5.9 ± 1.1	50.9 ± 6.9	85.2 ± 15.5	27.3 ± 3.8
Acetone								
Pyrazole-induced	8.1 ± 1.7	108.5 ± 17.9	77.7 ± 16.3	46.6 ± 7.2	11.3 ± 2.3	183.3 ± 33.1	113.9 ± 17.8	66.6 ± 10.2
Control	11.7 ± 1.9	59.2 ± 9.8	39.3 ± 6.7	20.1 ± 4.8	12.7 ± 2.3	77.4 ± 17.5	50.1 ± 7.3	28.1 ± 4.4
Propionaldehyde								
Pyrazole-induced	0.8 ± 0.2	11.7 ± 2.3	15.9 ± 2.3	4.5 ± 0.8	0.8 ± 0.2	15.6 ± 3.1	21.5 ± 3.7	7.1 ± 1.3
Control	0.5 ± 0.1	4.4 ± 0.8	8.8 ± 1.6	2.4 ± 0.4	0.5 ± 0.1	6.7 ± 1.2	10.1 ± 1.8	3.7 ± 0.6
TOTAL								
Pyrazole-induced	19.0 ± 4.4	379.1 ± 59.1	404.2 ± 58.8	124.5 ± 21.1	25.0 ± 4.4	559.1 ± 108.4	578.5 ± 103.7	183.8 ± 19.8
Control	22.1 ± 5.3	189.4 ± 22.3	213.8 ± 25.9	54.8 ± 8.9	25.6 ± 4.1	253.0 ± 33.2	269.5 ± 37.4	82.9 ± 13.4

TABLE D1
Comparison of Lipid Peroxidation Products Formed from Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Control or Pyrazole-Induced Male B6C3F$_1$ Mouse Liver Microsomes

Lipid Peroxidation Product	10-Minute Incubation				15-Minute Incubation			
	Water	Chloral Hydrate	TCA	TCE	Water	Chloral Hydrate	TCA	TCE
1.0 mg/mL microsomal protein								
Malondialdehyde								
Pyrazole-induced	1.7 ± 0.4	81.8 ± 17.3	75.8 ± 13.7	13.1 ± 2.3	2.3 ± 0.3	119.1 ± 26.7	109.2 ± 22.7	18.1 ± 3.9
Control	1.9 ± 0.4	37.7 ± 7.1	38.7 ± 6.8	6.9 ± 1.2	2.6 ± 0.5	51.8 ± 7.9	47.5 ± 8.3	9.5 ± 1.9
Formaldehyde								
Pyrazole-induced	2.8 ± 0.6	55.8 ± 10.7	80.1 ± 15.5	18.5 ± 2.9	4.1 ± 0.9	83.3 ± 20.1	116.4 ± 24.9	24.6 ± 4.1
Control	2.5 ± 0.5	27.5 ± 5.6	37.5 ± 6.9	8.8 ± 1.4	3.7 ± 0.7	38.7 ± 6.3	52.9 ± 7.4	11.7 ± 2.0
Acetaldehyde								
Pyrazole-induced	3.9 ± 0.8	69.6 ± 13.4	96.4 ± 19.3	27.3 ± 4.4	5.5 ± 1.4	99.4 ± 23.3	141.3 ± 28.8	43.6 ± 6.8
Control	3.5 ± 0.7	33.3 ± 7.2	49.9 ± 7.1	11.4 ± 1.9	4.9 ± 1.1	42.7 ± 5.8	67.3 ± 10.7	19.8 ± 3.7
Acetone								
Pyrazole-induced	7.3 ± 1.4	94.9 ± 19.1	61.1 ± 11.5	35.7 ± 5.6	10.7 ± 2.8	143.0 ± 31.7	95.7 ± 17.8	51.3 ± 6.9
Control	10.1 ± 2.1	45.3 ± 6.8	27.3 ± 4.8	17.6 ± 2.7	13.5 ± 3.3	59.6 ± 8.5	41.4 ± 6.6	21.5 ± 3.5
Propionaldehyde								
Pyrazole-induced	0.5 ± 0.1	8.9 ± 2.0	12.3 ± 2.3	3.7 ± 0.9	0.6 ± 0.2	12.9 ± 3.1	20.5 ± 4.1	5.5 ± 1.1
Control	0.3 ± 0.1	3.8 ± 0.8	6.9 ± 1.5	1.8 ± 0.3	0.4 ± 0.1	5.4 ± 1.0	8.9 ± 1.7	2.4 ± 0.5
TOTAL								
Pyrazole-induced	16.2 ± 3.3	311.0 ± 55.8	325.7 ± 57.1	98.3 ± 16.6	23.2 ± 4.6	457.7 ± 93.8	483.1 ± 97.7	143.1 ± 20.9
Control	18.3 ± 4.5	147.6 ± 19.7	160.3 ± 20.8	46.5 ± 7.4	25.1 ± 4.3	198.2 ± 21.7	218.0 ± 24.3	64.9 ± 9.3

[a] 0.1 mg/mL active microsomal protein plus 0.9 mg/mL heat-killed microsomal protein. TCA=trichloroacetic acid, TCE=trichloroethanol. The concentrations of chloral hydrate, TCA, and TCE were 5 mmol. Data are presented as nmol lipid peroxidation product/mg microsomal protein (mean ± standard deviation)

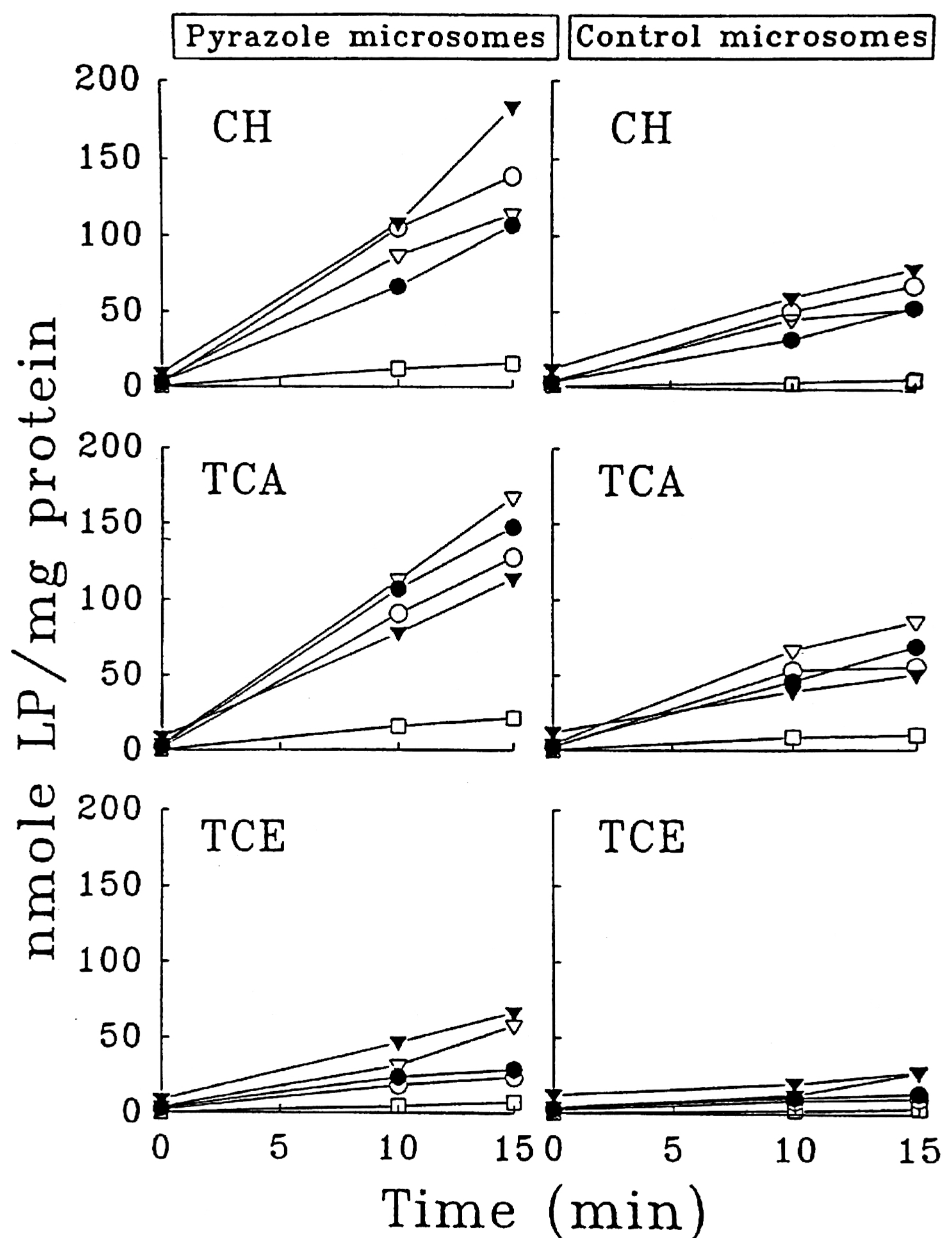

FIGURE D4
Lipid Peroxidation Products Formed from the Metabolism of Chloral Hydrate (CH), Trichloroacetic Acid (TCA), and Trichloroethanol (TCE) by Mouse Control Microsomes and Pyrazole-Induced Mouse Liver Microsomes (0.1 nmol/mg microsomal protein) (○ malondialdehyde, ● formaldehyde, ▽ acetaldehyde, ▲ acetone, □ propionaldehyde)

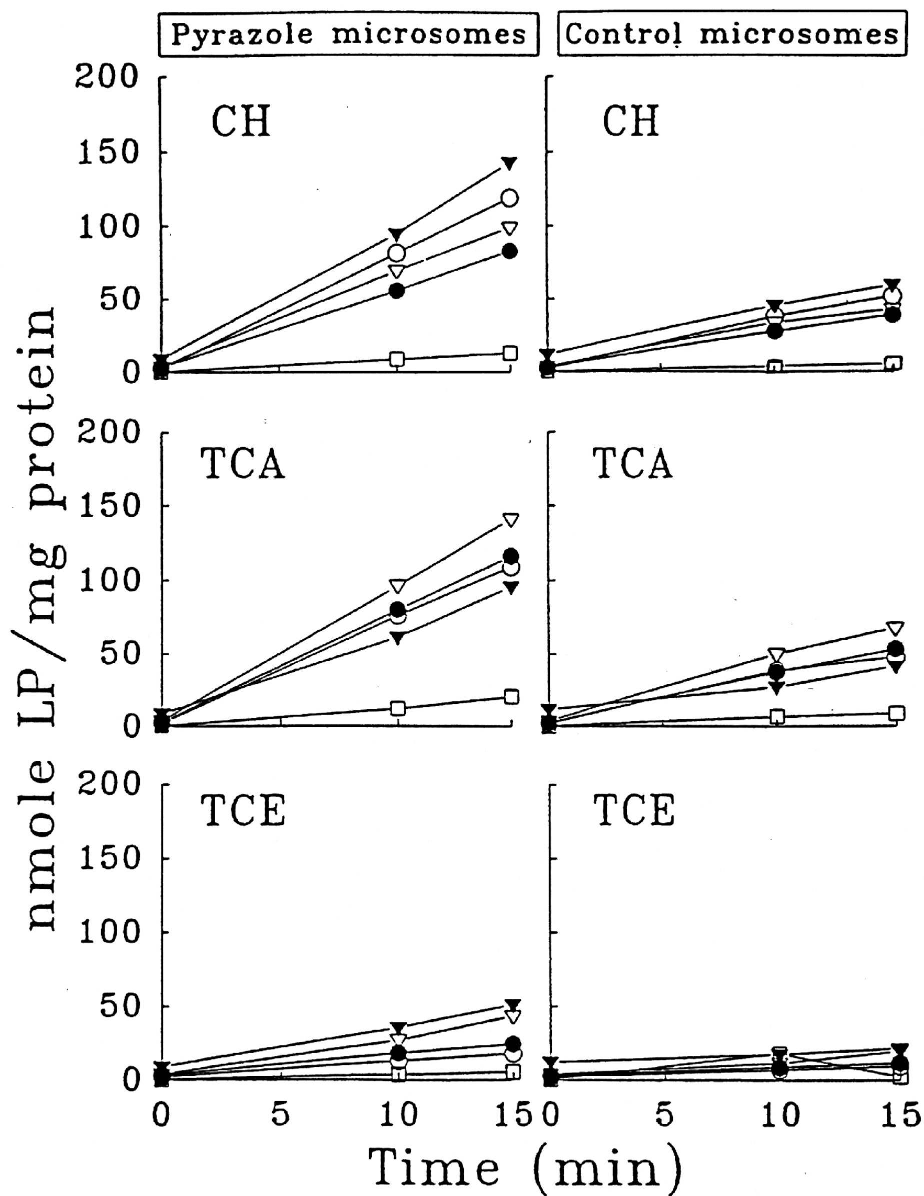

FIGURE D5
Lipid Peroxidation Products Formed from the Metabolism of Chloral Hydrate (CH), Trichloroacetic Acid (TCA), and Trichloroethanol (TCE) by Mouse Control Microsomes and Pyrazole-Induced Mouse Liver Microsomes (1.0 nmol/mg microsomal protein)
(○ malondialdehyde, ● formaldehyde, ▽ acetaldehyde, ▲ acetone, □ propionaldehyde)

TABLE D2
Inhibitory Effect of 2,4-Dichloro-6-phenylphenoxyethylamine on Chloral Hydrate-Induced Lipid Peroxidation in Mouse Liver Microsomal Metabolism[a]

Lipid Peroxidation Product	nmol Lipid Peroxidation Product/mg Microsomal Protein					
	Microsomes Control	Microsomes+ 100 μM DPEA	Microsomes+ 500 μM DPEA	Microsomes+ Chloral Hydrate	Microsomes+ Chloral Hydrate+ 100 μM DPEA	Microsomes+ Chloral Hydrate+ 500 μM DPEA
Malondialdehyde						
Pyrazole-induced	2.1 ± 0.3	2.7 ± 0.4	5.8 ± 0.9	121.7 ± 26.3	97.3 ± 21.1	49.9 ± 6.3
Control	1.8 ± 0.3	3.4 ± 0.5	5.0 ± 0.8	53.1 ± 6.9	38.7 ± 5.6	15.3 ± 2.1
Formaldehyde						
Pyrazole-induced	2.9 ± 0.4	5.3 ± 0.6	8.3 ± 1.1	87.2 ± 20.4	74.4 ± 12.5	39.6 ± 5.5
Control	3.3 ± 0.5	5.5 ± 0.6	8.7 ± 1.2	41.5 ± 7.3	30.1 ± 4.4	13.4 ± 2.0
Acetaldehyde						
Pyrazole-induced	4.7 ± 1.0	6.1 ± 1.2	9.1 ± 1.3	93.3 ± 20.7	69.9 ± 11.7	35.7 ± 5.6
Control	4.4 ± 0.9	7.9 ± 1.6	9.4 ± 1.2	49.6 ± 8.1	33.2 ± 5.1	18.4 ± 2.7
Acetone						
Pyrazole-induced	7.7 ± 1.4	8.5 ± 1.6	10.2 ± 1.3	133.4 ± 27.8	107.8 ± 19.3	59.8 ± 10.1
Control	9.3 ± 1.7	10.7 ± 1.9	11.6 ± 1.7	61.7 ± 6.8	44.6 ± 6.7	21.6 ± 3.3
Propionaldehyde						
Pyrazole-induced	0.6 ± 0.1	1.0 ± 0.2	1.4 ± 0.3	8.9 ± 2.1	6.5 ± 1.1	4.1 ± 0.7
Control	0.4 ± 0.1	0.7 ± 0.1	1.2 ± 0.2	5.3 ± 1.0	3.7 ± 0.6	2.4 ± 0.3
TOTAL						
Pyrazole-induced	18.0 ± 3.4	23.6 ± 3.6	34.8 ± 5.4	444.5 ± 91.6	355.9 ± 51.3	189.1 ± 27.8
Control	19.2 ± 4.1	28.2 ± 4.4	35.9 ± 5.3	211.2 ± 24.7	150.3 ± 23.1	71.1 ± 10.6

[a] The concentration of chloral hydrate was 200 μM. Data are presented as mean ± standard deviation. DPEA=2,4-dichloro-6-phenylphenoxyethylamine

TABLE D3
Effect of 2,4-Dichloro-6-phenylphenoxyethylamine on 6-Hydroxylation of Chlorzoxazone[a]

DPEA (μM)	Control Microsomes		Pyrazole-Induced Microsomes	
	6-Hydroxy-CZX (nmol/mg/min)	Inhibition (%)	6-Hydroxy-CZX (nmol/mg/min)	Inhibition (%)
0	1.9 ± 0.3	0.0	4.2 ± 0.7	0.0
100	1.2 ± 0.2	36.8	3.0 ± 0.4	28.6
500	0.4 ± 0.1	78.9	1.2 ± 0.2	71.4

[a] Data obtained from two independent experiments and are expressed as mean ± standard deviation. DPEA=2,4-dichloro-6-phenylphenoxyethylamine; CZX=chlorzoxazone

TABLE D4
Comparison of the Effects of NADPH and NADH on Lipid Peroxidation Induced by Chloral Hydrate[a]

	nmol Total Lipid Peroxidation Product/mg Microsomal Protein/min[b]			
	Control Microsomes	Control Microsomes+ Chloral Hydrate	Pyrazole-Induced Microsomes	Pyrazole-Induced Microsomes+ Chloral Hydrate
NADPH	1.3 ± 0.2	14.1 ± 2.1	1.2 ± 0.2	19.6 ± 4.6
NADH	1.1 ± 0.2	1.4 ± 0.3	1.2 ± 0.2	1.6 ± 0.3

[a] The concentration of NADPH and NADH was 300 μM; the concentration of chloral hydrate was 200 μM.
[b] Data are from two experiments and are expressed as mean ± standard deviation; isolated as derivatives formed by trapping the lipid peroxidation products with 2,4-dinitrophenylhydrazine and quantified by HPLC

TABLE D5
Effect of Xanthine Oxidase, Allopurinol, and NADPH on Lipid Peroxidation Induced by Microsomal Metabolism of Chloral Hydrate

	nmol Total Lipid Peroxidation Product/mg Microsomal Protein/min[a]		
	Microsomes	Microsomes+ Chloral Hydrate	Microsomes+ NADPH+ Chloral Hydrate
Dimethylsulfoxide[b]			
Pyrazole-induced	1.3 ± 0.2	1.6 ± 0.3	23.2 ± 3.5
Control	1.4 ± 0.2	1.7 ± 0.3	10.5 ± 1.7
Hypoxanthine (200 μM)			
Pyrazole-induced	1.2 ± 0.2	1.3 ± 0.2	22.1 ± 3.5
Control	1.0 ± 0.2	1.5 ± 0.3	11.9 ± 1.8
Allopurinol (100 μM)			
Pyrazole-induced	1.6 ± 0.3	1.7 ± 0.3	23.5 ± 3.7
Control	1.3 ± 0.2	1.4 ± 0.3	11.1 ± 1.7
Allopurinol (1,000 μM)			
Pyrazole-induced	1.5 ± 0.3	1.8 ± 0.4	24.0 ± 3.8
Control	1.4 ± 0.3	1.5 ± 0.3	10.2 ± 1.6

[a] Data are from two experiments and are expressed as mean ± standard deviation.
[b] Solvent control

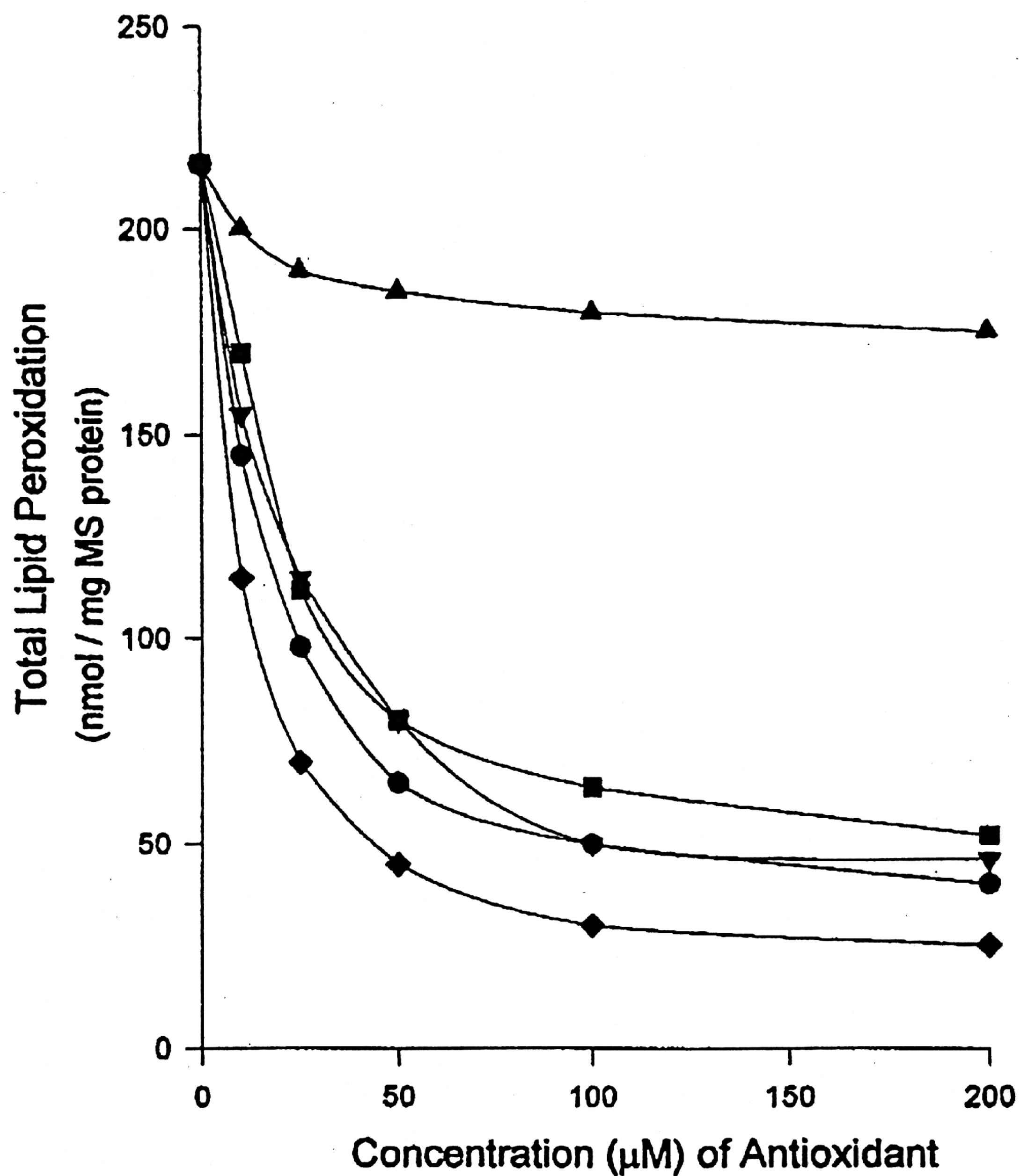

Figure D6
Inhibitory Effects of α-Tocopherol (●), ß-Carotene (■), Ascorbic Acid (▲), Menadione (▼), and a Combination of Ascorbic Acid and α-Tocopherol (◆) on Chloral Hydrate-Induced Lipid Peroxidation

TABLE D6
Effect of Antioxidants on Lipid Peroxidation from Metabolism of Chloral Hydrate by Male B6C3F$_1$ Mouse Liver Control Microsomes

Antioxidant/μM	Total Production (nmol/mg microsomal protein)[a] 0	10	25	50	100	200
α-Tocopherol	217 ± 27	146 ± 19	107 ± 13	66 ± 8	36 ± 5	34 ± 5
β-Carotene	217 ± 27	169 ± 22	135 ± 18	83 ± 11	47 ± 7	40 ± 6
Ascorbic acid	217 ± 27	189 ± 26	193 ± 24	178 ± 23	156 ± 22	170 ± 22
α-Tocopherol and ascorbic acid	217 ± 27	116 ± 16	68 ± 9	37 ± 6	22 ± 4	20 ± 4
Menadione	217 ± 27	154 ± 17	120 ± 17	78 ± 10	46 ± 6	41 ± 5

[a] The concentration of chloral hydrate was 5 mM. The data are from two experiments and are expressed as mean ± standard deviation.

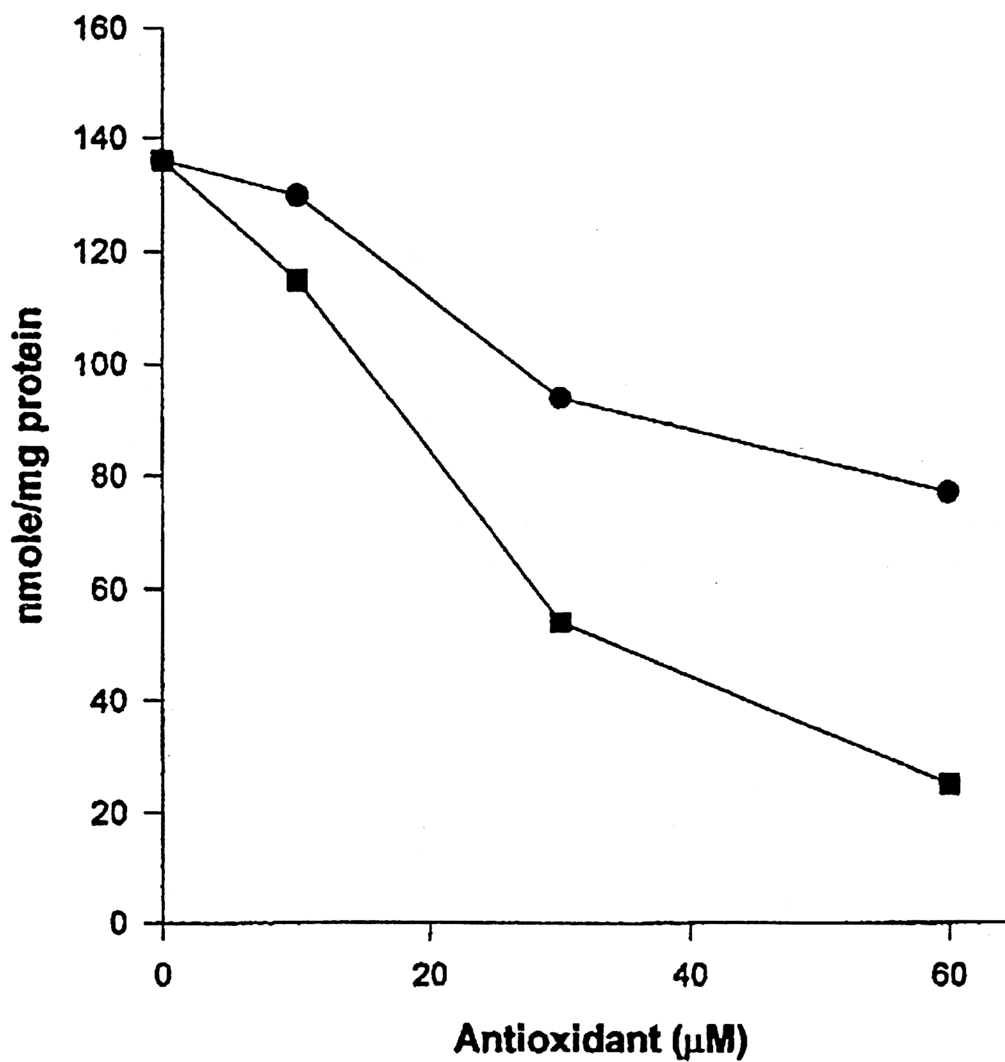

FIGURE D7
Inhibitory Effects of α-Tocopherol (■), and Menadione (●) on Trichloroacetic Acid-Induced Lipid Peroxidation

TABLE D7
Inhibitory Effect of α-Tocopherol and Menadione on Trichloroacetic Acid-Induced Lipid Peroxidation[a]

Trichloroacetic Acid (μM)	α-Tocopherol (μM)	Menadione (μM)	Malon-dialdehyde (nmol/mg microsomal protein)	Formaldehyde	Acetaldehyde	Acetone	Propion-aldehyde	Total
0	0	0	2.2 ± 0.0	3.8 ± 0.1	1.1 ± 0.1	7.8 ± 0.4	0	14.9 ± 0.1
2	0	0	18.5 ± 0.3	38.2 ± 1.1	23.2 ± 2.2	46.8 ± 6.6	7.3 ± 0.1	133.9 ± 7.4
2	10	0	14.2 ± 0.3	31.3 ± 2.6	19.2 ± 1.8	44.7 ± 3.8	5.1 ± 0.1	114.5 ± 6.8
2	30	0	6.2 ± 0.1	19.6 ± 2.2	5.3 ± 0.1	23.8 ± 0.8	0	54.9 ± 3.3
2	60	0	6.8 ± 0.1	11.2 ± 2.0	7.4 ± 0.2	12.6 ± 0.1	0	38.0 ± 1.7
0	0	0	1.7 ± 0.1	3.0 ± 0.1	0.8 ± 0.1	4.5 ± 0.2	0	10.0 ± 0.1
2	0	0	12.2 ± 0.7	26.0 ± 0.9	21.0 ± 1.8	38.0 ± 7.0	3.0 ± 0.1	100.2 ± 12.6
2	0	5	12.6 ± 0.2	26.4 ± 1.1	22.2 ± 1.1	35.0 ± 7.0	1.2 ± 0.1	92.4 ± 10.8
2	0	20	11.8 ± 0.2	18.0 ± 0.7	17.0 ± 1.7	23.6 ± 2.2	0	70.4 ± 13.7
2	0	50	9.2 ± 0.1	18.2 ± 0.2	10.5 ± 0.1	21.1 ± 0.2	0	59.0 ± 8.8

[a] Data are from two experiments and are expressed as mean ± standard deviation.

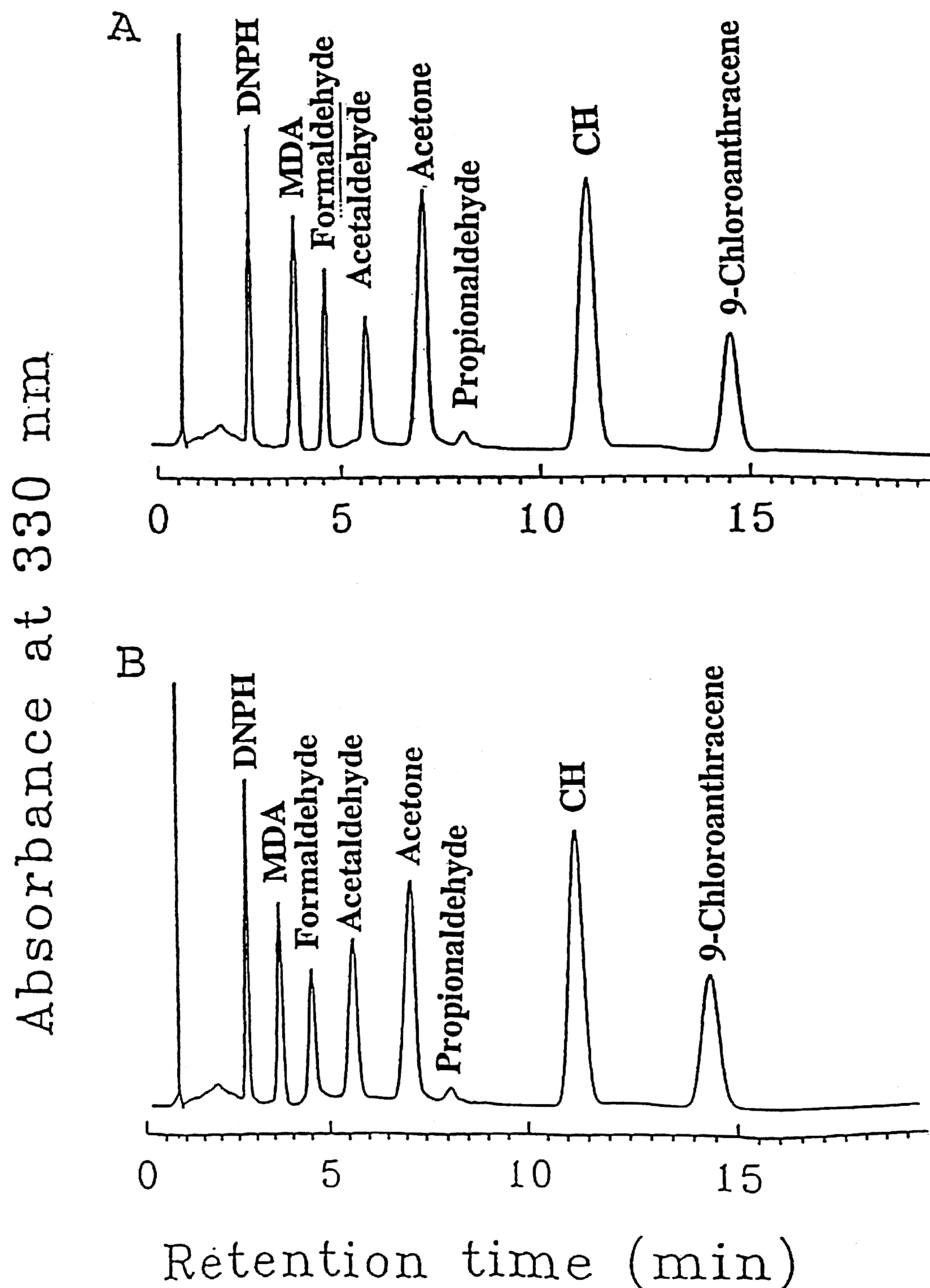

FIGURE D8
High-Performance Liquid Chromatography Profiles of 2,4,-Dinotrophenylhydrazine-Derived Lipid Peroxidation Products Formed from the Incubation of Chloral Hydrate with (A) Rat Liver Microsomes or (B) Human Liver Microsomes

TABLE D8
Lipid Peroxidation Products Formed from Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Male F344/N Rat Liver Microsomes and Human Liver Microsomes[a]

Lipid Peroxidation Products	nmol Lipid Peroxidation Product/mg of Microsomal Protein							
	Rat Liver Microsomes				Human Liver Microsomes			
	Water	Chloral Hydrate	TCA	TCE	Water	Chloral Hydrate	TCA	TCE
Malondialdehyde	1.9 ± 0.3	36.9 ± 5.9	30.4 ± 5.5	12.1 ± 2.1	2.5 ± 0.4	31.1 ± 5.7	37.8 ± 6.1	14.5 ± 2.6
Formaldehyde	2.2 ± 0.4	30.5 ± 5.4	31.3 ± 5.1	14.7 ± 2.3	1.8 ± 0.3	19.5 ± 3.1	22.6 ± 3.3	9.6 ± 1.8
Acetaldehyde	3.7 ± 0.7	19.8 ± 3.7	35.7 ± 6.2	17.3 ± 3.1	3.5 ± 0.5	24.3 ± 3.5	14.3 ± 2.8	11.5 ± 1.9
Acetone	4.8 ± 1.1	38.4 ± 5.7	27.6 ± 4.7	19.6 ± 3.4	5.2 ± 0.7	33.7 ± 4.4	34.7 ± 5.6	19.7 ± 3.3
Propionaldehyde	0.4 ± 0.1	3.6 ± 0.5	3.1 ± 0.5	1.5 ± 0.3	0.5 ± 0.1	2.8 ± 0.6	3.3 ± 0.6	2.2 ± 0.5
TOTAL	13.0 ± 2.4	129.2 ± 21.8	128.1 ± 19.3	65.2 ± 8.8	13.5 ± 2.2	111.4 ± 17.3	112.7 ± 18.5	57.5 ± 9.1

[a] Data are from two experiments and are expressed as mean ± standard deviation. TCA=trichloroacetic acid; TCE=trichloroethanol. The concentrations of chloral hydrate, TCA, and TCE were 1 mM.

Table D9
Malondialdehyde and Malondialdehyde-Modified DNA Adducts Formed from the Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Mouse and Rat Liver Microsomes[a]

	Malondialdehyde (nmol/mg microsomal protein)	Malondialdehyde-Modified DNA Adducts (adducts/10^7 nucleotides)
Pyrazole-Induced Mouse Liver Microsomes		
Water	2.6 ± 0.5	—[b]
Chloral hydrate	121.1 ± 31.3	7.5 ± 1.9
Trichloroacetic acid	127.7 ± 29.6	8.2 ± 2.1
Trichloroethanol	41.8 ± 8.7	2.1 ± 0.4
Untreated Mouse Liver Microsomes		
Water	2.1 ± 0.4	—
Chloral hydrate	57.6 ± 13.7	4.3 ± 1.2
Trichloroacetic acid	53.8 ± 16.5	4.7 ± 1.6
Trichloroethanol	17.3 ± 3.9	1.1 ± 0.3
Untreated Rat Liver Microsomes		
Water	1.9 ± 0.3	—
Chloral hydrate	36.9 ± 5.9	2.4 ± 0.5
Trichloroacetic acid	30.4 ± 5.5	2.8 ± 0.5
Trichloroethanol	12.1 ± 2.1	0.7 ± 0.2

[a] Data are from two experiments and are expressed as mean ± standard deviation. The concentrations of chloral hydrate, trichloroacetic acid, and trichloroethanol were 1 mM. Measurements were made for malondialdehyde formation in the absence of calf thymus DNA and measurements of malondialdehyde-modified DNA adduct formation were made in the presence of calf thymus DNA.

[b] Not detectable (<0.1 adduct/10^7 nucleotides)

TABLE D10
Malondialdehyde and Malondialdehyde-Modified DNA Adducts Formed from the Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Human Liver Microsomes[a]

Age (years)	Chemical	Malondialdehyde (nmol/mg microsomal protein)[b]	Malondialdehyde-modified DNA adducts (adducts/10^7 nucleotides)
Male			
65	Water	2.5 ± 0.4	—[c]
	Chloral hydrate	31.4 ± 6.3	2.1 ± 0.3
	Trichloroacetic acid	38.7 ± 7.2	2.4 ± 0.4
	Trichloroethanol	16.1 ± 2.7	1.5 ± 0.3
57	Water	2.6 ± 0.4	—
	Chloral hydrate	29.7 ± 5.3	2.3 ± 0.5
	Trichloroacetic acid	43.9 ± 6.9	2.5 ± 0.7
	Trichloroethanol	15.8 ± 2.4	1.5 ± 0.3
57	Water	2.8 ± 0.6	—
	Chloral hydrate	32.1 ± 7.1	2.2 ± 0.4
	Trichloroacetic acid	49.3 ± 7.7	2.8 ± 0.6
	Trichloroethanol	18.9 ± 3.1	1.1 ± 0.3
55	Water	2.4 ± 0.5	—
	Chloral hydrate	30.1 ± 5.5	2.6 ± 0.5
	Trichloroacetic acid	31.8 ± 6.1	2.3 ± 0.4
	Trichloroethanol	12.3 ± 2.3	0.8 ± 0.2
35	Water	3.3 ± 0.5	—
	Chloral hydrate	28.7 ± 6.1	2.1 ± 0.4
	Trichloroacetic acid	34.8 ± 3.9	2.7 ± 0.6
	Trichloroethanol	11.9 ± 2.3	0.8 ± 0.2
27	Water	2.1 ± 0.2	—
	Chloral hydrate	33.1 ± 4.5	2.1 ± 0.4
	Trichloroacetic acid	33.9 ± 3.8	1.7 ± 0.4
	Trichloroethanol	10.3 ± 2.1	0.7 ± 0.2

TABLE D10
Malondialdehyde and Malondialdehyde-Modified DNA Adducts Formed from the Metabolism of Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol by Human Liver Microsomes

Age (years)	Chemical	Malondialdehyde (nmol/mg microsomal protein)	Malondialdehyde-modified DNA adducts (adducts/10^7 nucleotides)
Female			
69	Water	2.8 ± 0.2	—
	Chloral hydrate	44.5 ± 8.6	3.5 ± 0.6
	Trichloroacetic acid	48.3 ± 9.4	3.8 ± 0.8
	Trichloroethanol	18.4 ± 2.8	0.8 ± 0.2
67	Water	2.3 ± 0.3	—
	Chloral hydrate	33.3 ± 3.5	1.5 ± 0.3
	Trichloroacetic acid	39.7 ± 5.1	2.4 ± 0.5
	Trichloroethanol	14.4 ± 2.6	1.3 ± 0.4
63	Water	2.8 ± 0.7	—
	Chloral hydrate	31.2 ± 5.4	2.1 ± 0.5
	Trichloroacetic acid	43.8 ± 6.9	2.0 ± 0.4
	Trichloroethanol	11.3 ± 2.8	0.9 ± 0.2
60	Water	2.1 ± 0.4	—
	Chloral hydrate	35.1 ± 7.1	2.9 ± 0.5
	Trichloroacetic acid	32.6 ± 5.8	2.2 ± 0.5
	Trichloroethanol	12.4 ± 3.0	1.1 ± 0.2
35	Water	2.4 ± 0.3	—
	Chloral hydrate	29.8 ± 5.9	2.1 ± 0.4
	Trichloroacetic acid	31.7 ± 5.7	2.3 ± 0.5
	Trichloroethanol	16.3 ± 2.8	1.3 ± 0.3
32	Water	1.9 ± 0.3	—
	Chloral hydrate	21.4 ± 3.1	1.3 ± 0.3
	Trichloroacetic acid	25.1 ± 4.7	1.7 ± 0.4
	Trichloroethanol	14.7 ± 2.2	0.8 ± 0.2
Average (n = 12)	Water	2.5 ± 0.4	—
	Chloral hydrate	31.7 ± 5.3	2.2 ± 0.6
	Trichloroacetic acid	37.8 ± 7.4	2.4 ± 0.6
	Trichloroethanol	14.4 ± 2.8	1.0 ± 0.3

[a] Data are from two independent experiments and are expressed as mean ± standard deviation. The concentrations of chloral hydrate, trichloroacetic acid, and trichloroethanol were 1 mM. Measurements were made for malondialdehyde formation in the absence of calf thymus DNA and measurements of malondialdehyde-modified DNA adduct formation were made in the presence of calf thymus DNA.

[b] Isolated as the derivatives formed from reacting with 2,4-dinitrophenylhydrazine

[c] Not detectable (<0.1 adduct/10^7 nucleotides)

Table D11
Inhibition of Malondialdehyde and Malondialdehyde-Modified DNA Adduct Formation from Incubation of Chloral Hydrate with Pyrazole-Induced Mouse Liver Microsomes in the Absence or Presence of Calf Thymus DNA by Antioxidants[a]

	α-Tocopherol (μM)	Ascorbic Acid (μM)	Malondialdehyde[b] (nmol/mg microsomal protein)	MDA-MG-1 (adducts/10^7 nucleotides)
Control	0	0	91.3 ± 21.1	7.9 ± 1.5
Ascorbic acid	0	25	87.5 ± 22.6	7.3 ± 0.9
	0	50	81.6 ± 19.5	6.8 ± 0.4
	0	100	84.7 ± 20.3	5.6 ± 0.6
	0	200	69.9 ± 15.7	5.7 ± 0.8
α-Tocopherol	25	0	58.7 ± 13.9	5.4 ± 0.7
	50	0	33.7 ± 8.5	2.6 ± 0.4
	100	0	31.1 ± 6.7	2.4 ± 0.7
	200	0	27.5 ± 7.2	2.2 ± 0.4
Ascorbic acid and α-tocopherol	25	25	40.9 ± 11.3	3.7 ± 0.6
	50	50	24.4 ± 5.3	1.7 ± 0.3
	100	100	6.8 ± 5.3	0.7 ± 0.2
	200	200	8.4 ± 2.7	0.7 ± 0.3

[a] Data are from two experiments and are expressed as mean ± standard deviation. The concentration of chloral hydrate was 200 μM.
[b] Isolated as the derivatives formed from reacting with 2,4-dinitrophenylhydrazine

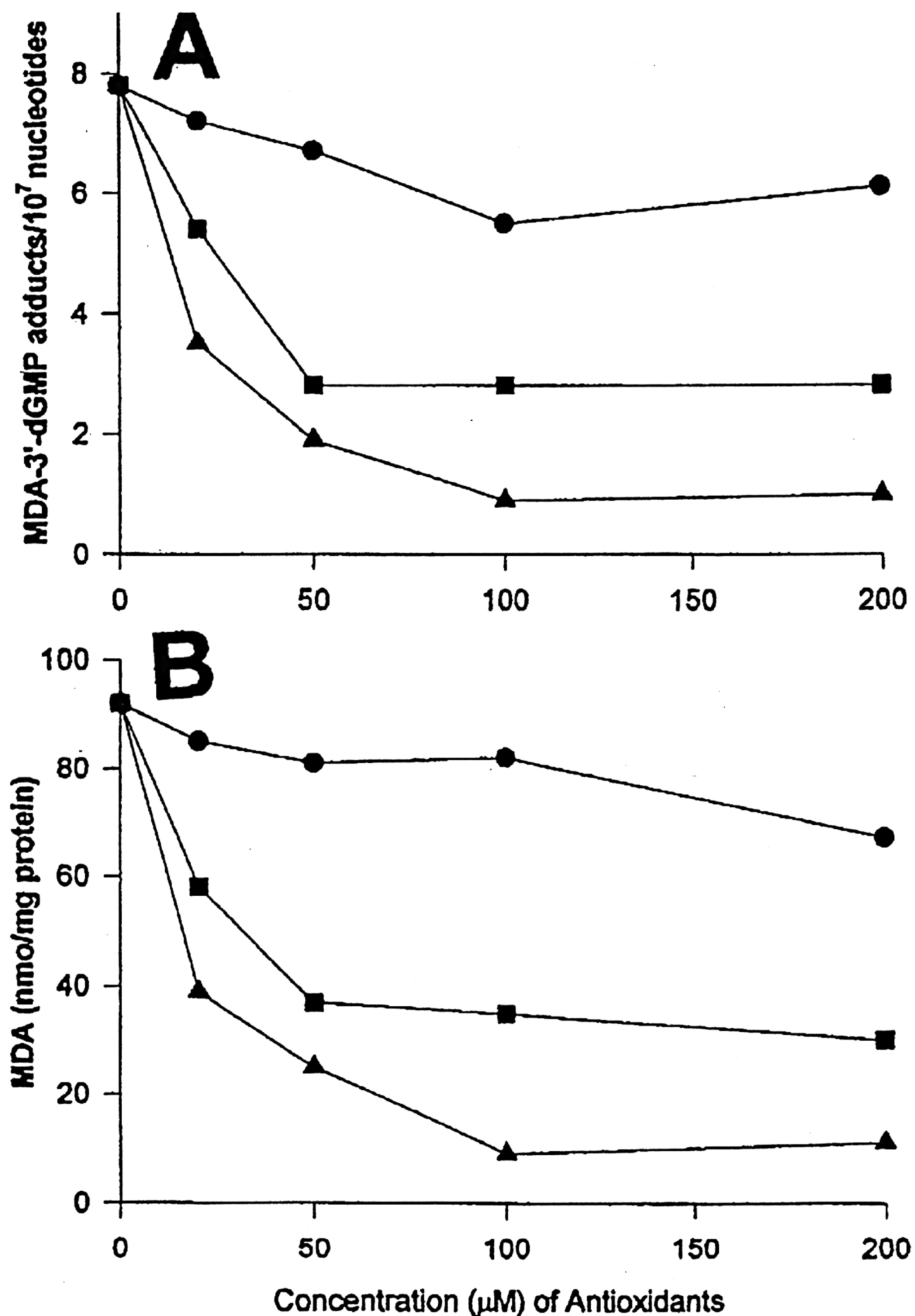

FIGURE D9
(A) Inhibition of Chloral Hydrate-Induced, Malondialdehyde-Modified DNA Adducts by α-Tocopherol (■), Ascorbic Acid (●), and a Combination of α-Tocopherol and Ascorbic Acid (▲); (B) Inhibition of Chloral Hydrate-Induced Malondialdehyde Formation by α-Tocopherol (■), Ascorbic Acid (●), and a Combination of a-Tocopherol and Ascorbic Acid (▲)

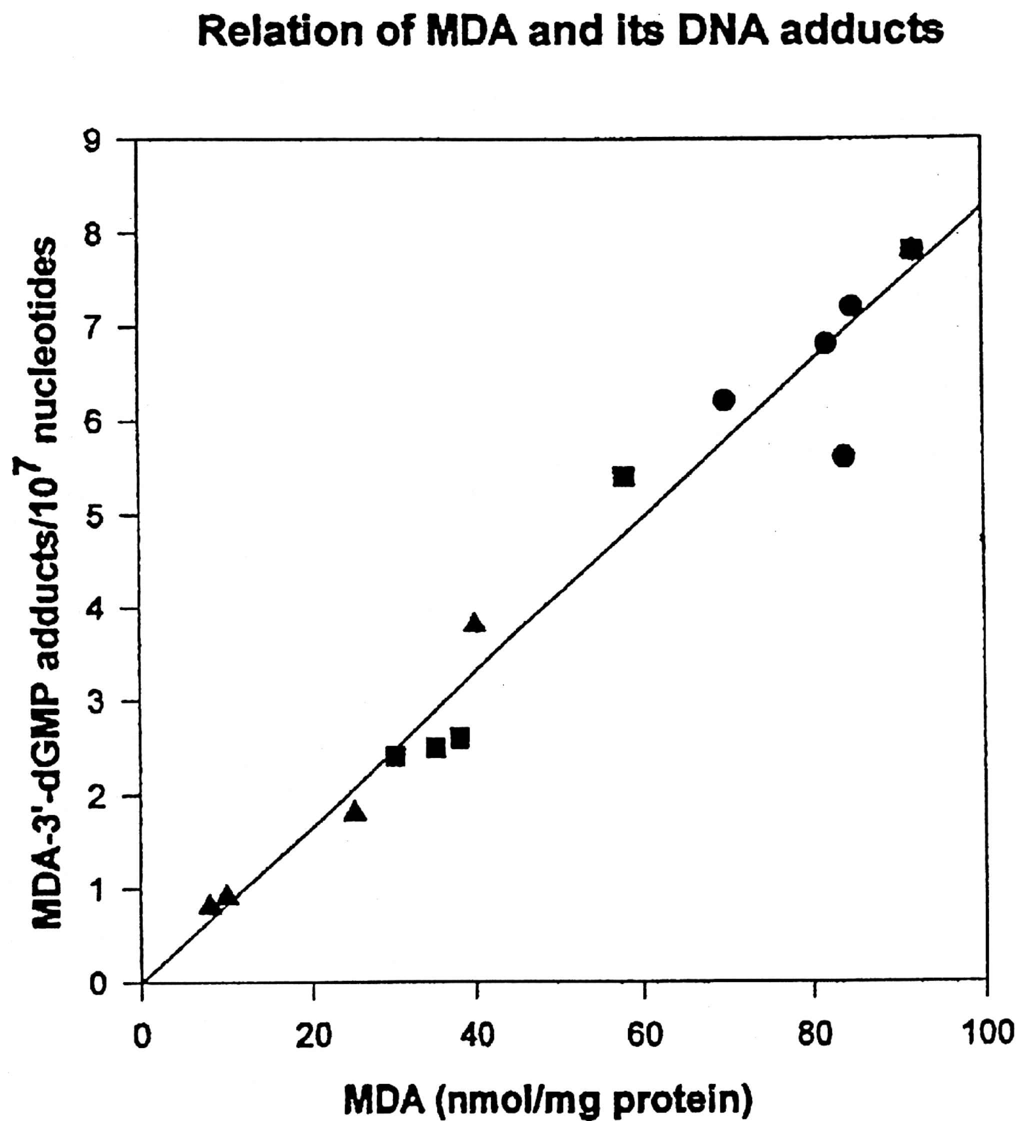

FIGURE D10
Relationship Between the Quantity of Chloral Hydrate-Induced Malondialdehyde and the Quantity of Chloral Hydrate-Induced, Malondialdehyde-Modified DNA Adducts Mediated by Pyrazole-Induced Mouse Liver Microsomes
[incubation with α-tocopherol (■), ascorbic acid (●), and a combination of α-tocopherol and ascorbic acid (▲)]

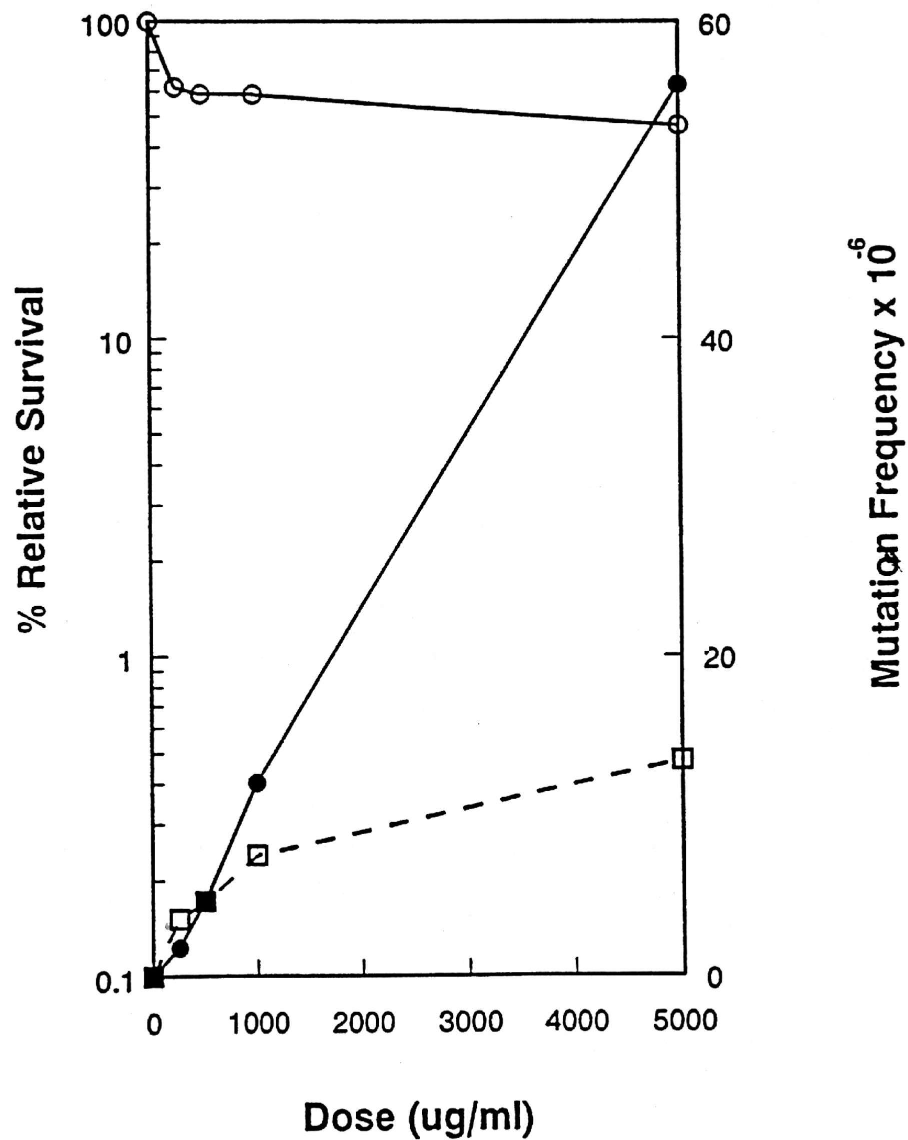

FIGURE D11
Mutagenicity of Chloral Hydrate at the *tk* and *hprt* Loci in H2E1 V2 Human Lymphoblastoid Cells Expressing Cytochrome P_{450} 2E1
(The data represent means from duplicate experiments; ❍ = cytotoxicity of chloral hydrate, ● = mutant fraction recovered at the *tk* locus, ❒ = mutant fraction recovered at the *hprt* locus)

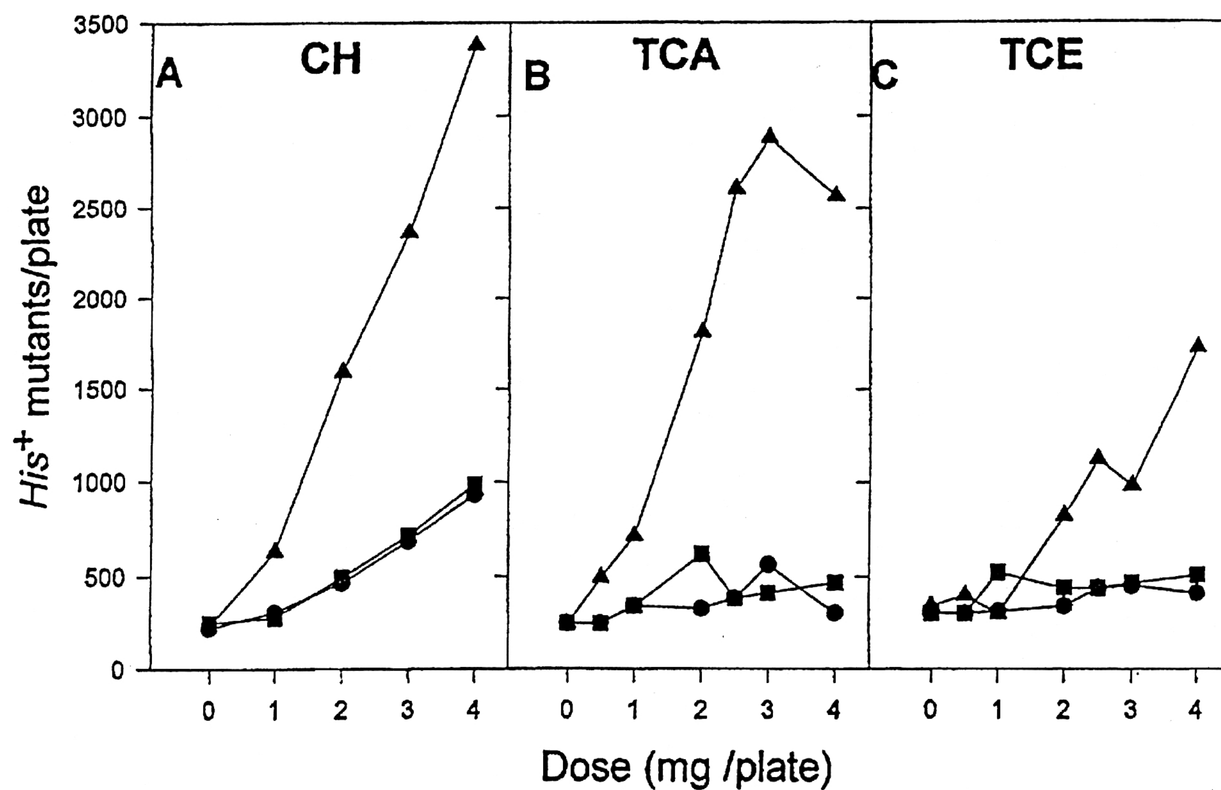

FIGURE D12
Mutagenicity of (A) Chloral Hydrate (CH), (B) Trichloroacetic Acid (TCA), and (C) Trichloroethanol (TCE) in *Salmonella typhimurium* Test Strain TA104 Tested with S9 (■), Without S9 (●), and Preincubated with S9 for 3 Minutes Prior to Addition of *S. typhimurium* (▲)

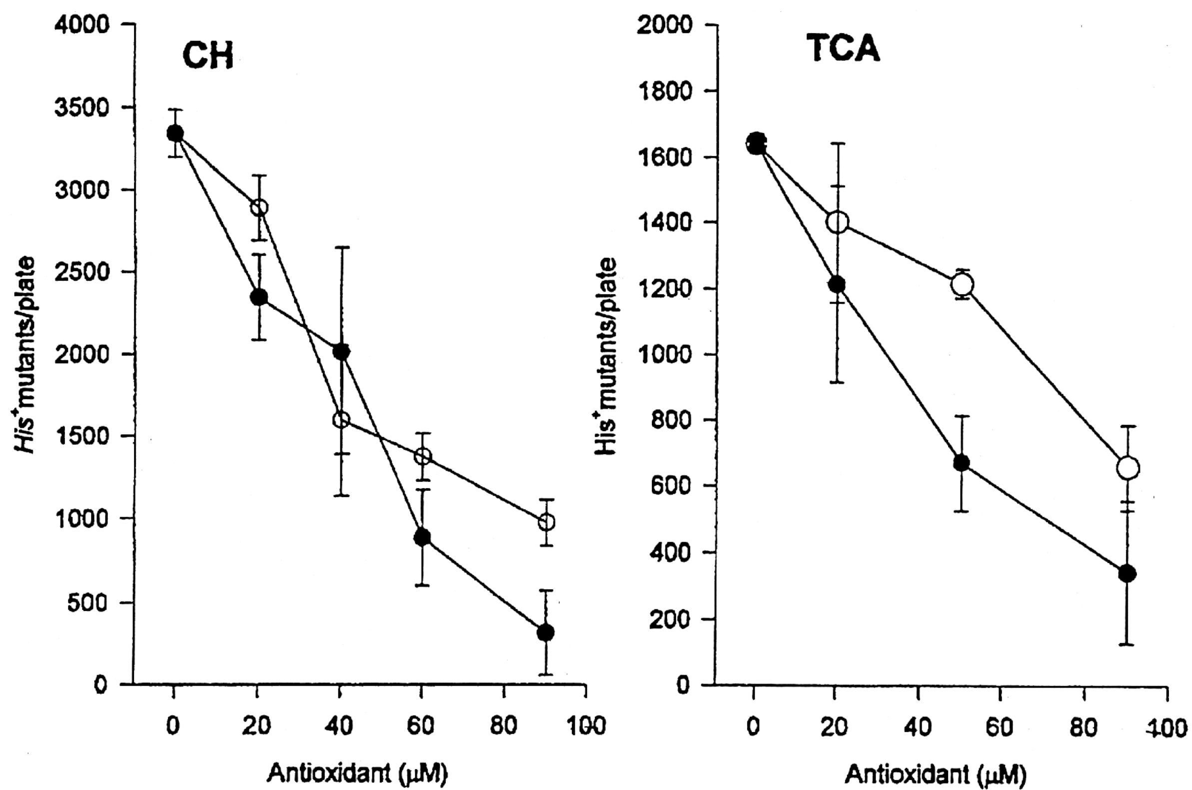

FIGURE D13

Inhibition of (A) Chloral Hydrate-Induced (CH) and (B) Trichloroacetic Acid-Induced (TCA) *Salmonella typhimurium* Mutagenicity by α-Tocopherol (●) and Menadione (○)
(Data are presented as the mean ± standard deviation.)

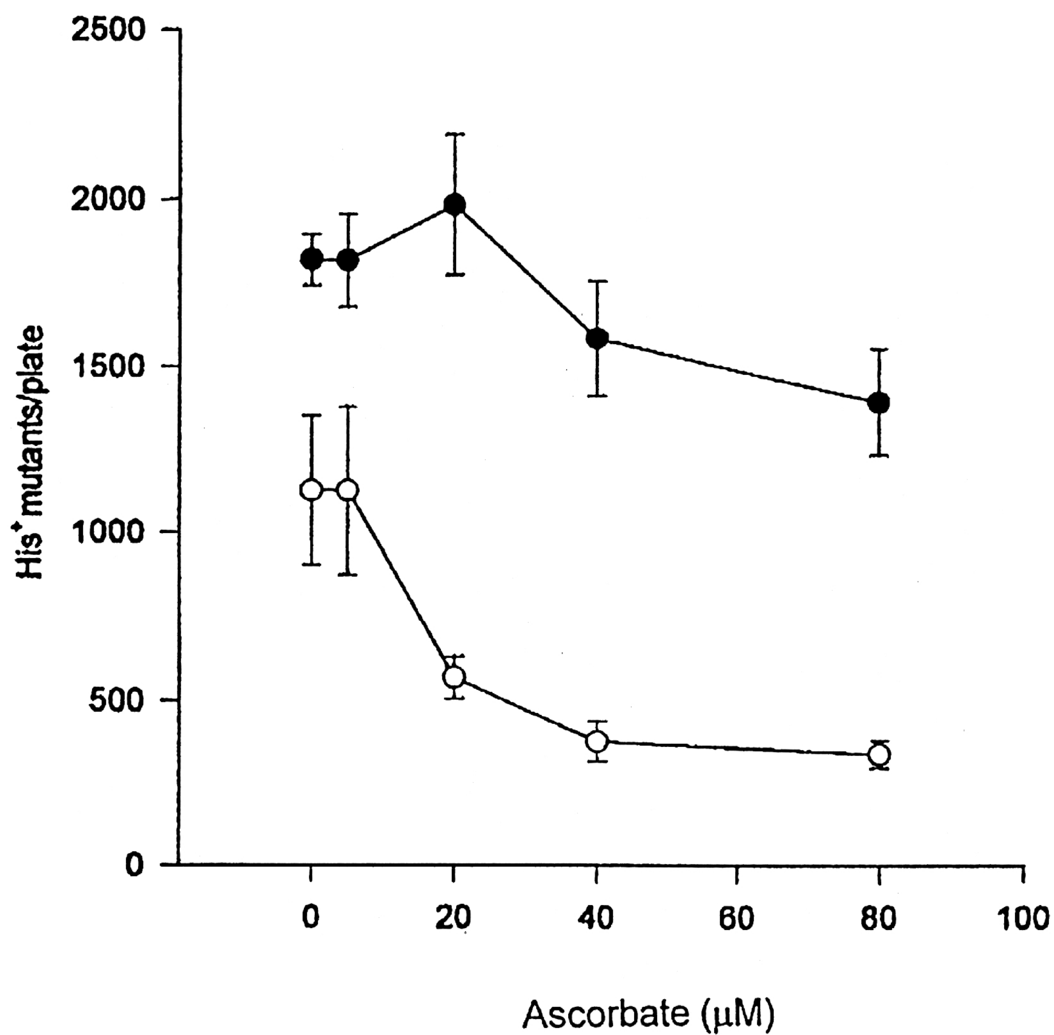

Figure D14
Inhibition of Trichloroacetic Acid-Induced *Salmonella typhimurium* Mutagenicity by Ascorbic Acid (●) and a Combination of Ascorbic Acid and α-Tocopherol (○)
(Data are presented as the mean ± standard deviation.)

APPENDIX E
GENETIC TOXICOLOGY

TABLE E1 **Mutagenicity of Chloral Hydrate in *Salmonella typhimurium*** **E-2**
TABLE E2 **Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Chloral Hydrate** **E-4**
TABLE E3 **Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Chloral Hydrate** **E-6**
TABLE E4 **Induction of Sex-Linked Recessive Lethal Mutations in *Drosophila melanogaster* by Chloral Hydrate** **E-7**
TABLE E5 **Induction of Micronuclei in Polychromatic Bone Marrow Cells of Male Mice Treated Three Times with Chloral Hydrate by Intraperitoneal Injection** **E-7**

TABLE E1
Mutagenicity of Chloral Hydrate in *Salmonella typhimurium*[a]

Strain	Dose (μg/plate)	Revertants/plate[b]					
		-S9		+10% hamster S9		+10% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA100							
	0	134 ± 7.4	125 ± 8.7	139 ± 2.4	125 ± 5.8	126 ± 5.8	142 ± 5.5
	100	141 ± 3.9	163 ± 6.6	161 ± 1.3	153 ± 3.9	149 ± 4.4	159 ± 2.3
	333	197 ± 16.3		255 ± 7.6		200 ± 11.8	
	1,000	306 ± 6.0	297 ± 1.2	366 ± 17.5	351 ± 19.3	327 ± 4.2	306 ± 3.5
	3,333	423 ± 17.1	422 ± 12.7	530 ± 8.2	524 ± 20.0	494 ± 9.0	495 ± 3.4
	4,000		456 ± 5.6				
	5,000		501 ± 1.0		584 ± 22.5		565 ± 12.9
	6,667	468 ± 17.4					
	7,500		518 ± 7.2				
	10,000			689 ± 37.8	632 ± 12.8	704 ± 66.8	650 ± 8.6
Trial summary		Positive	Positive	Positive	Positive	Positive	Positive
Positive control[c]		1,894 ± 86.8	2,025 ± 20.2	1,431 ± 42.4	2,068 ± 92.8	1,485 ± 83.6	1,612 ± 23.0

Strain	Dose (μg/plate)	-S9	+10% hamster S9	+10% rat S9
TA1535				
	0	17 ± 3.2	12 ± 5.2	10 ± 3.1
	100	19 ± 0.6	10 ± 2.0	15 ± 4.6
	333	19 ± 1.7	14 ± 0.7	7 ± 0.9
	1,000	18 ± 1.5	12 ± 0.7	11 ± 1.0
	3,333	15 ± 1.2	13 ± 0.9	15 ± 1.0
	6,667	13 ± 2.9		
	10,000		9 ± 0.6	11 ± 1.5
Trial summary		Negative	Negative	Negative
Positive control		1,156 ± 37.8	126 ± 9.6	109 ± 4.0
TA1537				
	0	7 ± 1.2	7 ± 1.5	6 ± 1.5
	100	9 ± 2.5	7 ± 1.3	8 ± 0.9
	333	5 ± 0.6	5 ± 1.2	7 ± 1.2
	1,000	11 ± 1.8	6 ± 0.6	7 ± 0.6
	3,333	11 ± 1.8	9 ± 1.3	5 ± 0.7
	6,667	12 ± 2.9		
	10,000		4 ± 0.3	5 ± 0.9
Trial summary		Negative	Negative	Negative
Positive control		478 ± 54.9	133 ± 4.0	124 ± 12.8

TABLE E1
Mutagenicity of Chloral Hydrate in *Salmonella typhimurium*

Strain	Dose (μg/plate)	Revertants/plate					
		-S9		+10% hamster S9		+10% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA98							
	0	21 ± 2.0	22 ± 3.7	29 ± 2.0	31 ± 3.2	30 ± 0.9	37 ± 4.7
	100	20 ± 6.0	21 ± 2.3	24 ± 2.3	26 ± 1.7	29 ± 2.9	29 ± 1.5
	333	21 ± 0.7		33 ± 1.5		31 ± 3.6	
	1,000	31 ± 0.3	27 ± 2.5	28 ± 5.0	32 ± 2.3	30 ± 1.2	38 ± 4.7
	3,333	46 ± 5.6	42 ± 4.0	42 ± 5.2	43 ± 0.6	39 ± 0.6	45 ± 3.7
	4,000		43 ± 1.5				
	5,000		41 ± 3.1		45 ± 1.3		46 ± 1.5
	6,667	39 ± 4.1					
	7,500		27 ± 6.9[d]				
	10,000			27 ± 4.6	43 ± 2.8	31 ± 2.3	38 ± 1.7
Trial summary		Equivocal	Equivocal	Negative	Equivocal	Negative	Negative
Positive control		1,526 ± 14.2	1,738 ± 33.8	1,345 ± 157.3	1,775 ± 14.9	1,245 ± 89.2	1,476 ± 27.3

[a] Study was performed at EG&G Mason. The detailed protocol and these data are presented in Haworth *et al.* (1983). 0 μg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.
[d] Slight toxicity

TABLE E2
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Chloral Hydrate[a]

Compound	Dose (μg/mL)	Total Cells Scored	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome[b] (%)
-S9								
Trial 1								
Summary: Weakly positive								
Dimethylsulfoxide		50	1,039	488	0.46	9.8	26.0	
Mitomycin-C	0.001	50	1,040	635	0.61	12.7	26.0	30.00
	0.010	5	105	267	2.54	53.4	26.0	441.40
Chloral hydrate	16.7	50	1,017	463	0.45	9.3	26.0	−3.07
	50.0	50	1,040	520	0.50	10.4	26.0	6.46
	167.0	50	1,042	747	0.71	14.9	26.0	52.63*
	500.0	0					26.0	
					P<0.001[c]			
Trial 2								
Summary: Positive								
Dimethylsulfoxide		50	1,041	415	0.39	8.3	26.0	
Mitomycin-C	0.001	50	1,035	515	0.49	10.3	26.0	24.82
	0.010	5	103	196	1.90	39.2	26.0	377.34
Chloral hydrate	100	50	1,023	593	0.57	11.9	26.0	45.41*
	150	50	1,032	625	0.60	12.5	26.0	51.92*
	200	50	1,033	831	0.80	16.6	34.8[d]	101.79*
	350	0					34.8[d]	
					P<0.001			

TABLE E2
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Chloral Hydrate

Compound	Dose (μg/mL)	Total Cells Scored	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome (%)
+S9								
Trial 1								
Summary: Weakly positive								
Dimethylsulfoxide		50	1,036	464	0.44	9.3	26.0	
Cyclophosphamide	0.4	50	1,042	651	0.62	13.0	26.0	39.50
	2.0	5	105	194	1.84	38.8	26.0	312.53
Chloral hydrate	167	50	1,037	391	0.37	7.8	26.0	−15.82
	500	50	1,017	386	0.37	7.7	26.0	−15.26
	1,700	50	1,040	567	0.54	11.3	26.0	21.73*
	5,000	0					26.0	
					P<0.001			
Trial 2								
Summary: Positive								
Dimethylsulfoxide		50	1,040	365	0.35	7.3	26.0	
Cyclophosphamide	0.4	50	1,020	629	0.61	12.6	26.0	75.71
	2.0	5	105	162	1.54	32.4	26.0	339.61
Chloral hydrate	3,000	50	1,030	504	0.48	10.1	26.0	39.42*
	4,000	50	1,033	587	0.56	11.7	26.0	61.91*
	5,000	50	1,036	712	0.68	14.2	34.8[d]	95.82*
					P<0.001			

* Positive response (≥20% increase over solvent control)
[a] Study was performed at Litton Bionetics, Inc. The detailed protocol is presented in Galloway *et al.* (1987). SCE=sister chromatid exchange; BrdU=bromodeoxyuridine
[b] SCEs/chromosome in treated cells versus SCEs/chromosome in solvent control cells
[c] Significance of SCEs/chromosome tested by the linear regression trend test versus log of the dose
[d] Because chloral hydrate induced a delay in the cell division cycle, harvest time was extended to maximize the number of second-division metaphase cells available for analysis.

TABLE E3
Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Chloral Hydrate[a]

-S9

	Dose (μg/mL)	Total Cells	No. of Abs	Abs/ Cell	Cells with Abs (%)
Trial 1 - Harvest time: 20.5 hours[b]					
Summary: Negative					
Dimethylsulfoxide					
		100	3	0.03	2.0
Mitomycin-C					
	0.0400	100	23	0.23	13.0
	0.0625	25	28	1.12	36.0
Chloral hydrate					
	400	100	2	0.02	2.0
	600	100	0	0.00	0.0
	800	100	3	0.03	3.0
					P=0.473[c]
Trial 2 - Harvest time: 20.5 hours[b]					
Summary: Positive					
Dimethylsulfoxide					
		100	1	0.01	1.0
Mitomycin-C					
	0.0400	100	7	0.07	7.0
	0.0625	25	15	0.60	48.0
Chloral hydrate					
	750	100	6	0.06	6.0
	1,000	100	23	0.23	18.0*
	1,250	100	57	0.57	36.0*
	1,500	0			
					P<0.001

+S9

	Dose (μg/mL)	Total Cells	No. of Abs	Abs/ Cell	Cells with Abs (%)
Trial 1 - Harvest time: 12.5					
Summary: Positive					
Dimethylsulfoxide					
		100	1	0.01	1.0
Cyclophosphamide					
	7.5	100	14	0.14	11.0
	37.5	25	14	0.56	28.0
Chloral hydrate					
	3,000	100	30	0.30	24.0*
	3,500	50	10	0.20	20.0*
	4,000	25	8	0.32	24.0*
	4,500	0			
					P<0.001
Trial 2 - Harvest time: 12.5 hours					
Summary: Weakly positive					
Dimethylsulfoxide					
		100	3	0.03	3.0
Cyclophosphamide					
	7.5	100	13	0.13	10.0
	37.5	25	13	0.52	32.0
Chloral hydrate					
	2,500	100	9	0.09	7.0
	3,000	100	46	0.46	33.0*
	3,500	0			
					P<0.001

* Positive (P≤0.05)
[a] Study was performed at Litton Bionetics, Inc. The detailed protocol is presented in Galloway *et al*. (1987). Abs=aberrations
[b] Because chloral hydrate induced a delay in the cell division cycle, incubation time prior to addition of Colcemid was lengthened to provide sufficient first-division metaphase cells at harvest.
[c] Significance of percent cells with aberrations tested by the linear regression trend test versus log of the dose

TABLE E4
Induction of Sex-Linked Recessive Lethal Mutations in *Drosophila melanogaster* by Chloral Hydrate[a]

Route of Exposure	Dose (ppm)	Incidence of Death (%)	Incidence of Sterility (%)	No. of Lethals/No. of X Chromosomes Tested			Total[b]
				Mating 1	Mating 2	Mating 3	
Feeding[c]	5,500	6	0	4/2,140	2/2,315	3/2,287	9/6,742 (0.13%)
	0			1/2,167	1/2,362	1/2,260	3/6,789 (0.04%)
Injection[c]	10,000	10	0	0/1,910	2/2,394	1/2,183	3/6,487 (0.05%)
	0[d]			2/1,370	0/3,003	1/2,120	3/6,493 (0.05%)

[a] Study was performed at Brown University. The detailed protocol and these data are presented in Yoon *et al*. (1985).
[b] Total number of lethal mutations/total number of X chromosomes tested for three mating trials
[c] Significance of total number of lethal mutations/total number of X chromosomes tested by a normal approximation to the binomial test (Margolin *et al.*, 1983). Results of the feeding experiment were inconclusive. Results of the injection experiment were negative at the 5% level.
[d] Solvent control

TABLE E5
Induction of Micronuclei in Polychromatic Bone Marrow Cells of Male Mice Treated Three Times with Chloral Hydrate by Intraperitoneal Injection[a]

Compound	Dose (mg/kg)	Number of Mice	Micronucleated PCEs/1,000 PCEs[b]
Trial 1			
Phosphate-buffered saline[c]		4	2.9 ± 0.5
Cyclophosphamide[d]	15	4	19.1 ± 2.2
Chloral hydrate	125	5	2.1 ± 0.5
	250	5	2.7 ± 0.6
	500	5	4.4 ± 0.8
			P=0.006[e]
Trial 2			
Phosphate-buffered saline		5	1.7 ± 0.3
Cyclophosphamide	15	5	17.4 ± 1.7
Chloral hydrate	125	5	2.2 ± 0.5
	250	5	2.1 ± 0.3
	500	5	3.5 ± 0.5
			P=0.004

[a] Study was performed at Oak Ridge Associated Universities. The detailed protocol is presented in Shelby *et al*. (1993). PCE=polychromatic erythrocyte; 2,000 PCEs were scored in each animal in each dose group.
[b] Mean ± standard error
[c] Solvent control
[d] Positive control
[e] Significance of micronucleated PCEs/1,000 cells PCEs by the one-tailed trend test (ILS, 1990); significant at $P \leq 0.025$