NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDY OF CHLORAL HYDRATE

(*AD LIBITUM* AND DIETARY CONTROLLED)

(CAS NO. 302-17-0)

IN MALE B6C3F$_1$ MICE

(GAVAGE STUDY)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

December 2002

NTP TR 503

NIH Publication No. 03-4437

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

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.

The studies described in this Technical Report were performed under the direction of the NCTR and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The prechronic and chronic studies were conducted in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations, and all aspects of the chronic studies were subjected to retrospective quality assurance audits before being presented for public review.

These studies are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology and carcinogenesis studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. The interpretive conclusions presented in this Technical Report are based only on the results of these NTP studies. Extrapolation of these results to other species and quantitative risk analyses for humans require wider analyses beyond the purview of these studies. Selection *per se* is not an indicator of a chemical's carcinogenic potential.

Details about ongoing and completed NTP studies are available at the NTP's World Wide Web site: http://ntp-server.niehs.nih.gov. Abstracts of all NTP Technical Reports and full versions of the most recent reports and other publications are available from the NIEHS' Environmental Health Perspectives (EHP) http://ehp.niehs.nih.gov (800-315-3010 or 919-541-3841). In addition, printed copies of these reports are available from EHP as supplies last. A listing of all the NTP Technical Reports printed since 1982 appears on the inside back cover.

NTP TECHNICAL REPORT

ON THE

TOXICOLOGY AND CARCINOGENESIS

STUDY OF CHLORAL HYDRATE

(*AD LIBITUM* AND DIETARY CONTROLLED)

(CAS NO. 302-17-0)

IN MALE B6C3F$_1$ MICE

(GAVAGE STUDY)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

December 2002

NTP TR 503

NIH Publication No. 03-4437

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

CONTRIBUTORS

The studies on chloral hydrate were conducted at the FDA's National Center for Toxicological Research under an interagency agreement between the FDA and the NIEHS. The studies were designed and monitored by a Toxicology Study Selection and Review Committee, composed of representatives from the NCTR and other FDA product centers, NIEHS, and other *ad hoc* members from other government agencies and academia. The interagency agreement was designed to use the staff and facilities of the NCTR in testing of FDA priority chemicals and to provide FDA scientists and regulatory policymakers information for hazard identification and risk assessment.

Toxicology Study Selection and Review Committee

B.A. Schwetz, D.V.M., Ph.D., Chairperson
National Center for Toxicological Research
W.T. Allaben, Ph.D.
National Center for Toxicological Research
F.A. Beland, Ph.D.
National Center for Toxicological Research
J.R. Bucher, Ph.D.
National Institute of Environmental Health Sciences
J.F. Contrera, Ph.D.
Center for Drug Evaluation, Food and Drug Administration
D.W. Gaylor, Ph.D.
National Center for Toxicological Research
K.J. Greenlees, Ph.D.
Center for Veterinarian Medicine, Food and Drug Administration
R.J. Lorentzen, Ph.D.
Center for Food Safety and Applied Nutrition, Food and Drug Administration
F.D. Sistare, Ph.D.
Center for Drug Evaluation, Food and Drug Administration

Bionetics

Prepared animal feed and cared for mice

J. Carson, B.S.
A. Matson, B.S.
M. Moore
M.L. Nichols

National Center for Toxicological Research, Food and Drug Administration

Conducted studies, evaluated and interpreted results and pathology findings, and reported findings

J.E.A. Leakey, Ph.D., Study Scientist
W.T. Allaben, Ph.D.
J.R. Appleget, B.S.
F.A. Beland, Ph.D.
D.W. Gaylor, Ph.D.
N.A. Littlefield, Ph.D.
J.M. Reed, M.S.
J.E. Seng, Ph.D.
K.L. Witt, M.S., Integrated Laboratory Systems, Inc.
W.M. Witt, D.V.M., Ph.D.

Conducted chemical analysis of feed and purity of the agent

W.M. Cooper, B.S.
F.E. Evans, Ph.D.
J.P. Freeman, Ph.D.
T.M. Heinze, M.S.
T.C. Schmitt, B.S.
P.H. Siitonen, B.S.

Pathology Associates International

Evaluated pathology findings

T.J. Bucci, V.M.D., Ph.D.
J.R. Latendresse, D.V.M., Ph.D.
C.V. Okerberg, D.V.M., Ph.D.

Experimental Pathology Laboratories, Inc.
Provided pathology quality assurance

J.F. Hardisty, D.V.M., Principal Investigator
C.C. Shackelford, D.V.M., M.S., Ph.D.

NTP Pathology Working Group
Evaluated slides, prepared pathology report (August 25, 1999)

C.C. Shackelford, D.V.M., M.S., Ph.D., Chairperson
Experimental Pathology Laboratories
T.J. Bucci, V.M.D., Ph.D.
Pathology Associates International
M.R. Elwell, D.V.M., Ph.D.
Covance Labs
R.A. Herbert, D.V.M., Ph.D.
National Institute of Environmental Health Sciences
J.F. Hardisty, D.V.M.
Experimental Pathology Laboratories
J.R. Latendresse, D.V.M., Ph.D.
Pathology Associates International
C.V. Okerberg, D.V.M., Ph.D.
Pathology Associates International
R.W. Trotter, D.V.M.
Pathology Associates International

R.O.W. Sciences, Inc.
Provided statistical analyses

M. Austen, M.S.
D.L. Barton, M.S.
B.D. Bryant
K. Carroll
S. Goldman
J.M. Gossett, M.S.
B. Hampton
C.C. McCarty, B.S.
W.A. McCracken, M.S.
B.T. Thorn, M.S.
C.C. Ulmer, M.S.

Biotechnical Services, Inc.
Prepared Technical Report

S.R. Gunnels, M.A., Principal Investigator
L.M. Harper, B.S.
E.S. Paal, M.S.J.
D.C. Serbus, Ph.D.
W.D. Sharp, B.A., B.S.
R.A. Willis, B.A., B.S.
P.A. Yount, B.S.

CONTENTS

SUMMARY

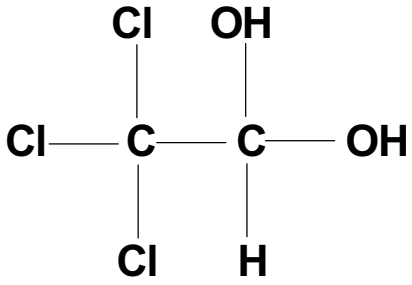
Background: Chloral hydrate is used as a sedative and a sleep aid for children and as an anesthetic for large animals. We studied the effects of chloral hydrate in male mice to identify potential toxic or carcinogenic hazards to humans. Because it is known that higher body weight causes a greater risk for some kinds of cancers, we also examined if the effects of chloral hydrate changed when we reduced the amount of food eaten by the mice.

Methods: We gave the mice doses of 25, 50, or 100 mg of chloral hydrate per kg of body weight by depositing the chemical dissolved in water directly into the animals' stomachs by a tube. Control mice were dosed with water. In one study, the mice had free access to feed; in a second study, the animals' feed was restricted to maintain lower body weights. Groups of 50 animals were dosed five times per week for two years, and tissues from more than 40 sites were examined for every animal.

Results: In the study where mice ate freely, the animals receiving 25 mg chloral hydrate per kg body weight had a higher rate of liver neoplasms than did the control animals. In the study where feed was restricted, the incidence of liver carcinomas was increased in the groups receiving chloral hydrate compared to the control group.

Conclusions: We conclude that chloral hydrate caused some liver neoplasms in male mice, whether or not the animals ate restricted diets.

ABSTRACT



CHLORAL HYDRATE

CAS No. 302-17-0

Chemical Formula: $C_2H_3Cl_3O_2$ Molecular Weight: 165.42

Synonyms: Trichloracetaldehyde monohydrate; 1,1,1-trichloro-2,2-ethanediol; 2,2,2-trichloro-1,1-ethanediol
Trade names: Aquachloral Supprettes; Noctec; Somnos

Chloral hydrate is used medically as a sedative or hypnotic and as a rubefacient in topical preparations, and it is often given to children as a sedative during dental and other medical procedures. Chloral hydrate is used as a central nervous system depressant and sedative in veterinary medicine and as a general anesthetic in cattle and horses. It is a byproduct of the chlorination of water and has been detected in plant effluent after the bleaching of softwood pulp. Chloral, the anhydrous form of chloral hydrate, is used as a synthetic intermediate in the production of insecticides and herbicides. Chloral hydrate was nominated for study by the Food and Drug Administration based upon widespread human exposure and its potential hepatotoxicity and the toxicity of related chemicals. A dietary control component was incorporated in response to concerns within the regulatory community relating to increased background neoplasm incidences in rodent strains used for toxicity testing and to the proposed use of dietary restriction to control background neoplasm incidence in rodent cancer studies. Male B6C3F$_1$ mice (*ad libitum*-fed or dietary-controlled) received chloral hydrate (99% pure) by gavage for 2 years.

2-YEAR STUDY IN MALE MICE

Groups of 120 male mice received chloral hydrate in distilled water by gavage at doses of 0, 25, 50, or 100 mg/kg 5 days per week for 104 to 105 weeks. Each dose group was divided into two dietary groups of 60 mice. The *ad libitum*-fed mice had free access to feed, and the dietary-controlled mice received feed in measured daily amounts calculated to maintain body weight on a previously computed idealized body weight curve. Twelve mice from each diet and dose group were evaluated at 15 months.

Survival, Feed Consumption, and Body Weights

Survival of dosed groups of *ad libitum*-fed and dietary-controlled mice was similar to that of the corresponding vehicle controls. When compared to the *ad libitum*-fed groups, dietary control significantly increased survival in the vehicle controls and 25 and 50 mg/kg groups. Mean body weights of all dosed groups were similar to those of the vehicle control groups throughout the study. The dietary-controlled mice were successfully maintained at or near their target idealized body

weights. There was less individual variation in body weights in the dietary-controlled groups than in the corresponding *ad libitum*-fed groups. Feed consumption by 25 and 50 mg/kg *ad libitum*-fed mice was generally similar to that by the vehicle controls throughout the study. Feed consumption by 100 mg/kg *ad libitum*-fed mice was slightly less than that by the vehicle controls throughout the study.

Hepatic Enzyme Analysis

Chloral hydrate did not significantly induce either lauric acid ω-hydroxylase activity or CYP4A immunoreactive protein in any of the dosed groups of *ad libitum*-fed mice. However, 100 mg/kg did significantly induce both lauric acid ω-hydroxylase activity and CYP4A immunoreactive protein in the dietary-controlled mice. Moreover, the induction response profile of CYP4A was similar to the increase in the incidence of liver neoplasms at 2 years in the dietary-controlled mice with the major effect occurring in the 100 mg/kg group.

The serum enzymes alanine aminotransferase, amylase, aspartate aminotransferase, and lactate dehydrogenase were also assayed at 2 years. In the *ad libitum*-fed groups there was a significant increase in aspartate aminotransferase activity in the 50 mg/kg group. There were no other significant effects in any dosed group, but in general the dietary-controlled groups exhibited lower values than the corresponding *ad libitum*-fed groups.

Organ Weights and Pathology Findings

The heart weight of *ad libitum*-fed male mice administered 100 mg/kg and the kidney weights of 50 and 100 mg/kg *ad libitum*-fed mice were significantly less than those of the vehicle controls at 2 years. The liver weights of all dosed groups of *ad libitum*-fed and dietary-controlled mice were greater than those of the vehicle control groups at 2 years, but the increases were not statistically significant.

The incidence of hepatocellular adenoma or carcinoma (combined) in *ad libitum*-fed mice administered 25 mg/kg was significantly greater than that in the vehicle controls at 2 years. The incidences of hepatocellular carcinoma and of hepatocellular adenoma or carcinoma (combined) occurred with positive trends in dietary-controlled male mice at 2 years, and the incidence of hepatocellular carcinoma in 100 mg/kg dietary-controlled mice was significantly increased.

CONCLUSIONS

Under the conditions used in this 2-year gavage study, there was *some evidence of carcinogenic activity** of chloral hydrate in male $B6C3F_1$ mice based on increased incidences of hepatocellular adenoma or carcinoma (combined) in *ad libitum*-fed mice and on increased incidences of hepatocellular carcinoma in dietary-controlled mice. In the dietary-controlled mice, induction of enzymes associated with peroxisome proliferation was observed at higher doses.

---

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 10. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 12.

Summary of the 2-Year Carcinogenesis Study of Chloral Hydrate in B6C3F$_1$ Mice

	Ad libitum-Fed Groups	Dietary-Controlled Groups
Doses in water by gavage	Vehicle control, 25, 50, or 100 mg/kg	Vehicle control, 25, 50, or 100 mg/kg
Body weights	Dosed groups similar to vehicle control group	Dosed groups similar to vehicle control group
Survival rates	41/48, 37/48, 36/48, 44/48	45/48, 44/48, 47/48, 41/48
Nonneoplastic effects	None	None
Neoplastic effects	Liver: hepatocellular adenoma or carcinoma (16/48, 25/48, 23/47, 22/48)	Liver: hepatocellular carcinoma (2/48, 5/48, 4/48, 8/48)
Level of evidence of carcinogenic activity for *ad libitum*-fed and dietary-controlled groups	Some evidence	

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence** and **some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as "were also related" to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as "may have been" related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on chloral hydrate on May 18, 2000, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

A. John Bailer, Ph.D., Chairperson
Department of Mathematics and Statistics
Miami University
Oxford, OH

James S. Bus, Ph.D.
Health and Environmental Sciences
Dow Chemical Company
Midland, MI

Linda A. Chatman, D.V.M., Principal Reviewer
Pfizer, Inc.
Groton, CT

John M. Cullen, Ph.D., V.M.D.
Department of Microbiology, Parasitology, and Pathology
College of Veterinary Medicine
North Carolina State University
Raleigh, NC

Harold Davis, Ph.D.*
Director of Toxicology
Amgen, Inc.
Thousand Oaks, CA

Norman R. Drinkwater, Ph.D., Principal Reviewer
McArdle Laboratory for Cancer Research
University of Wisconsin-Madison
Madison, WI

Susan M. Fischer, Ph.D.*
M.D. Anderson Cancer Center
The University of Texas
Smithville, TX

Stephen S. Hecht, Ph.D.
University of Minnesota Cancer Centers
Minneapolis, MN

Michele Medinsky, Ph.D.
Durham, NC

Jose Russo, M.D.*
Fox Chase Cancer Center
Philadelphia, PA

Special Reviewer

D. Gail McCarver, M.D., Principal Reviewer
Department of Pediatrics
Medical College of Wisconsin
Milwaukee, WI

---

* Did not attend

SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On May 18, 2000, the draft Technical Report on the toxicology and carcinogenesis study of chloral hydrate (*ad libitum* and feed restricted) received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.E.A. Leakey, NCTR, introduced the toxicology and carcinogenesis study of chloral hydrate by discussing the uses of the chemical and the rationale for study, describing the experimental design, reporting on survival and body weight effects, and commenting on compound-related neoplasms in male mice. The proposed conclusion for the 2-year study was *some evidence of carcinogenic activity* in male B6C3F$_1$ mice.

Dr. Leakey noted that the FDA has been interested in the effects of dietary restriction on neoplasm incidences in test animals because several corporations are using dietary restriction to improve survival in long-term bioassays. Dr. Leakey used data on liver neoplasm risk in animals whose body weight was reduced by a restricted diet or treatment with a noncarcinogenic chemical to illustrate the concern that dietary restriction will desensitize bioassays by decreasing the rates of chemical-induced neoplasms. He described the model that controls body weight to give a predicted neoplasm rate as well as controlling survival. Dr. Leakey also discussed the mechanistic studies of liver enzyme induction and the pharmacokinetic studies of the metabolite trichloroacetic acid.

Dr. Drinkwater, a principal reviewer, agreed with the proposed conclusions. However, he thought that the last sentence, which implies that induction of liver neoplasms in dietary-controlled mice was a result of peroxisome proliferation, was not proven. Dr. Leakey responded that a higher top dose would have been desirable and further study was needed to confirm the mechanism.

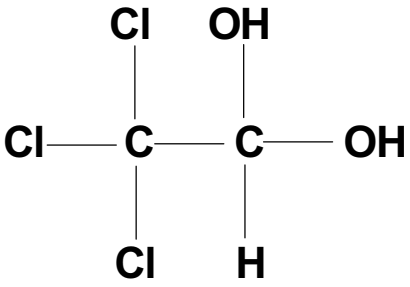
Dr. Chatman, the second principal reviewer, agreed with the proposed conclusions. She also agreed that the last sentence was not fully supported and should be removed. Dr. W.T. Allaben, NCTR, suggested limiting such speculation to the Discussion, and Dr. Leakey agreed. Dr. Chatman asked if incidences of preneoplastic lesions such as foci or hypertrophy in the liver were increased. Dr. T.J. Bucci, Pathology Associates International, said that preneoplastic lesion incidences were not increased.

Dr. McCarver, the third principal reviewer, agreed in principle with the proposed conclusions, and suggested including the doses at which the increased neoplasms occurred. She also suggested softening the last sentence by inserting "somewhat" in front of "similar."

Dr. Drinkwater wondered if the intermittent bolus dosing in the current study, compared to the continuous drinking water exposure in the Daniel *et al.* (1992a) study, was responsible for the lower neoplasm response. Dr. Leakey replied that this could be the case because trichloroacetic acid plasma levels would fall between doses, and peroxisome proliferation is quite reversible. Dr. Bailer suggested that the dose-response model that looks at concentration gradient could include body weight as a covariable, and the modeling mechanism might provide insight into the impact of body weight.

Dr. Drinkwater moved that the sentence of the proposed conclusions on peroxisome proliferation be removed and the remainder of the proposed conclusions be accepted as written; Dr. Chatman seconded the motion. Dr. Bus moved to amend the motion by changing *some evidence of carcinogenic activity* to *equivocal evidence of carcinogenic activity*; Dr. Hecht seconded the amended motion. After discussion of the definitions of levels of evidence, the amended motion was defeated by four no votes to two yes votes. Dr. Bus then argued for retaining some version of the last sentence because of current scientific debate about the relationship between peroxisome proliferation and human cancers. Dr. Drinkwater agreed to modify his motion with a revised sentence reading "In the dietary-controlled mice, induction of enzymes associated with peroxisome proliferation was observed at higher doses." The motion with the revised sentence was accepted by five yes votes to one no vote.

INTRODUCTION



CHLORAL HYDRATE

CAS No. 302-17-0

Chemical Formula: $C_2H_3Cl_3O_2$ Molecular Weight: 165.42

Synonyms: Trichloracetaldehyde monohydrate; 1,1,1-trichloro-2,2-ethanediol; 2,2,2-trichloro-1,1-ethanediol
Trade names: Aquachloral Supprettes; Noctec; Somnos

CHEMICAL AND PHYSICAL PROPERTIES

Chloral hydrate is a clear, crystalline solid which is soluble in water, acetone, and methyl ethyl ketone and slightly soluble in turpentine, petroleum ether, carbon tetrachloride, benzene, and toluene (*Merck Index*, 1989). It has an aromatic, penetrating, and slightly acrid odor and a slightly bitter, caustic taste. It is stable in neutral aqueous solution (NTP, 1999), but under alkaline conditions it decomposes to form chloroform and formic acid (Butler, 1970).

PRODUCTION, USE, AND HUMAN EXPOSURE

Chloral hydrate's pharmacological effects were discovered by Buchheim in 1861 and it has been widely used in medicine for more than 130 years (Butler, 1970; Sourkes, 1992). It is produced by adding water to trichloroacetaldehyde (chloral), which is prepared by the chlorination of ethanol. In the United States, chloral hydrate is used mainly in pediatric medicine and dentistry as a hypnotic or sedative. Although its overall use has declined over the last 30 years, a recent survey of 238 United States hospitals showed it is still the most frequently prescribed sedative for children undergoing computerized tomography in hospital emergency departments (Krauss and Zurakowski, 1998). Chloral hydrate is also used to control agitation and to facilitate mechanical ventilation in preterm infants (Reimche *et al.*, 1989; Hindmarsh *et al.*, 1991; Mayers *et al.*, 1992; Heller *et al.*, 1997). In dentistry, chloral hydrate is used for conscious sedation to control agitation and to produce amnesia (Duncan *et al.*, 1994; Campbell *et al.*, 1998). As a hypnotic, chloral hydrate induces sleep without disrupting the frequency of rapid eye movement episodes (Leuschner and Beuscher, 1998), and it has been available as an over-the-counter sleep aid in several European countries and in Australia (Graham *et al.*, 1988; Ludwigs *et al.*, 1996). Pediatric sedative doses range from 25 to 100 mg/kg (Reimche *et al.*, 1989; American Academy of Pediatrics, 1993; Marti-Bonmati *et al.*, 1995; McCarver-May *et al.*, 1996).

In veterinary medicine, chloral hydrate is used as a hypnotic and sedative, and, in large animals such as cattle and horses, as a general anesthetic and an agent for euthanasia (Silverman and Muir, 1993). In rodents, chloral hydrate has a low therapeutic index; the

anesthetic dose for rats injected intraperitoneally is 200 to 300 mg/kg, and the LD_{50} is 500 mg/kg (Silverman and Muir, 1993). In mice, its efficacy as an anesthetic varies with species and strain (Baumgardner and Dewsbury, 1979; de Fiebre *et al.*, 1992; Wehner *et al.*, 1992).

Chloral hydrate is a byproduct of the chlorination of water, and concentrations in drinking water in the United States may reach 28 µg/L (IARC, 1995). It also has been detected as an environmental contaminant after the bleaching of softwood pulp (IARC, 1995). Chloral, the anhydrous form of chloral hydrate, is used as a synthetic intermediate in the production of insecticides such as DDT, methoxychlor, naled, trichlorfon, and dichlorvos, and the herbicide trichloroacetic acid (IARC, 1995). Chloral hydrate exposure also occurs because it is the primary metabolite of trichloroethylene, a potential human carcinogen that has been used extensively in industry as a solvent and degreasing agent, which has resulted in occupational exposure and widespread environmental contamination (Anonymous, 1988; IARC, 1995; Burg and Gist, 1999).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

The absorption and systemic distribution of chloral hydrate are rapid. In $B6C3F_1$ mice, peak plasma values were observed within 15 minutes of oral dosing and the half-life ($t_{1/2}$) of the drug was 2.7 minutes (Beland *et al.*, 1998). After intravenous dosing, the $t_{1/2}$ of chloral hydrate in plasma was 5 to 24 minutes in $B6C3F_1$ mice (Abbas *et al.*, 1996); likewise, the whole-body clearance $t_{1/2}$ value for chloral hydrate in Swiss-Webster mice treated intraperitoneally was 12 minutes (Cabana and Gessner, 1970). In F344 rats treated orally, the $t_{1/2}$ in plasma was 3.8 minutes (Beland *et al.*, 1998). A biphasic elimination profile has been observed after intravenous administration, with $t_{1/2}$ values of 5.4 and 4.5 minutes (Merdink *et al.*, 1999). A similar rapid clearance of intravenously injected chloral hydrate has been reported in dogs ($t_{1/2}$ = 4 minutes, Breimer *et al.*, 1974; Hobara *et al.*, 1987).

Chloral hydrate is rapidly metabolized by mammalian tissues; its major metabolites are trichloroacetic acid and trichloroethanol; the latter compound can be conjugated to give trichloroethanol glucuronide (Figure 1). Chloral hydrate is reduced to trichloroethanol by isoforms of NADPH-dependent aldehyde reductases [EC 1.1.1.2], NADPH-dependent 3α-hydroxysteroid oxidoreductase [EC 1.1.1.50], and NADH-dependent alcohol dehydrogenases [EC 1.1.1.1.] (Ikeda *et al.*, 1980, 1981a,b). It is oxidized to trichloroacetic acid by isoforms of NAD(P)-dependent aldehyde dehydrogenase [EC 1.2.1.3/5]. Although chloral hydrate is metabolized predominantly in the liver, it is also metabolized extrahepatically to a significant extent (Tabakoff *et al.*, 1974; Hobara *et al.*, 1987; Ogino *et al.*, 1990a; Lipscomb *et al.*, 1996). For example, chloral hydrate reductase activity is higher in the rat adrenal gland than in the liver (Ogino *et al.*, 1990a), possibly due to the presence of high NADPH-dependent 3α-hydroxysteroid oxidoreductase activity. Blood also reduces and oxidizes chloral hydrate to a significant extent (Lipscomb *et al.*, 1996). Pulmonary carbonyl reductase [EC 1.1.1.184] has also been reported to both reduce and oxidize chloral hydrate to trichloroethanol and trichloroacetic acid respectively (Hara *et al.*, 1991). Perfused rat liver generally produces slightly more trichloroacetic acid than trichloroethanol, but under anoxic conditions, the ratio of these two metabolites changes so that trichloroethanol predominates (Kawamoto *et al.*, 1987a; Kawamoto *et al.*, 1988). Trichloroethanol is rapidly conjugated by hepatic UDP-glucuronosyltransferase [EC 2.4.1.17] to trichloroethanol glucuronide, and minimal free trichloroethanol is excreted (Hobara *et al.*, 1987; Abbas *et al.*, 1996). In rodents, trichloroethanol glucuronide is predominantly excreted in the urine and the fraction eliminated in the bile undergoes enterohepatic recirculation (Stenner *et al.*, 1997). Although trichloroacetic acid is mainly excreted unchanged in the urine, bile cannulation experiments have shown that significant amounts pass from the liver into the intestine in rats and mice (Green and Prout, 1985). Minimal trichloroacetate is recovered in the feces, suggesting either enterohepatic recirculation or metabolism by gut bacteria (Green and Prout, 1985).

In $B6C3F_1$ mice and F344 rats dosed orally, peak plasma levels of trichloroethanol occurred in 15 minutes and the plasma $t_{1/2}$ was 4.5 and 8.0 minutes, respectively (Beland *et al.*, 1998). A longer plasma $t_{1/2}$ of trichloroethanol has been observed in $B6C3F_1$ mice

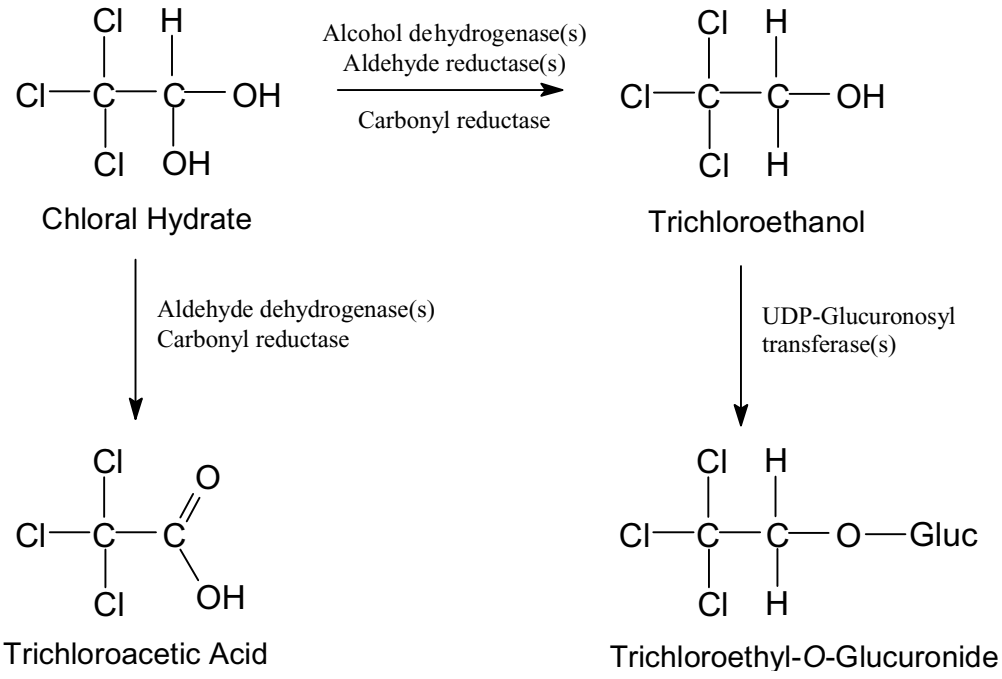


FIGURE 1
Metabolism of Chloral Hydrate

(15.6 to 21.6 minutes; Abbas *et al.*, 1996) and F344 rats (43 minutes; Merdink *et al.*, 1999) after intravenous dosing with chloral hydrate. In B6C3F$_1$ mice dosed orally, the $t_{1/2}$ in plasma for trichloroethanol glucuronide was 7.3 minutes (Beland *et al.*, 1998). In B6C3F$_1$ mice dosed intravenously, the $t_{1/2}$ in plasma for trichloroethanol glucuronide was 12.6 to 43.2 minutes (Abbas *et al.*, 1996). In Swiss-Webster mice, the whole-body clearance $t_{1/2}$ for combined trichloroethanol and trichloroethanol glucuronide was 211 minutes (Cabana and Gessner, 1970). The $t_{1/2}$ for the clearance of trichloroethanol glucuronide in F344 rats (Beland *et al.*, 1998) and dogs (Breimer *et al.*, 1974) was 24 and 52 minutes, respectively.

Trichloroacetic acid is the most persistent metabolite detected in B6C3F$_1$ mice (Abbas *et al.*, 1996), with a $t_{1/2}$ in plasma of 8.5 hours after oral administration (Beland, *et al.*, 1998); this value decreased after repeated dosing. Similarly, in F344 rats dosed orally with chloral hydrate, trichloroacetic acid was the most persistent metabolite, with a $t_{1/2}$ of 11.2 hours (Beland *et al.*, 1998). Trichloroacetic acid also persists in dogs, where a $t_{1/2}$ of 5.5 days has been reported (Breimer *et al.*, 1974). Other studies report similar results regarding the persistence of trichloroacetic acid in dogs (Marshall and Owens, 1954; Owens and Marshall, 1955; Hobara *et al.*, 1987). Studies with perfused rat liver showed that trichloroacetic acid does not appear to be metabolized by the liver and only about 1% of circulating concentrations are excreted unchanged in the bile (Toxopeus and Frazier, 1998).

Trichloroethylene is oxidized to chloral hydrate by cytochrome P450 (Miller and Guengerich, 1983). In rodents, hepatic isoform CYP2E1 appears to be the predominant cytochrome P450 isoform catalyzing this reaction (Nakajima *et al.*, 1990, 1993). However, trichloroethylene appears to be a suicide substrate for this isoform (Halmes *et al.*, 1997), so other isoforms may play a greater role during prolonged exposure. Chloral hydrate formed from trichloroethylene was rapidly metabolized to trichloroethanol and trichloroacetic acid (Green and Prout, 1985; Kawamoto *et al.*, 1987b; Abbas and Fisher, 1997; Stenner *et al.*, 1998; Greenberg *et al.*, 1999). The rate of trichloroethanol formation was very similar in trichloroethylene-treated rats and mice; however, higher concentrations of trichloroacetic acid occurred in mice, reflecting a faster metabolism of trichloroethylene in mice compared to rats (Miller and Guengerich, 1983; Prout *et al.*, 1985). B6C3F$_1$ mice metabolize inhaled trichloroethylene to a greater extent than 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 (saturated) 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 the rats (Dekant *et al.*, 1986). A pronounced gender difference in the elimination of trichloroacetic acid has been observed in B6C3F$_1$ mice administered trichloroethylene, with the rate in females ($t_{1/2}$ = 2.2 to 11.2 hours) twice that of males ($t_{1/2}$ = 5.6 to 24.8 hours) (Fisher *et al.*, 1991; Fisher and Allen, 1993).

Although trichloroethylene and chloral hydrate share the common metabolic pathway outlined above, trichloroethylene is metabolized by additional pathways of toxicological importance which are not available to chloral hydrate. For example, trichloroethylene is partly dehalogenated and conjugated with glutathione by hepatic and renal glutathione *S*-transferase [EC 2.5.1.18] (reviewed by Goeptar *et al.*, 1995, Figure 2.). The resulting *S*-(1,2-dichlorovinyl) glutathione is metabolized via γ-glutamyltransferase [EC 2.3.22], and cysteinylglycinedipeptidase [EC 3.4.13.6] to *S*-(1,2-dichlorovinyl)cysteine which is activated by renal β-lyase [EC 4.4.1.13] to reactive sulfur-containing metabolites such as chlorothioketene and thionacyl chloride. Alternatively, *S*-(1,2-dichlorovinyl)cysteine can be *N*-acetylated by L-cysteine *S*-conjugate *N*-acetyltransferase [EC 2.3.1.80] to produce a mercapturic acid conjugate which is excreted in urine.

A minor cytochrome P450-mediated pathway also oxidizes trichloroethylene to trichlorethylene oxide (epoxide), which is further metabolized into oxalate, *N*-(hydroxyacetyl)aminoethanol, or dichloroacetyl chloride (Figure 2); the latter may be converted into dichloroacetic acid (Hathway, 1980; Miller and Guengerich, 1983; Prout *et al.,* 1985; Goeptar *et al.,* 1995; IARC, 1995).

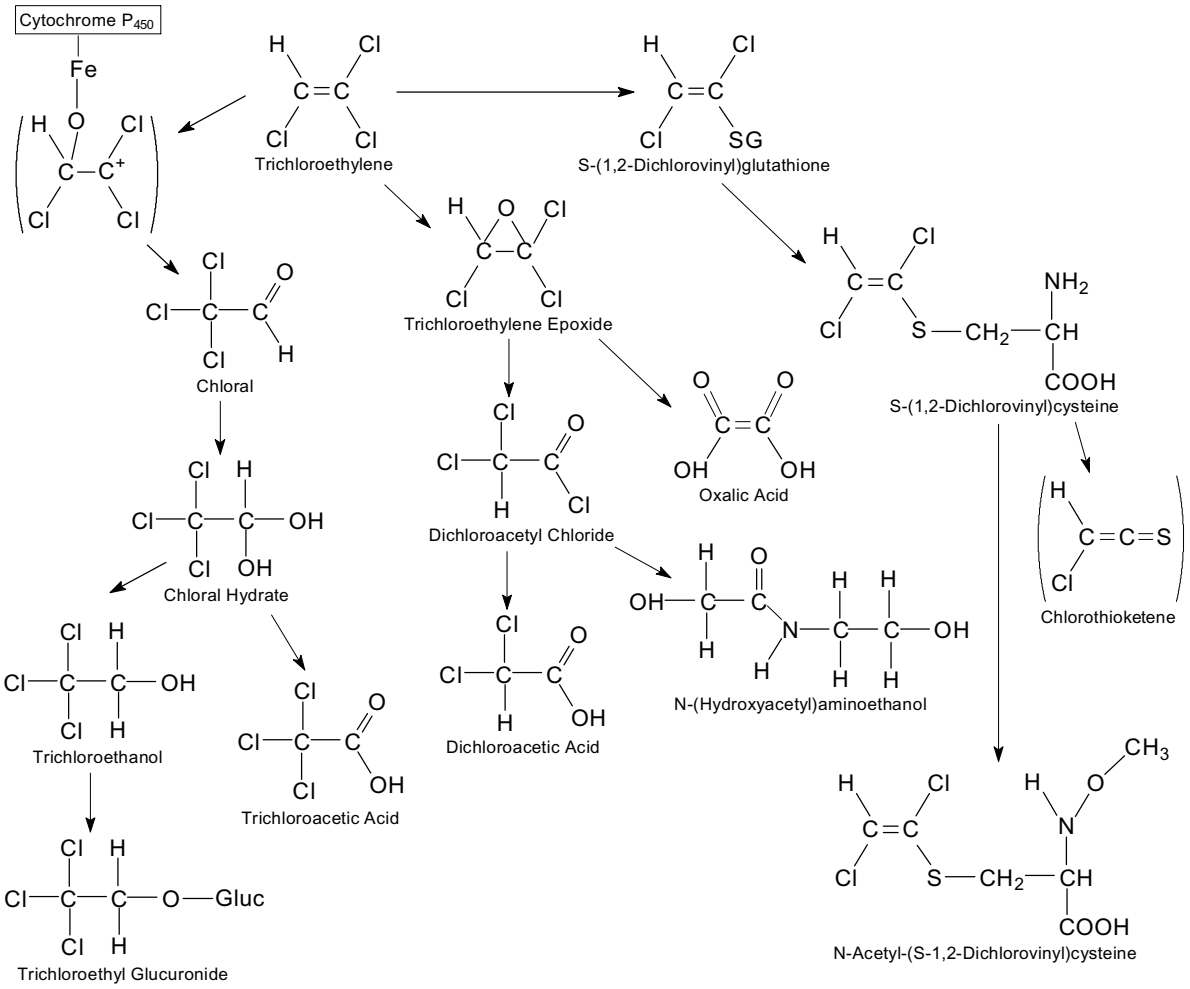


FIGURE 2
Metabolism of Trichloroethylene

Dichloroacetic acid has been reported to be present in blood from animals treated with trichloroethylene, chloral hydrate, or trichloroacetic acid (Prout *et al.*, 1985; Larson and Bull, 1992a,b; Abbas *et al.*, 1996). When administered to rats, dichloroacetic acid was eliminated much more rapidly than trichloroacetic acid ($t_{1/2}$ = 0.9 to 1.6 hours) and appeared to be metabolized in the liver to glyoxylic acid and CO_2. Although initial studies reported blood concentrations approaching those of trichloroacetic acid in B6C3F$_1$ mice administered chloral hydrate intravenously (Abbas *et al.*, 1996), this metabolite was not found in B6C3F$_1$ mice or F344 rats treated orally (Beland *et al.*, 1998) or intravenously (Merdink *et al.*, 1998, 1999). In addition, other studies suggest that previous reports of this metabolite in blood may be based on an experimental artifact, because trichloroacetic acid was converted into dichloroacetic acid under the acid conditions used in assay extraction procedures if reducing agents such as Fe^{2+} from hemoglobin are also present (Ketcha *et al.*, 1996; Fisher, 1997). Trichloroacetic acid was not dehalogenated by perfused rat liver (Toxopeus and Frazier, 1998), but was converted into dichloroacetic acid by intestinal bacteria (Hardman, 1991; Moghaddam *et al.*, 1996). However, more recent studies employing methods that should not produce artifactual dichloroacetate have reported that gut microflora do not appear to contribute to serum dichloroacetic acid observed in trichlorethylene-treated mice (Moghaddam *et al.*, 1997).

Humans

In humans, chloral hydrate and trichloroethylene are metabolized by the same enzyme systems utilized in rodents (Gorecki *et al.*, 1990; Mayers *et al.*, 1991; Lipscomb *et al.*, 1996, 1998; Henderson *et al.*, 1997; Lash *et al.*, 1999a,b). Trichloroacetic acid, trichloroethanol, and trichloroethanol glucuronide were the major metabolites excreted in the urine (Marshall and Owens, 1954; Owens and Marshall, 1955; Blacow, 1972). When chloral hydrate was administered orally, a minimal amount of the parent compound was found in the blood, while the metabolites trichloroethanol, trichloroethanol glucuronide, and trichloroacetic acid rose to peak concentrations within an hour of administration (Breimer *et al.*, 1974). The estimated $t_{1/2}$ of trichloroethanol and trichloroethanol glucuronide was about 7 hours, while the $t_{1/2}$ of trichloroacetic acid was 4 to 5 days. In later work (Breimer, 1977), chloral hydrate could not be detected and the peak concentrations of trichloroethanol and trichloroethanol glucuronide were observed after 20 to 60 minutes. The $t_{1/2}$ for trichloroethanol ranged from 7 to 9.5 hours (mean = 8.0 hours), the $t_{1/2}$ for trichloroethanol glucuronide ranged from 6.0 to 8.0 hours (mean = 6.7 hours), and the $t_{1/2}$ for trichloroacetic acid was approximately 4 days. Zimmermann *et al.* (1998) conducted a similar study and obtained similar values; i.e., chloral hydrate could only be detected 8 to 60 minutes after dosing, the peak plasma concentration of trichloroethanol occurred at 0.67 hours, and the peak trichloroacetic acid concentration was found at 32 hours. The $t_{1/2}$ for trichloroethanol was 9.3 to 10.2 hours and the $t_{1/2}$ for trichloroacetic acid was 89 to 94 hours. The same metabolites were identified in the blood of adult and infant males 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. The $t_{1/2}$ of trichloroethanol and trichloroethanol glucuronide was greatest in newborn infants and decreased with time, which is probably a reflection of a decreased capacity for glucuronidation in neonates (Reimche *et al.*, 1989; Gorecki *et al.*, 1990; Hindmarsh *et al.*, 1991; Mayers *et al.*, 1991). Dichloroacetic acid has been reported as a metabolite of chloral hydrate in humans (Henderson *et al.*, 1997); however, as noted above, it is possible that this was an experimental artifact. Chloral hydrate has also been shown to cross the placenta and to be secreted in breast milk (Bernstine *et al.*, 1954, 1956; MSDS, 1991).

Because trichloroethylene is metabolized in humans by the same pathways as in rodents (Figure 2), trichloroacetic acid levels in blood or urine are frequently used to monitor occupational exposures (Chia *et al.*, 1996). *S*-(1,2-Dichlorovinyl)glutathione and *N*-acetyl-(1,2-dichlorovinyl)cysteine have been detected in micromolar concentrations in blood and urine, respectively, of human volunteers exposed to 50 or 100 ppm trichloroethylene by inhalation (Lash *et al.*, 1999a,b), or after oral ingestion (Bruning *et al.*, 1998).

TOXICITY

The toxicity and potential carcinogeneity of chloral hydrate and its major metabolites have been reviewed in detail (IARC, 1995; Benson, 2000).

Experimental Animals

The LD_{50} of chloral hydrate in CD-1 mice following a single acute dose was 1,442 mg/kg in males and 1,265 mg/kg in females (Sanders *et al.*, 1982). In a 14-day gavage study in male CD-1 mice, increased liver weights, decreased spleen weights, and decreased blood lactate dehydrogenase concentrations were observed in mice administered daily doses of 144 mg/kg chloral hydrate (Sanders *et al.*, 1982). When CD-1 mice were given 0, 70, or 700 ppm chloral hydrate (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 depressed in exposed males. Exposure-related increases in the activities of liver microsomal enzymes cytochrome b5, aniline hydroxylase, and aminopyrine-*N*-demethylase were observed in exposed males. Increased aniline hydroxylase activity was also observed in 700 ppm females. Serum lactate dehydrogenase and aspartate aminotransferase activities were also increased in males exposed to 700 ppm, while blood urea nitrogen concentrations decreased with increasing exposure.

In another study (Beland *et al.*, 1998; NTP, 1999), eight male and eight female F344 rats and $B6C3F_1$ mice were administered 0, 50, 100, 200, 400, or 800 mg chloral hydrate/kg body weight in water by gavage 5 days per week for 16 (mice) or 17 (rats) days for a total of 12 doses. One male mouse in each group except the 400 mg/kg group died and two females in the 800 mg/kg group died. One male and two female 800 mg/kg rats died. Mean body weight gains of all groups of dosed male mice were significantly greater than those of the vehicle control group, mean body weight gains of 400 and 800 mg/kg male rats were significantly less than those of the vehicle controls. Chloral hydrate did not significantly alter body weight gain in females of either rats or mice. The only clinical findings attributed to chloral hydrate treatment were light sedation in the 400 mg/kg groups and heavy sedation in the 800 mg/kg groups; sedation subsided within 30 minutes and 3 hours, respectively. Liver weights of the 800 mg/kg male rats and mice, 400 mg/kg male mice, and 800 mg/kg female mice were significantly greater than those of the vehicle controls. No chemical-related histopathologic lesions were observed.

In contrast, Ogino *et al.* (1990b) reported that chloral hydrate administered to male rats at doses above 400 mg/kg intraperitoneally or 600 mg/kg subcutaneously produced severe gastric ulcers. Fleischman *et al.* (1977) reported that a single intraperitoneal dose at 400 mg/kg produced adynamic ileus in female Crl:COBS CD(SD)BR rats, which resulted in death or morbidity in 14 of 27 females within 36 days of treatment. The oral LD_{50} of chloral hydrate in rats following an acute dose was 480 mg/kg (Goldenthal, 1971). In male Sprague-Dawley rats exposed to daily doses of 24, 48, 96, or 168 mg/kg chloral hydrate 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.*, 1992b). Blood activities of lactate dehydrogenase and alanine aminotransferase were increased in all exposed groups, particularly in the 168 mg/kg group; aspartate aminotransferase activities were significantly increased in all exposed groups. Hepatocellular necrosis was observed in all but the 48 mg/kg group. No effects were observed in females that received up to 288 mg/kg per day (Daniel *et al.*, 1992b).

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. Because there was 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 the decreased rate of formation of trichloroacetic acid in rats 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 also found to induce peroxisomes more readily in mice compared to rats and this result correlated with much higher levels of circulating trichloroacetic acid in mice than in rats (Odum *et al.*, 1988). Conversely, dichloroacetic acid was a more potent inducer of peroxisomal marker enzymes in rats than in mice (DeAngelo *et al.*, 1989; Mather *et al.*, 1990). These results, together with the well-established role of peroxisome proliferators as rodent hepatocarcinogens (Grasso and Hinton, 1991; Nilsson *et al.*,

1991), have led to the suggestion that peroxisome induction by trichloroacetic acid has a causal role in liver carcinogenesis in trichloroethylene-, chloral hydrate-, and trichloroacetic acid-treated mice (Green and Prout, 1985; Elcombe *et al.*, 1987; DeAngelo *et al.*, 1989).

Acute exposure of male B6C3F$_1$ mice to trichloroacetic acid or dichloroacetic acid stimulates hepatic lipid peroxidation when assayed by tissue concentrations of thiobarbituric acid-reactive substances (TBARS) (Larson and Bull, 1992a; Austin *et al.*, 1995). However, 14 days of pretreatment with either trichloroacetic acid or the potent peroxisome proliferator clofibric acid inhibited TBARS levels (Austin *et al.*, 1995). Peroxisome proliferators generally reduce hepatic lipid peroxidation possibly due to induction of aldehyde dehydrogenase and catalase activities (Antonenkov *et al.*, 1985, 1988; Hawkins *et al.*, 1987). Incubation, *in vitro*, of rodent liver microsomes with chloral hydrate, trichloroethanol, or trichloroacetic acid, an NADPH regenerating system, and calf thymus DNA, resulted in lipid peroxidation (Ni *et al.*, 1994, 1996) and the resultant formation of a DNA adduct [3-(2-deoxy-β-D-erythro-pentofuranosyl) pyrimido [1,2α]purin-10(3H)-one] from the reaction of malondialdehyde with deoxyguanosine (Ni *et al.*, 1995). Although this DNA adduct has been detected *in vivo* (Chaudhary *et al.*, 1994), it is not known if treatment with chloral hydrate, trichloroethanol, or trichloroacetic acid will increase its concentration. When male B6C3F$_1$ mice were treated with trichloroethylene by gavage for up to 6 weeks, hepatic TBARS increased, indicating increased lipid peroxidation; only marginal increases in nuclear 8-hydroxydeoxyguanosine were observed (Channel *et al.*, 1998). Acute treatment with trichloroacetic acid also caused small increases in hepatic nuclear 8-hydroxydeoxyguanosine concentrations, whereas dichloroacetic acid caused larger increases (Austin *et al.*, 1996). Electron spin resonance experiments using either liver slices or microsomes from B6C3F$_1$ mice have demonstrated the formation of free radicals following treatment with trichloroethylene or chloral hydrate (Ni *et al.*, 1994; Steel-Goodwin *et al.*, 1996), whereas other studies using rat liver microsomes failed to detect free radical formation following trichloroethylene or trichloroethanol treatment but did show an increase with trichloroethanol (Gonthier and Barret, 1989). Larson and Bull (1992a) proposed that trichloroacetic acid generated free radicals during a postulated hepatic cytochrome P450-mediated dehalogenation reaction that formed dichloro- and monochloroacetic acids. However, liver perfusion experiments have not detected significant hepatic metabolism of trichloroacetic acid (Toxopeus and Frazier, 1998), and although dichloroacetic acid does appear to be dehalogenated by rodent liver, cytosolic rather than cytochrome P450 enzymes, were reported to be responsible for this reaction (Lipscomb *et al.*, 1995). Several isoforms of aldehyde dehydrogenase are important in detoxicating reactive aldehydes during lipid peroxidation (Mitchell and Petersen, 1987; Canuto *et al.*, 1994) and it is conceivable that chloral hydrate and trichloroacetic acid stimulate lipid peroxidation by inhibiting these enzymes.

In vitro exposure to low concentrations of chloral hydrate increase intracellular Ca^{2+} concentrations for several cell types, which may in turn inhibit intracellular spindle formation and disrupt mitosis (Lee *et al.*, 1987) or de-ciliate ciliated embryos or microorganisms (Chakrabarti *et al.*, 1998). This effect may result from inhibition of Ca^{2+}-dependent ATPase in the cell membranes (Bergesse *et al.*, 1983).

Klaunig *et al.* (1989) exposed isolated hepatocytes from male F344 rats or B6C3F$_1$ mice to a range of concentrations of chloral hydrate, trichloroethanol, or trichloroacetic acid. Cytotoxicity (measured by lactate dehydrogenase release) occurred at concentrations above 0.5 mM for trichloroethanol and above 5 mM for chloral hydrate or trichloroacetic acid in the mouse hepatocytes. Cytotoxicity was observed in rat hepatocytes at doses greater than 1 mM and 5 mM for chloral hydrate and trichloroethanol respectively, but was not observed with trichloroacetic acid. Trichloroacetic acid, but not chloral hydrate or trichloroethanol, inhibited gap-junction-mediated intracellular communication (measured by dye coupling) in mouse hepatocytes at concentrations between 0.5 and 5 mM. Cell communication was not inhibited by trichloroacetic acid, chloral hydrate, or trichloroethanol in rat hepatocytes.

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 (Blacow,

1972). Hepatic damage with jaundice, renal damage with albuminuria, or heart damage may occur. The lethal human dose is estimated to be 5 to 10 g (MSDS, 1991); however, there have been reports of survival occurring after a 30-g dose and death following a 4-g dose (IARC, 1995). The toxic effects of an overdose of chloral hydrate are characterized by respiratory depression and hypotension. The latter may be caused by depressed contractility of the myocardium, and chloral hydrate overdose has been reported to cause cardiac arrhythmias in patients predisposed to heart disease (Gustafson *et al.*, 1977). In premature infants, chloral hydrate or its metabolites have been reported to competitively inhibit binding of bilirubin to serum albumin, increasing the risk of kernicterus in jaundiced individuals (Onks *et al.*, 1992). Repeated dosing with chloral hydrate is also associated with cholestasis in newborn infants, which decreases the rate of clearance of conjugated bilirubin (Lambert *et al.*, 1990).

IMMUNOTOXICITY

Experimental Animals

No alteration in humoral or cell-mediated immunity was observed in male CD-1 mice administered 14.4 or 144 mg chloral hydrate/kg body weight 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 CD-1 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 both exposed groups of females, but the number of antibody-forming cells per million cells was significantly reduced only in the 0.7 mg/mL group.

Trichloroacetic acid, administered in drinking water at 5,000 ppm for 90 days, did not significantly alter immunological parameters such as delayed sensitivity, antibody production, or natural killer cell cytotoxicity in male Sprague-Dawley rats (Mather *et al.*, 1990). However, both cell-mediated and humoral immunity were depressed by trichloroethylene in CD-1 mice, particularly in females (reviewed in Davidson and Beliles, 1991).

Humans

In humans, sensitization is rare, but may occur from repeated topical application of chloral hydrate (MSDS, 1991). The most common reported reactions include erythema predominantly affecting the face, neck, and chest, and scarlatiniform eruptions, urticaria, angio-edema, purpura, eczema, bullous lesions, and erythema multiforme (Almeyda and Levantine, 1972).

NEUROBEHAVIORAL TOXICITY

Experimental Animals

Chloral hydrate did not produce dependence or tolerance when administered orally for 6 weeks to cynomolgus monkeys (Leuschner and Zimmermann, 1996). Male CD-1 mice administered chloral hydrate by gavage for 14 days or for in drinking water for 90 days exhibited no behavioral responses other than those attributed to the acute effects of chemical exposure (Kallman *et al.*, 1984). Pups of CD-1 mice exposed to 205 mg/kg chloral hydrate in drinking water throughout gestation and until weaning demonstrated a depressed retention of passive avoidance learning shortly after weaning; pups of dams exposed to 21 mg/kg were not affected (Kallman *et al.*, 1984). Clinical signs of central nervous system toxicity, including ataxia, lethargy, convulsions, and hindlimb paralysis have been reported in rats exposed chronically to trichloroethylene (NTP, 1988); mice had a period of excitement followed by a 15 to 30 minute subanesthetic state (Henschler *et al.*, 1984).

A neurotoxic carboline, 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline (TaClo), has been identified in very low concentrations in the blood of rats concurrently administered chloral hydrate and tryptamine (Bringmann *et al.*, 1996). TaClo is readily formed from chloral and tryptamine under acid conditions *in vitro*, and it is reported to possess dopaminergic neurotoxic activity analogous to structurally similar 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Gerlach *et al.*, 1998).

Humans

In a review of hospital records, Miller and Greenblatt (1979) found depression to be the most common adverse central nervous system effect of chloral hydrate exposure, with an incidence of 1.1%. Central nervous

system excitement occurred at a lower frequency (0.22%). Evidence suggests neurotoxicity may result from chronic exposure to trichloroethylene (Juntunen, 1986; Feldman *et al.*, 1988; Davidson and Beliles, 1991). Bioavailability studies failed to identify TaClo in blood from volunteers administered several concentrations of chloral hydrate (Leuschner *et al.*, 1998), but another study using a more sensitive GC-MS analysis technique detected TaClo at concentrations of 10 to 20 ng/mL in the blood of elderly patients who had taken multiple doses of chloral hydrate (Bringmann *et al.*, 1999).

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

Chloral hydrate crosses the placenta and can be converted into trichloroacetic acid in the fetal compartment (IARC, 1995). Kallman *et al.* (1984) exposed CD-1 mice to 21 or 205 mg/kg chloral hydrate 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 other than the behavioral effects previously noted.

Johnson *et al.* (1998a) exposed female Sprague-Dawley rats to 151 mg/kg chloral hydrate in the drinking water from gestation day 1 to 22. The treatment did not cause any evidence of maternal toxicity or changes in the number of implantation or resorption sites, number of live or dead fetuses, placental or fetal weight, crown-rump length, or incidence of morphological changes. Similar experiments were conducted with trichloroethanol (153 mg/kg) and trichloroacetic acid (291 mg/kg). No developmental effects were observed with trichloroethanol; however, trichloroacetic acid caused an increase in resorptions, implantations, and cardiac anomalies. In an earlier study with trichloroethanol administered during pregnancy, Crebelli and Carere (1989) did not observe embryotoxicity or teratogenicity but did find a developmental delay. Adverse developmental effects of trichloroacetic acid have also been reported by Smith *et al.* (1989), who dosed Long-Evans rats for 10 days beginning on day 6 of pregnancy. Trichloroacetic acid caused soft tissue malformations at incidences ranging from 9% at 330 mg/kg to 97% at 1,800 mg/kg. These malformations were primarily in the cardiovascular system. Similar cardiovascular malformations have been reported in Sprague-Dawley rats (Dawson *et al.*, 1990) and chick embryos (Loeber *et al.*, 1988) exposed to trichloroethylene.

The developmental toxicity of chloral hydrate has also been investigated *in vitro* using a rat whole embryo culture system (Saillenfait *et al.*, 1995). No adverse effects were observed when Sprague-Dawley rat embryos were explanted on gestational day 10 and exposed to 0.5 mM chloral hydrate for 46 hours; however, 1 to 2 mM chloral hydrate caused dose-related increases in malformations, and 2.5 mM was lethal. The effects of chloral hydrate on sperm morphology and motility was examined in F344 rats administered 0, 55, or 188 mg/kg in drinking water for 52 weeks (Klinefelter *et al.*, 1995). The highest exposure caused a decrease in the percent of motile sperm but did not affect epididymal or testicular histopathology. Also, as described below, chloral hydrate has been reported to cause aneuploidy in mouse spermatocytes.

Humans

Although exposure to trichloroethylene-contaminated drinking water has been associated with increased incidence of congenital heart disease in humans (Goldberg *et al.*, 1990), and trichloroacetic acid has been implicated as the causative agent (Johnson *et al.*, 1998b), the possibility that similar congenital defects might result from chloral hydrate exposure during pregnancy does not appear to have been investigated.

CARCINOGENICITY

Experimental Animals

In a skin paint study in "S" strain albino mice, the incidence of skin neoplasms was not significantly increased 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 15-day-old 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.* (1992a) reported that 1,000 ppm chloral hydrate (166 mg/kg) was hepatocarcinogenic when administered to male B6C3F$_1$ mice in drinking water. The incidence of hepatocellular adenoma was 7/24 (29%) in treated mice versus 1/20 (5%) in control mice; the incidences of carcinoma were 11/24 (46%) in the 1,000 ppm group and 2/20 (10%) in controls. The incidences of adenoma or carcinoma combined were 17/24 (71%) and 3/20 (15%) for the treated and control groups, respectively. In another study, reported in abstract form only, chloral hydrate at doses of 800 and 1,400 ppm in drinking water increased the incidence of hepatocellular carcinoma compared to controls when administered for 2 years (DeAngelo and George, 1995). An increased incidence was not observed at 50 ppm. However, this study was partly compromised due to low survival and high rates of background liver tumors (A. DeAngelo, personal communication).

Male and female Sprague-Dawley rats were administered up to 135 mg chloral hydrate/kg body weight per day in drinking water for 124 and 128 weeks, respectively, and the highest dose resulted in a statistically significant increase in hepatocellular hypertrophy but there was no evidence of neoplasia in any organ (Leuschner and Beuscher, 1998). A similar lack of neoplasia was reported for 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).

Both trichloroacetic acid and dichloroacetic acid were hepatocarcinogens in B6C3F$_1$ mice when administered in drinking water (Herren-Freund *et al.*, 1987; Bull *et al.*, 1990; Daniel *et al.*, 1992a; Pereira, 1996). Dichloroacetic acid appeared to be more potent than trichloracetic acid and differences existed in their mechanism of action. Livers from dichloroacetic acid-treated mice exhibited cytomegaly, increased disposition of glycogen, and produced foci and tumors which were predominantly H-*ras* positive and expressed *c-Jun*-related proteins (Bull *et al.,* 1993; Stauber and Bull, 1997). In contrast, livers from mice treated chronically with trichloroacetic acid exhibited peroxisomal proliferation and disposition of lipofuscin and produced foci and tumors which did not express *c-Jun*-related proteins (Bull *et al.*, 1993; Tao *et al.*, 1996; Stauber and Bull, 1997). In male F344/N rats, dichloroacetic acid (administered in drinking water at 50 and 160 ppm) resulted in increased incidence of hepatocellular adenomas and carcinomas after 100 weeks, suggesting that it is a more potent hepatocarcinogen in rats than in mice (DeAngelo *et al.*, 1996). Conversely, trichloroacetic acid administered to F344/N rats at equivalent or higher doses did not induce liver tumors (DeAngelo *et al.*, 1997). Trichloroacetic acid (administered in drinking water at 50 to 5,000 ppm for 6 months) did, however, promote the formation of hepatic preneoplastic foci in male Sprague-Dawley rats that were previously initiated with diethylnitrosamine and partial hepatectomy (Parnell *et al.*, 1986).

Trichloroethylene has also been shown to be a hepatocellular carcinogen in male and female B6C3F$_1$ mice (NCI, 1976; NTP, 1983; Herren-Freund *et al.*, 1987). Forestomach papillomas and carcinomas were noted in Ha:ICR mice given 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; IARC, 1995). In other mouse inhalation studies, the incidence of lung adenocarcinoma was increased in exposed male and female ICR and Swiss mice and in female B6C3F$_1$ mice, and hepatocellular carcinoma was induced in male Swiss mice and male and female B6C3F$_1$ mice (Maltoni *et al.*, 1988). Trichloroethylene was not hepatocarcinogenic in Osborne-Mendel, Sprague-Dawley, or Wistar rats (NTP, 1983, 1988; Crebelli and Carere, 1989). However, male F344/N and Osborne-Mendel rats exposed to trichloroethylene by gavage had significantly increased incidences of renal adenocarcinoma (NTP, 1988, 1990). Increased incidences of renal adenocarcinoma and Leydig cell tumors were observed in another study in trichloroethylene-exposed Sprague-Dawley rats (Maltoni *et al.*, 1988). Trichloroethanol does not appear to have been tested for carcinogenicity (Bruckner *et al.*, 1989; IARC, 1995).

Humans

No epidemiologic studies of chloral hydrate were found in a review of the literature. Occupational exposure to trichloroethylene has been associated with an increased incidence of renal cell carcinoma, but not liver cancer (Henschler *et al.*, 1995; IARC, 1995).

INFLUENCE OF BODY WEIGHT AND FEED CONSUMPTION ON HEPATOCARCINOGENESIS IN MALE MICE

The $B6C3F_1$ mouse is one of several mouse strains that are highly susceptible to liver neoplasms (Carmichael *et al.*, 1997). This susceptibility appears to be linked to the C3H/He parent strain, which has been reported to be approximately 50-fold more susceptible than the C57BL/6 parent strain (Bennett *et al.*, 1995). The high susceptibility of the $B6C3F_1$ mouse to hepatocarcinogenicity has made the strain a sensitive biomarker for both genotoxic and nongenotoxic hepatocarcinogens and, unlike insensitive mouse strains or rats, the $B6C3F_1$ mouse shows positive results with nongenotoxic tumor promoters without the need for an initiating chemical or partial hepatectomy (Parodi *et al.*, 1992; Williams *et al.*, 1996; Carmichael *et al.*, 1997).

Over the past three decades, improvements in diet formulations and animal husbandry techniques and commercial breeding considerations have resulted in a general drift towards increased body weights for all the major rodent strains used in toxicity testing (Nohynek *et al.*, 1993; Roe, 1993; Hart *et al.*, 1995; Seilkop, 1995). Body weight increases in rodent strains used in toxicity testing are frequently associated with decreased survival and increased susceptibility to neoplastic and degenerative diseases (Nohynek *et al.*, 1993; Roe, 1993; Keenan *et al.*, 1995). These effects have compromised 2-year bioassays because of insufficient survival until the end of the studies and excessively high background neoplasm incidences that prevent the demonstration of positive dose response relationships (Roe, 1981, 1993; Keenan *et al.*, 1997).

Such effects have been observed in $B6C3F_1$ mice used in recent NTP bioassays (Seilkop, 1995). In control groups of male mice, the background incidence of liver neoplasms increased at a rate of 3.9% per year during the 1980s; for females, the increase was 7.3% (Turturro *et al.*, 1996). These increases were associated with concurrent increases in body weights of control groups. It was originally argued that the increased incidences were mainly due to the switch from group to single-animal housing that occurred during this period (Haseman *et al.*, 1994), but data from a recent NTP study that reverted to group housing for female mice suggests that this may not be the correct explanation (NTP, 2000).

This drift towards increasing body weight of the experimental animal population has established a positive correlation between early body weight gain and liver neoplasm incidence in $B6C3F_1$ mice (Turturro *et al.*, 1993). This had not been apparent from analysis of earlier NTP studies (Rao *et al.*, 1990). Data from NTP studies using the mean body weights of control groups from individual studies (Turturro *et al.*, 1993, 1996; Seilkop, 1995) or weights of individual mice from groups of studies (Seilkop, 1995; Leakey *et al.*, 1998) have demonstrated that body weights between 6 and 14 months of age can predict the risk of development of liver adenomas or carcinomas at the conclusion of a 2-year chronic study (Figures 3 and 4).

Dietary and caloric restriction reduce body weight and spontaneous neoplasm incidence in rodents such as the $B6C3F_1$ mouse (see Leakey *et al.*, 1998 for a comprehensive review). Moderate dietary restriction has therefore been proposed as a means to control survival and background neoplasm incidences in chronic cancer bioassays (Roe, 1993; Keenan *et al.*1994, 1997; Hart *et al.*, 1995). However, dietary restriction also inhibits chemical-induced hepatocarcinogenesis in rodents, and in the case of relatively lean NCTR $B6C3F_1$ mice, 40% caloric restriction completely inhibited the hepatocarcinogenicity of known genotoxic carcinogens when tested in the newborn mouse assay (Fu *et al.*, 1994). Dietary restriction is generally implemented by limiting food consumption to a fixed percentage of *ad libitum* food consumption, and this may vary between rodent populations of the same strain in different laboratories (Turturro *et al.*, 1993, 1996).

Further difficulties arise with this approach when exposure to the test chemical causes additional body weight decreases. This frequently occurs due to toxic responses to the test chemical, and a 10% reduction in body weight has been used as a criterion for achieving a minimally toxic dose (Haseman, 1985). Chemical-induced body weight reductions can arise for several reasons, including decreased food consumption due to palatability problems in feed studies, anorexia due to toxic stress, disrupted intestinal absorption, or toxic wasting syndromes resulting from disruption of metabolism or endocrine systems (Leakey *et al.*, 1998).

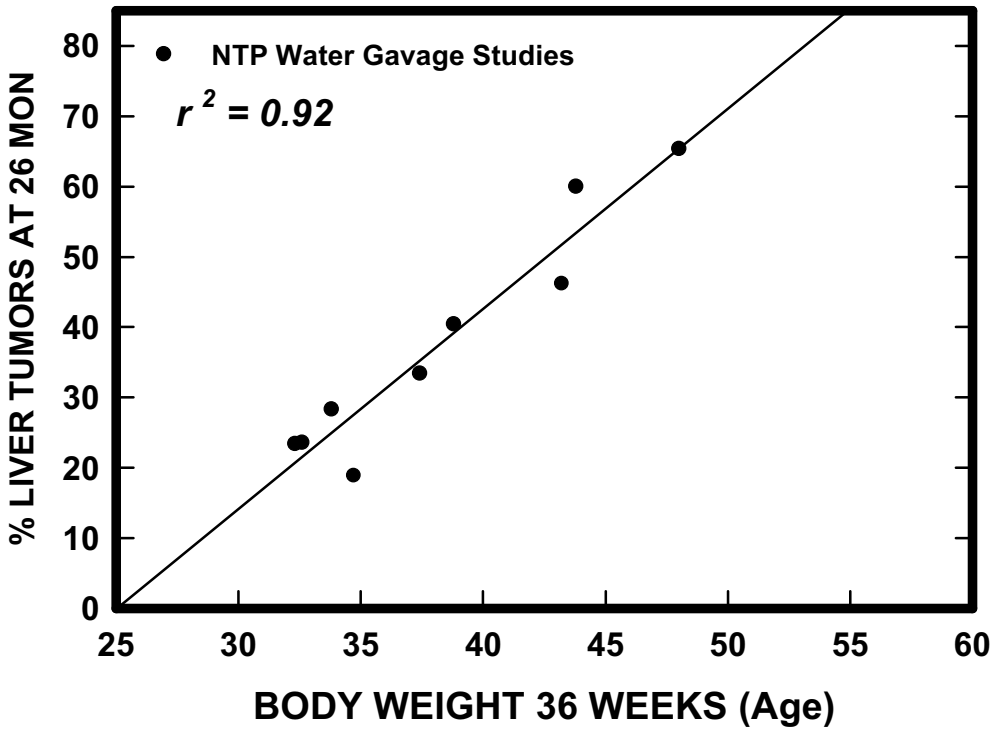


FIGURE 3
Association Between Body Weight and Liver Neoplasm Incidence in Male B6C3F$_1$ Mice
Data from recent NTP studies that used water-based gavage. The liver neoplasm values are the survival-adjusted rates of hepatocellular adenoma or carcinoma. See Appendix D for details.

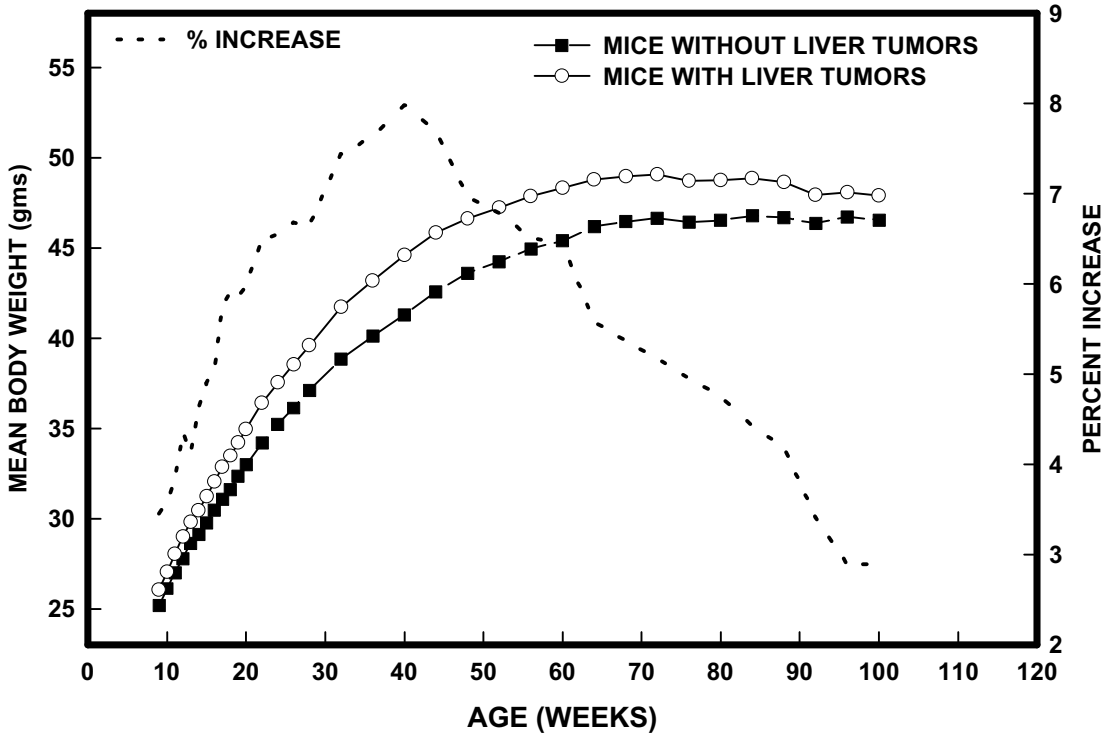


FIGURE 4
Influence of Body Weight on Liver Neoplasm Incidence as a Function of Age
Individual body weight and liver pathology data were obtained from the control male mice groups of 24 recent NTP 2-year bioassays (Appendix D). The weight values were grouped by whether or not the mice developed a liver neoplasm (adenoma or carcinoma) and the calculated means for each group were plotted as a function of age of the animals. The dashed line represents the percentage difference between the two groups, and identifies the period when body weight has the greatest influence on liver neoplasm development.

In many cases, chemical-induced body weight decreases also have been shown to reduce liver neoplasm incidence in $B6C3F_1$ mice (e.g., NTP, 1997). Chemical-induced body weight decreases may possibly mask positive neoplasm responses (see Appendix D). Although the use of simple dietary restriction, or selective breeding of smaller mouse stocks, may solve the problem of survival and high background incidences of liver neoplasms, they will not address the issues of altered sensitivity due to chemical-induced body weight changes or the standardization of background incidences of neoplasms throughout the regulatory community.

An alternative approach involves using dietary control to manipulate body growth in all $B6C3F_1$ mice used in bioassays to fit a standardized weight curve. Such standardized or idealized weight curves could be created for male and female $B6C3F_1$ mice, as well as for other rodent strains used for chronic bioassays. These idealized growth curves could then be used throughout industry and the regulatory community to standardize background neoplasm incidences between laboratories. The body weights of mice used for both control and treatment groups in future bioassays could be manipulated to fit these growth curves by moderate feed restriction or dietary supplementation. For $B6C3F_1$ mice, use of the idealized weight curves could produce a spontaneous liver tumor incidence of approximately 15% to 20% at 26 months of age. A 15% to 20% liver neoplasm incidence is sufficiently high to guarantee that the sensitivity of the mouse to chemical carcinogenesis has not been compromised, and it is low enough to ensure that the spontaneous neoplasms will not obscure any chemical-induced liver tumors and that sufficient mice will survive to the end of a 2-year study. For this study, data from mice used in NTP and NCTR chronic bioassays and aging studies were used to construct idealized weight curves for male and female $B6C3F_1$ mice that predicted a liver neoplasm incidence of 15% to 20%.

GENETIC TOXICITY

Although chloral, like most aldehydes, will react with a variety of nucleophiles and would be expected to directly react with DNA, chloral hydrate is so strongly hydrated in aqueous solution that it does not appear to react at all with nucleophiles and does not form DNA-protein cross-links (Keller and Heck, 1988). Nevertheless, chloral hydrate has frequently been reported to be genotoxic both *in vitro* and *in vivo* in a variety of assays, particularly in those measuring aneuploidy. These effects may result from disrupted calcium homeostasis (Lee *et al.*, 1987), while stimulation of lipid peroxidation may also play a role in other effects (Ni *et al.*, 1995). Thorough reviews of genetic toxicology data are available (IARC, 1995; Benson, 2000).

Chloral hydrate gave positive results in the *Salmonella typhimurium* gene mutation assay in strains TA98, TA100, and TA104, with and without S9 activation (Waskell, 1978; Bruce and Heddle, 1979; Bignami *et al.*, 1980; Haworth *et al.*, 1983; Ni *et al.*, 1994). However, no mutagenic activity was detected in these strains in other studies (e.g., Leuschner and Leuschner, 1991). Positive results have 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 nondisjunction 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 fed chloral hydrate (5,500 ppm in 5% sucrose); chloral hydrate administered 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). Chloral hydrate has also been shown to be weakly clastogenic in L5178Y mouse lymphoma cells (Harrington-Brock *et al.*, 1998).

Several *in vivo* 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; Russo and Levis, 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). Nutley *et al.* (1996) used two methods of aneuploidy assessment (anti-kinetochore antibody staining and fluorescence *in situ* hybridization with centromeric DNA probes), and both methods 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. Another 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 later 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).

Trichloroethanol and trichloroacetic acid have also been reported to induce strand breaks in DNA. Although several studies have demonstrated covalent binding of trichloroethylene to DNA when incubated *in vitro* in the presence of a 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). 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), but the metabolite responsible for this induction has not been established. *S*-(1,2-dichlorovinyl)cysteine and related compounds have been reported to be mutagenic in Ames tests using the TA100 strain (Goeptar *et al.*, 1995).

STUDY RATIONALE

The Food and Drug Administration (FDA) nominated chloral hydrate for testing based upon widespread human exposure, its potential hepatotoxicity in rodents, and the toxicity of related chemicals (Smith, 1990). 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. Data from the studies will be used to augment the regulatory decision process in terms of accurately assessing human health risk.

The study was conducted in B6C3F$_1$ mice because previous studies reported hepatocarcinogenicity in this mouse strain (Rijsinghani *et al.*, 1986; Daniel *et al.*, 1992a). Oral gavage was the route of administration because it mimics the route of exposure in humans. The study was divided into two portions and is presented in separate NTP Technical Reports (NTP, 2002, and the current report). The primary study focused on the potential genotoxicity of chloral hydrate and the possibility that developmental exposure might induce liver neoplasms. That portion, which included the 2-year chronic exposure study in female B6C3F$_1$ mice, is published as a companion report (NTP, 2002). The second portion focused on the potential role of feed consumption and body weight on the incidence of liver neoplasms and includes the 2-year chronic exposure study in male B6C3F$_1$ mice and is presented here. Other objectives of the study in male mice were to confirm whether chloral hydrate was hepatocarcinogenic in male B6C3F$_1$ mice and to provide additional biochemical and toxicokinetic data to aid in the understanding of the mechanism(s) through which chloral hydrate induces liver neoplasms in mice.

The FDA is concerned about the potential impact of experimental dietary restriction on bioassay sensitivity because it has received several drug applications where the chronic toxicity studies were carried out in dietary-controlled rodents, and the agency is developing guidelines for how such studies should be interpreted (Allaben *et al.*, 1996). A major objective of this study

was to test the feasibility of using dietary control to normalize body weight in a carcinogenicity bioassay and the ability of the idealized weight curve to accurately predict liver neoplasm incidence.

Chloral hydrate is an appropriate compound to use in testing for body weight effects because: previous studies suggested chloral hydrate is hepatocarcinogenic in male $B6C3F_1$ mice, so a positive effect on liver neoplasms was likely; aqueous gavage could be used for administration, which would be less complicated than a feed-based exposure for dose comparisons between *ad libitum*-fed and dietary restricted mice and would not involve corn oil administration, which might influence caloric consumption in both groups; and initial range finding studies (NTP, 1999) suggested chloral hydrate treatment would not cause large changes in body weight. Conversely, use of dietary control to weight-match the treatment groups was expected to increase the sensitivity of the bioassay, which was advantageous because chloral hydrate dose selection required relatively low doses.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF CHLORAL HYDRATE

Chloral hydrate was obtained from Amend Drug and Chemical Company, Inc. (Irvington, NJ), in one lot (Z57601P28). Identity and purity analyses were conducted by the study laboratory (Appendix F).

The chemical, a white crystalline powder, was identified as chloral hydrate by gas chromatography/ mass spectrometry (GC/MS) and ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy (NMR). Purity of lot Z57601P28 was determined by gas chromatography and high-performance liquid chromatography. Gas chromatography indicated one major peak and one impurity with an area of approximately 0.18% of the total peak area. The overall purity was determined to be 99%. High-performance liquid chromatography confirmed the presence of one impurity.

Stability of the bulk chemical was analyzed at the end of the 2-year study using gas chromatography, GC/MS, and ^{1}H-NMR. These analyses indicated chloral hydrate had remained stable as a bulk chemical throughout the study. The bulk chemical was stored at room temperature in amber glass bottles.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared every 4 weeks by dissolving chloral hydrate crystals in distilled water (Table F2). Stability studies of 0.85, 1.4, 2.5, and 5 mg/mL formulations were performed by the study laboratory using gas chromatography. The stability of the formulations was confirmed for at least 24 days when stored in amber glass vials at room temperature.

Periodic analyses of the dose formulations were conducted at the study laboratory using gas chromatography. Of the dose formulations analyzed, 41 of 42 were within 10% of the target concentrations with no value greater than 103% or less than 87% of the target concentration.

CONSTRUCTION OF IDEALIZED BODY WEIGHT CURVES

Individual body weight data and neoplasm incidences for male B6C3F$_1$ mice were obtained from 18 NTP single-housing bioassays in which the mice weighed more than 36 grams at 52 weeks of age; from two NTP studies that used dietary restriction (NTP, 1997a); from an NTP study where the test chemical caused large decreases in body weight and liver neoplasm incidence and did not appear to be carcinogenic (NTP, 1996); and from a large NCTR caloric restriction study that used 40% caloric restriction in male B6C3F$_1$ mice (Witt *et al.*, 1991; Sheldon *et al.*, 1995). This mouse population included animals that had been subjected to forced body weight reduction resulting from either dietary restriction or chemical exposure. A full discussion of the rationale for using this approach and the justification for using this mouse population is given in Appendix D.

Data from mice that had survived less than 500 days and from animals with grossly abnormal body weight values were eliminated. The body weight values correlated with the actual age of the mice. The body weight values were then adjusted to estimate weight values at weekly, biweekly or 4-week intervals so they could be compared directly. Starting with the initial 9-week age point, the mice from all the studies were assigned to one of 17 consecutive weight groups ranging from 20 to 57.5 g in 2.5 g intervals with extra groups for high and low outliers. The mice in each weight group were then sorted according to whether they developed a liver neoplasm so the relative neoplasm risk could be calculated for each group. Mice that were reported to have either a hepatocellular adenoma, carcinoma, or hepatoblastoma were designated positive; mice with no reported liver neoplasms were designated negative. The process was repeated for each age point.

At 44 weeks of age, the mouse population exhibited a body-weight-to-liver-neoplasm relationship that was reasonably linear through the entire weight range (Figure 5). The idealized body weight curve for male $B6C3F_1$ mice was constructed by determining which weight group best corresponded to a 15% to 20% liver neoplasm risk at each age point. These weight groups were then plotted against their age points to produce a crude idealized body weight curve. This curve was then smoothed and extrapolated back to 4 weeks of age by comparing it with the body weight curves of actual mice of similar body weight. The final idealized body weight curve is also shown in Figure 6. Full details of these procedures are given in Appendix D.

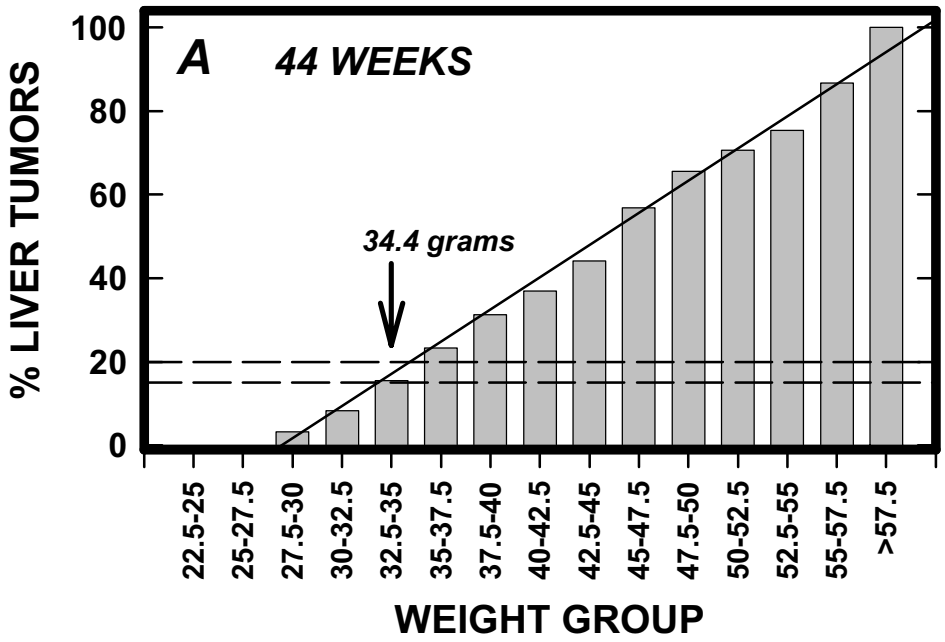


FIGURE 5
Relationship Between Body Weight at 44 Weeks of Age and Liver Neoplasm Risk in Male $B6C3F_1$ Mice
The mouse population used here included smaller mice that had been exposed to forced body weight reduction by either dietary restriction or chemical exposure. The dashed lines show the 15% to 20% liver neoplasm incidence range. The solid line shows the linear regression of weight groups between 25 and 55 g ($r^2 = 0.99$). The arrow marks the body weight which predicts a liver neoplasm incidence of 15% to 20%. Additional details and other age points are shown in Appendix D.

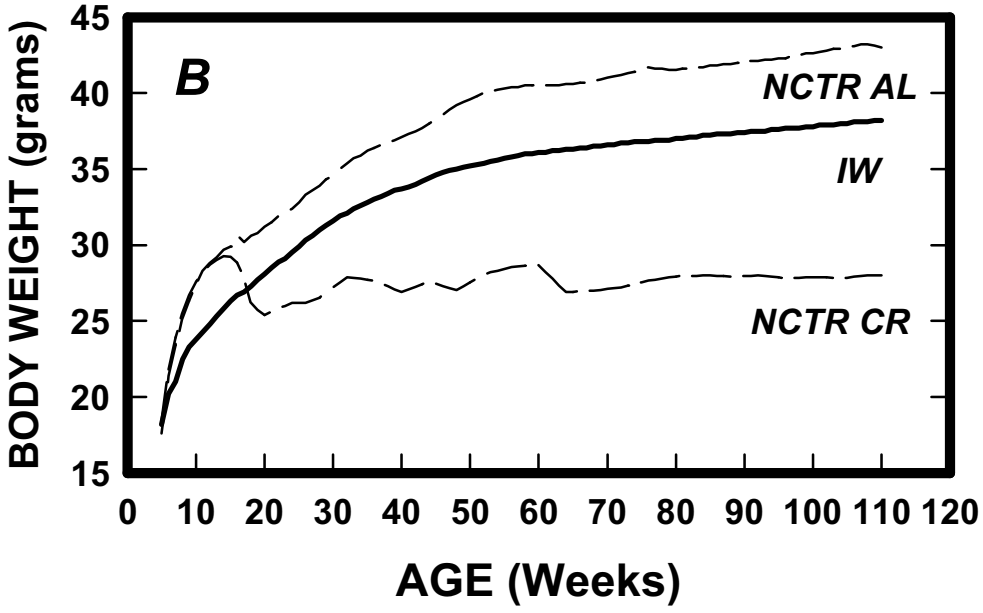


FIGURE 6
Idealized Body Weight Curve for Male B6C3F$_1$ Mice
The idealized body weight curve (IW) is shown with the solid line. Body weights for *ad libitum*-fed (AL) and 40% calorically restricted (CR) mice are shown for comparison (dashed lines). Feed consumption data from these mice were used to construct a feeding schedule for manipulating the animals' weights to fit the idealized body weight curve.

Proposed Feed Consumption for Dietary-Controlled B6C3F$_1$ Mice

Feed allocation values required to control body weight in mice to conform to the idealized body weight curve were calculated as grams of NIH-31 pellets per day and are shown in Appendix D. They were calculated by initially determining the weight reduction (as percentage of mean *ad libitum* weight) produced by 40% caloric restriction in the NCTR Project on Caloric Restriction animal colony for each week of age between 14 and 110 weeks. This was then used to calculate Pg, the percentage caloric restriction required to cause a 1 g decrease in *ad libitum* body weight for each week of age (values for weeks 6 through 17 were extrapolated from week 18 values). The proposed feed consumption to produce the idealized body weight curve was then calculated by multiplying the body weight differences between the mean *ad libitum* body weight values for male B6C3F$_1$/Nctr BR (C57BL/6N × C3H/HeN MTV$^-$) mice (Figure 6) and the idealized body weight values at each week by the corresponding Pg value. The resulting percentage restriction was subtracted from the historical feed consumption curve used to calculate feed allocation for the calorically restricted male B6C3F$_1$ mice in the NCTR colony. It was anticipated that individual mice would exhibit body weights that differed significantly from the idealized body weight curve at certain times during their growth. A computer program was developed as part of the *INLIFE* computer system to flag mice that were outside either 5% or 12% confidence limits of the idealized body weight curve. The program altered the feeding schedule for the following week in either 1.0 or 1.5 g increments to manipulate the body weight back onto the idealized body curve. Dietary control, as described in this study, is when variable amounts of dietary restriction or supplementation are utilized to maintain an animal at a set body weight.

2-YEAR STUDY

Study Design

Groups of 120 male mice received chloral hydrate in distilled water by gavage at doses of 0, 25, 50, or 100 mg/kg, 5 days per week for 104 to 105 weeks; vehicle controls received distilled water only. Each dose group was divided into two dietary groups of 60 mice. The *ad libitum*-fed mice had feed available *ad libitum* and the dietary-controlled mice received feed in measured daily amounts calculated to maintain body weight on a previously computed idealized body weight curve. Twelve mice from each diet/dose group were evaluated at 15 months.

Source and Specification of Animals

Male B6C3F$_1$/Nctr BR (C57BL/6N × C3H/HeN MTV$^-$) mice were obtained from the study laboratory's breeding colony. Mice were approximately 4 weeks old at receipt, 5 weeks old at the initiation of controlled feeding, and 6 weeks old on the first day of dosing. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix I).

Animal Maintenance

Mice were housed individually in divided cages. The animals were fed either *ad libitum* or daily in controlled amounts, and water was available *ad libitum* to all animals. Mice were fasted overnight before necropsy. Cages and racks were changed weekly. Further details of animal maintenance are given in Table 1. Information on feed composition and contaminants is provided in Appendix H.

Clinical Examinations and Pathology

All animals were observed daily. Clinical findings were recorded daily and body weights were recorded each weekday as part of the dosing procedures.

At the 15-month interim evaluation, animals were euthanized by decapitation, and at the end of the study, the animals were euthanized by exposure to carbon dioxide. The parameters measured are listed in Table 1.

Complete necropsies were performed on all mice. The brain and liver were weighed at 15 months, and the heart, right kidney, liver, lungs, right testis, and thymus were weighed at the end of the study. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in Tissue-Prep II, sectioned to a thickness of approximately 5 µm, and stained with hematoxylin and eosin for microscopic examination. The livers from all animals were weighed wet as soon as possible after dissection. For all paired organs (e.g., adrenal gland, kidney), samples from each organ were examined. Tissues examined microscopically are listed in Table 1.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the study laboratory's Micropath Data Collection System. The slides, paraffin blocks, and residual wet tissues were sent to the study laboratory's Quality Assurance Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were evaluated by an independent quality assessment group. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. A quality assessment pathologist evaluated slides from all tumors and the liver.

Differences of opinion were reconciled between the study and quality assessment pathologists. The quality assessment pathologist served as the Pathology Working Group (PWG) chairperson and presented histopathology slides containing the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologists, or lesions of general interest were presented by the chairperson to the PWG for review. The PWG consisted of the quality assessment pathologist, the study pathologist, and other pathologists experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of dose groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell *et al.* (1986).

TABLE 1
Experimental Design and Materials and Methods in the 2-Year Gavage Study of Chloral Hydrate

Study Laboratory
National Center for Toxicological Research (Jefferson, AR)

Strain and Species
$B6C3F_1$/Nctr BR (C57BL/6N × C3H/HeN MTV^-) mice

Animal Source
NCTR breeding colony (Jefferson, AR)

Time Held Before Study
Before dietary control: 1 week
Before dosing: 2 weeks

Average Age When Study Began
Beginning of dietary control: 5 weeks
Beginning of dosing: 6 weeks

Date of First Dose
15-Month interim evaluation: February 5 or 26, 1996
Terminal Sacrifice: January 29, February 12 or 19, or March 4, 1996

Duration of Dosing
Ad libitum: 5 days/week for 104 weeks
Dietary-controlled: 5 days/week for 104 to 105 weeks

Date of Last Dose
15-Month *ad libitum*: April 28 or 30 or May 19 or 21, 1997
2-Year *ad libitum*: January 23 or February 6, 13, or 27, 1998
15-Month dietary-controlled: April 29 or May 1, 20, or 22, 1997
2-Year dietary-controlled: January 23, February 6 or 16, or March 3, 1998

Necropsy Dates
15-Month *ad libitum*: April 29 or May 1, 20, or 22, 1997
2-Year *ad libitum*: January 25, February 8 or 16, or March 1, 1998
15-Month dietary-controlled: April 30 or May 2, 21, or 23, 1997
2-Year dietary-controlled: January 26, February 9 or 17, or March 4, 1998

Average Age at Necropsy
Ad libitum: 110 weeks
Dietary-controlled: 110 to 111 weeks

Size of Study Groups
15-Month interim evaluation: 12 males in each diet and dose group
Terminal sacrifice: 48 males in each diet and dose group

Method of Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weights.

Animals per Cage
1 (2 mice in each divided cage)

Method of Animal Identification
Ear clip

Diet
Autoclaved NIH-31 pellets (Purina Mills); dietary-controlled animals were given fortified feed. Feed was available *ad libitum* and changed each week to one group at each dose level. Feed was allocated to the dietary-controlled groups to maintain the animals' body weights in conformance with an idealized weight curve. Dietary-controlled mice were fed daily at the end of the light cycle.

TABLE 1
Experimental Design and Materials and Methods in the 2-Year Gavage Study of Chloral Hydrate

Water
Millipore-filtered tap water (Jefferson municipal supply) available *ad libitum*. Water bottles changed weekly.

Cages
Polycarbonate cages (Lab Products, Seaford, DE), changed weekly

Bedding
Hardwood chips (Northeastern Products, Warrensburg, NY)

Racks
Changed weekly

Animal Room Environment
Temperature: 23° to 25° C
Relative humidity: 38% to 62%
Room fluorescent light: 12 hours/day
Room air changes: 10 to 15/hour

Doses
0, 25, 50, or 100 mg/kg per day (dosing volume = 5 mL/kg)

Type and Frequency of Observation
Observed and clinical findings recorded daily; animals were weighed on days of dosing as part of the dosing procedure.

Method of Sacrifice
15-Month interim evaluation: decapitation
Terminal sacrifice: asphyxiation with carbon dioxide

Necropsy
Necropsy performed on all animals. The brain and liver were weighed at 15 months, and the heart, right kidney, liver, lung, right testis, and thymus were weighed at the end of the study.

Hepatic enzyme analysis
Blood samples were collected for serum enzyme analysis. The parameters evaluated included alanine aminotransferase, amylase, aspartate aminotransferase and lactate hydrogenase.

Histopathology
Complete histopathology was performed on vehicle control and 100 mg/kg mice at the end of the study and on all mice that died early. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, coagulating gland, esophagus, eye, gallbladder, harderian gland, heart with aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum, kidney, lacrimal gland, larynx, lung, lymph nodes (mandibular and mesenteric), muscle, nose, pancreas, parathyroid gland, prostate gland, salivary gland, skin, spleen, spinal cord, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thyroid gland, tongue, trachea, urinary bladder, and Zymbal's gland. In addition, the liver was examined in all dose groups at 15 months and 2 years.

STATISTICAL METHODS

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are one sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A4, B1, and B3 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A2 and B2) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. Tables A2 and B2 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, to animals that do not reach terminal sacrifice.

Analysis of Neoplasm and Nonneoplastic Lesion Incidences

The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk. For analysis of a given site, each animal is assigned a risk weight. This value is one if the animal had a lesion at that site or if it survived until terminal sacrifice; if the animal died prior to terminal sacrifice and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.

This method yields a lesion prevalence rate that depends only upon the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time (Bailer and Portier, 1988). Unless otherwise specified, a value of k=3 was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier (1988) following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats and $B6C3F_1$ mice (Portier *et al.*, 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).

Tests of significance included pairwise comparisons of each dosed group with controls and a test for an overall dose-related trend. Continuity-corrected Poly-3 tests were used in the analysis of neoplasm incidence, and reported P values are one sided. Positive trends are reported with right-tailed P values. Negative trends are reported with left-tailed P values, with the letter N added to indicate a lower incidence as dose increases. For neoplasms and nonneoplastic lesions detected at the interim evaluation, the Fisher exact test (Gart *et al.*, 1979), a procedure based on the overall proportion of affected animals, was used.

Analysis of Continuous Variables

Body weight comparisons were made at 3-month intervals. Body weights were analyzed separately for the interim and terminal sacrifice groups. A repeated measures mixed models analysis of variance approach was used. At each time interval, for each diet, tests were conducted for linear dose trends. Dunnett's test (1955) was used to compare the dosed group means to the vehicle control group mean. Analysis of variance procedures were used to analyze organ weights, terminal body weights, and the organ-weight-to-body-weight ratios for the terminal sacrifice animals. The organ weights and the organ-weight-to-brain-weight ratios were analyzed for the interim sacrifice animals. Dunnett's test (1955) was used to compare the dosed group means to the vehicle control group mean, for each diet. For hepatic enzyme activities, differences between experimental groups were analyzed using

PC-based SAS General Linear Models or ANOVA programs (SAS Institute, Inc., Cary, NC). Means from experimental dose groups were compared using Tukey's studentized range test or Duncan's multiple range test.

QUALITY ASSURANCE METHODS

The study was conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). The Quality Assurance Unit of the National Center for Toxicological Research performed audits and inspections of protocols, procedures, data, and reports throughout the course of the study. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at the NCTR. The audit findings were reviewed and assessed by the NCTR staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

RESULTS

The doses used in this study were the same as those selected for the companion study (NTP, 2002), in which the maximum dose of 100 mg/kg was selected because of concern that higher doses might be toxic in female mice. The lowest dose, 25 mg/kg, approximated the dose of chloral hydrate used in pediatric medicine.

2-YEAR STUDY

Survival

Estimates of 2-year survival probabilities for male *ad libitum*-fed and dietary-controlled mice are shown in Table 2 and in the Kaplan-Meier survival curves (Figure 7). Survival of dosed groups of *ad libitum*-fed male mice and of dietary-controlled male mice was similar to that of vehicle controls. When compared to the *ad libitum*-fed groups, dietary control significantly increased survival for the vehicle control, 25 mg/kg, and 50 mg/kg groups.

TABLE 2
Survival of Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
***Ad Libitum*-Fed**				
Animals initially in study	60	60	60	60
15-Month interim evaluation[a]	12	12	12	12
Moribund	0	4	2	1
Natural deaths	6	7	10	3
Animals surviving to study termination	42	37	36	44
Percent probability of survival at end of study[b]	88	77	75	92
Mean survival (days)[c]	706	702	697	707
Survival analysis[d]	P=0.209N	P=0.089	P=0.059	P=0.265N
Dietary-Controlled				
Animals initially in study	60	60	60	60
15-Month interim evaluation[a]	12	12	12	12
Moribund	1	1	1	0
Natural deaths	2	3	0	7
Animals surviving to study termination	45	44	47	41
Percent probability of survival at end of study	96	92	98	88
Mean survival (days)	705	697	708	695
Survival analysis	P=0.170	P=0.198	P=0.280N	P=0.148
Dietary-Controlled Compared to *Ad Libitum*-Fed Survival Analysis[e]	P=0.044	P=0.030	P=0.001	P=0.246N

[a] Censored from survival analyses
[b] Kaplan-Meier determinations
[c] Mean of all deaths (uncensored, censored, and terminal sacrifice).
[d] The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A negative trend or lower mortality in a dose group is indicated by **N**.
[e] One-tailed test shows the P value for the hypothesis that feed restriction increased survival as compared to an *ad libitum* diet.

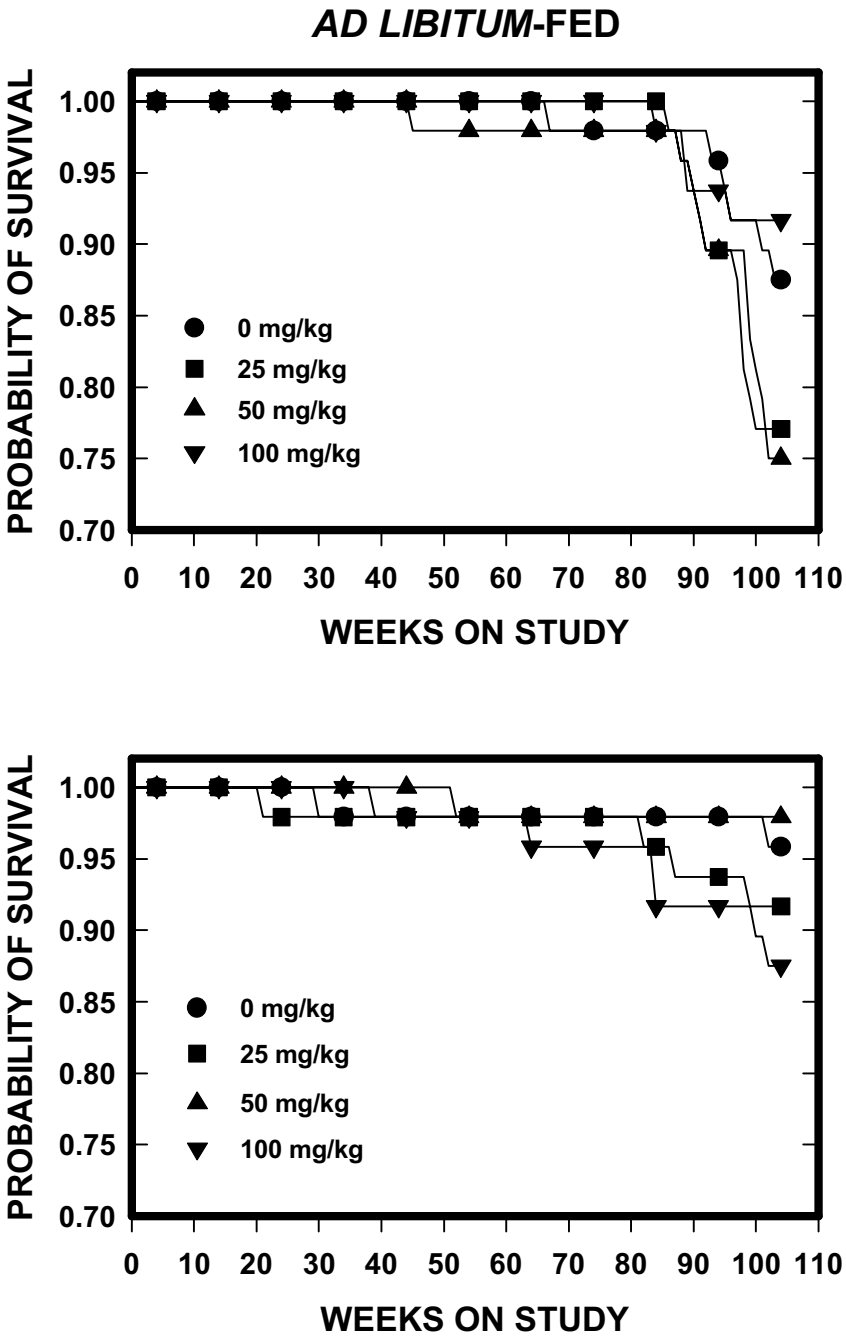


FIGURE 7
Kaplan-Meier Survival Curves for Male Mice Administered Chloral Hydrate by Gavage for 2 Years

Body Weights, Feed Consumption, and Clinical Findings

Mean body weights of all dosed groups were similar to those of the vehicle control groups throughout the study (Figure 8 and Tables 3 and 4). The dietary-controlled mice were successfully maintained at or near their target idealized body weights. The controlled feeding also reduced body weight variation between individual mice when compared to the *ad libitum*-fed mice. Figure 9 compares the growth curves of the *ad libitum*-fed vehicle control group with the growth curves of the dietary-controlled vehicle control group. The standard deviation is smaller for the dietary-controlled group than for the *ad libitum*-fed group. Figure 9 also compares the growth curve for the dietary-controlled vehicle control group with the idealized body weight curve. The mean body weight of this group was initially slightly less than the target body weight but remained within 5% of the target body weight throughout the study.

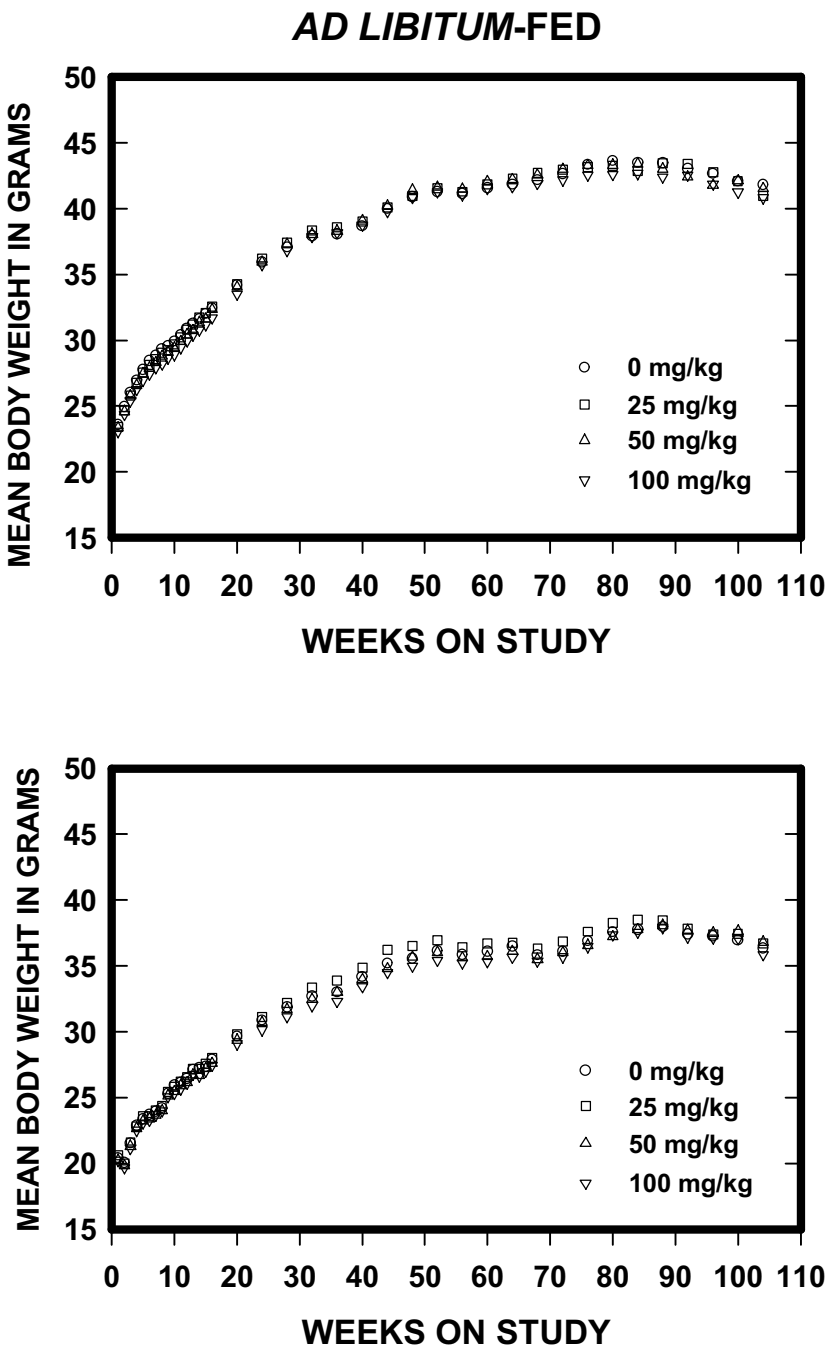


FIGURE 8
Growth Curves for Male Mice Administered Chloral Hydrate by Gavage for 2 Years

TABLE 3
Mean Body Weights and Survival of *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

Weeks on Study	Vehicle Control		25 mg/kg			50 mg/kg			100 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	23.5	60	23.4	100	60	23.3	99	60	23.1	98	60
2	24.8	60	24.7	100	60	24.5	99	60	24.4	98	60
3	26.0	60	25.8	99	60	25.7	99	60	25.4	98	60
4	26.9	60	26.7	99	60	26.5	99	60	26.2	97	60
5	27.7	60	27.5	99	60	27.4	99	60	27.0	97	60
6	28.3	60	28.1	99	60	27.7	98	60	27.4	97	60
7	28.7	60	28.5	99	60	28.2	98	60	27.9	97	60
8	29.1	60	29.0	100	60	28.7	99	60	28.2	97	60
9	29.4	60	29.3	100	60	29.0	99	60	28.6	97	60
10	29.8	60	29.7	100	60	29.4	99	60	28.9	97	60
11	30.3	60	30.2	100	60	29.8	98	60	29.4	97	60
12	30.8	60	30.7	100	60	30.3	98	60	29.9	97	60
13	31.1	60	31.1	100	60	30.7	99	60	30.4	98	60
14	31.4	60	31.7	101	60	31.2	99	60	30.8	98	60
15	31.9	60	32.0	100	60	31.5	99	60	31.2	98	60
16	32.3	60	32.5	101	60	32.2	100	60	31.8	98	60
20	34.0	60	34.3	101	60	33.9	100	60	33.5	99	60
24	35.7	60	36.3	102	60	35.8	100	60	35.7	100	60
28	36.9	60	37.5	102	60	37.2	101	60	36.8	100	60
32	37.8	60	38.4	102	60	38.0	101	60	37.9	100	60
36	37.9	60	38.7	102	60	38.2	101	60	38.2	101	60
40	38.6	60	39.1	101	60	39.0	101	60	38.8	101	60
44	39.9	60	40.2	101	60	40.2	101	60	39.8	100	60
48	40.8	60	41.0	100	60	41.3	101	59	40.8	100	60
52	41.2	60	41.6	101	60	41.6	101	59	41.3	100	60
56	41.1	60	41.3	100	60	41.4	101	59	41.1	100	60
60	41.5	60	41.9	101	60	42.0	101	59	41.6	100	60
64	41.8	60	42.3	101	60	42.2	101	59	41.7	100	60
68[a]	42.2	47	42.7	101	48	42.4	100	47	41.9	99	48
72	42.6	47	43.0	101	48	42.9	101	47	42.2	99	48
76	43.3	47	43.2	100	48	43.2	100	47	42.6	98	48
80	43.6	47	43.2	99	48	43.2	99	47	42.6	98	48
84	43.4	47	42.8	99	48	43.3	100	47	42.7	98	47
88	43.4	47	43.4	100	46	43.1	99	46	42.8	99	47
92	42.9	47	43.3	101	43	42.7	100	43	42.7	100	45
96	42.6	44	42.6	100	43	41.9	98	43	42.2	99	44
100	42.1	44	41.7	99	37	42.5	101	39	41.6	99	44
104	41.8	42	41.0	98	37	41.5	99	36	40.8	98	44
Mean for weeks											
1 to 13	28.2		28.1	100		27.8	99		27.4	97	
14 to 52	36.5		36.9	101		36.7	100		36.4	100	
53 to 104	42.5		42.5	100		42.5	100		42.0	99	

[a] Interim sacrifice occurred during week 65.

TABLE 4
Mean Body Weights and Survival of Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

Weeks on Study	Vehicle Control		25 mg/kg			50 mg/kg			100 mg/kg		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	20.5	60	20.6	100	60	20.2	99	60	20.1	98	60
2	20.1	60	20.2	100	60	19.9	99	60	19.9	99	60
3	21.6	60	21.5	100	60	21.4	99	60	21.2	98	60
4	22.9	60	22.9	100	60	22.6	99	60	22.5	98	60
5	23.4	60	23.6	101	60	23.2	99	60	23.1	99	60
6	23.7	60	23.6	100	60	23.4	99	60	23.3	98	60
7	24.0	60	24.0	100	60	23.6	98	60	23.7	99	60
8	24.2	60	24.4	101	60	24.0	99	60	24.0	99	60
9	25.3	60	25.4	100	60	25.2	100	60	25.1	99	60
10	25.9	60	25.8	100	60	25.5	98	60	25.4	98	60
11	26.2	60	26.1	100	60	25.9	99	60	25.7	98	60
12	26.4	60	26.5	100	60	26.0	98	60	26.1	99	60
13	27.1	60	27.1	100	60	26.7	99	60	26.7	99	60
14	27.5	60	27.4	100	60	27.3	99	60	26.9	98	60
15	27.5	60	27.6	100	60	27.5	100	60	27.2	99	60
16	28.3	60	28.3	100	60	28.1	99	60	27.8	98	60
20	29.7	60	29.7	100	60	29.4	99	60	29.0	98	60
24	30.9	60	31.1	101	59	30.7	99	60	30.1	97	60
28	31.9	60	32.2	101	59	31.7	99	60	31.1	97	60
32	32.7	59	33.4	102	59	32.5	99	60	31.9	98	60
36	33.0	59	34.0	103	59	33.0	100	60	32.2	98	60
40	34.2	59	34.9	102	59	34.0	99	60	33.4	98	59
44	35.2	59	36.2	103	59	34.8	99	60	34.5	98	59
48	35.7	59	36.6	103	59	35.7	100	60	34.9	98	59
52	36.2	59	37.0	102	59	36.1	100	59	35.3	98	59
56	36.0	59	36.4	101	59	35.6	99	59	35.2	98	59
60	36.2	59	36.7	101	59	35.7	99	59	35.3	98	59
64	36.6	59	36.8	101	59	36.1	99	59	35.7	98	58
68[a]	35.9	47	36.3	101	47	35.5	99	47	35.5	99	46
72	36.1	47	36.9	102	47	36.0	100	47	35.7	99	46
76	36.9	47	37.9	103	47	36.7	99	47	36.5	99	46
80	37.6	47	38.3	102	47	37.2	99	47	37.3	99	46
84	37.8	47	38.5	102	46	37.7	100	47	37.6	99	44
88	38.0	47	38.4	101	45	38.1	100	47	37.9	100	44
92	37.4	47	37.7	101	45	37.7	101	47	37.2	99	44
96	37.3	47	37.4	100	45	37.5	101	47	37.2	100	44
100	37.1	47	37.5	101	44	37.6	101	47	37.0	100	43
104	36.3	46	36.7	101	44	36.8	101	47	35.8	99	42
Mean for weeks											
1 to 13	23.9		24.0	100		23.7	99		23.6	99	
14 to 52	31.9		32.4	101		31.7	99		31.2	98	
53 to 104	36.9		37.3	101		36.8	100		36.5	99	

[a] Interim sacrifice occurred during week 65.

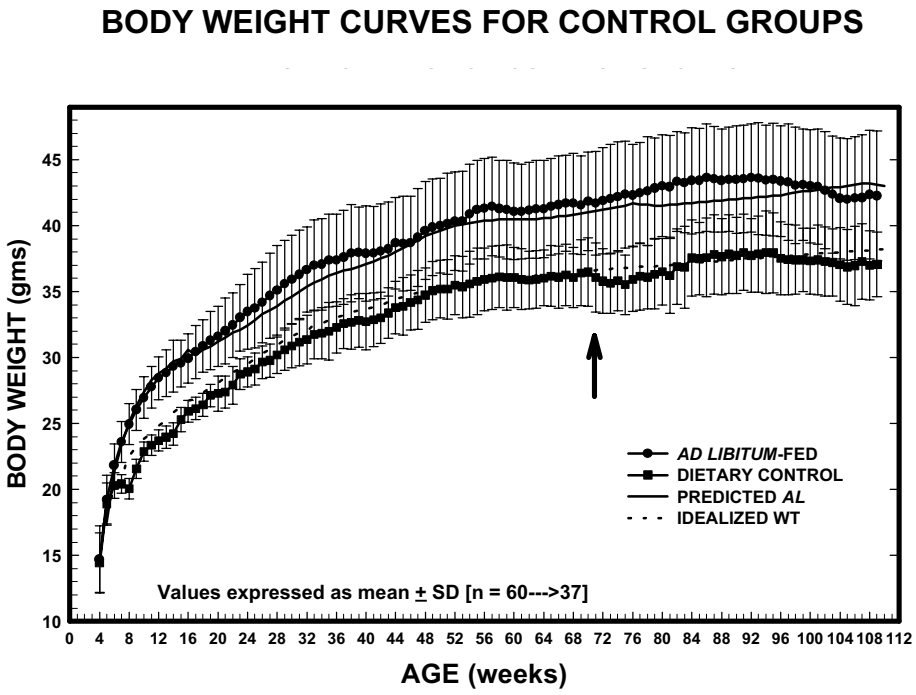


FIGURE 9
Growth Curves for *Ad Libitum*-Fed and Dietary-Controlled Vehicle Control Groups
The figure shows the mean and standard deviation of each weekly body weight value.
The arrow marks the time point at which 12 mice were removed for the interim evaluation.
The data for this figure are presented in Tables D5 and D6.

Feed consumption by 25 and 50 mg/kg *ad libitum*-fed mice was generally similar to that by the vehicle controls throughout the study (Table G1). Feed consumption by 100 mg/kg *ad libitum*-fed mice was less than that by the vehicle controls throughout the study. The feed consumption data suggest that the *ad libitum*-fed mice were eating up to 17% less than predicted from historical feed consumption data (Table 5). This might have been due to the effects of gavaging the mice daily with 5mL/kg body weight water or aqueous chloral hydrate. Because of this, the actual amount of feed consumed by many of the *ad libitum*-fed mice was less than the amount of feed allocated to the dietary-controlled mice. This suggests that many of the dietary-controlled mice were not calorically restricted. There were no clinical findings related to chloral hydrate administration.

The choral hydrate dosing also appeared to further decrease feed consumption in the *ad libitum*-fed mice (Table G1). This decrease was as great as 11% in the 100 mg/kg group. There were no significant dose effects on drinking water consumption (data not presented). The *ad libitum*-fed mice tended to drink more than the dietary-controlled mice. There was, therefore, no evidence of polydipsia, which has been reported to occur with 40% calorically restricted rodents (Duffy *et al.*, 1989).

TABLE 5
Comparison of Predicted and Observed Feed Consumption by *Ad Libitum*-Fed Vehicle Control Male Mice in the 2-Year Study of Chloral Hydrate

Weeks of Age	Predicted (g)	Observed (g)	Difference (%)	Minimum (g)	Maximum (g)	Idealized Wt Allocation[a]
6	4.00	4.22	105.50	3.72	5.01	3.50
18	6.10	5.05	82.79	4.37	5.59	4.50
30	5.80	4.91	84.66	4.01	5.32	5.00
42	5.80	5.00	86.21	4.59	5.37	5.00
54	5.70	4.97	87.19	4.37	5.70	5.00
66	5.70	4.84	84.91	4.22	5.28	5.00
78	6.00	5.00	83.33	4.18	5.55	5.00
90	6.10	5.07	83.11	4.31	5.64	5.50
102	6.00	5.03	83.83	4.27	5.86	5.50
110	5.40	5.24	97.04	4.39	6.52	4.50

[a] Feed allocated to dietary-controlled vehicle control mice, rounded to the nearest 0.5 g (see Appendix D)

Hepatic Enzyme Analysis

Liver microsomal samples from the supplemental study and the 15-month interim evaluation mice were assayed for total hepatic cytochrome P450 and several isoform-selective activities. Comprehensive details are give in Appendix E. Previous work suggested that chloral hydrate treatment induced cytochrome P450-dependent monooxygenase activities, which are partly catalyzed by cytochrome P450 isoforms from the CYP2B family (Sanders *et al.*, 1982). Induction of mouse hepatic CYP2B isoform has been associated with increased incidences of hepatocellular adenoma (Wolff *et al.*, 1991). However, there was no evidence of induction of either total cytochrome P450 or CYP2B isoforms by chloral hydrate in either the *ad libitum*-fed or dietary-controlled mice.

Cytochrome P450 isoforms belonging to the CYP4A family catalyze the ω-hydroxylation of fatty acids and are induced by peroxisome proliferators such as clofibrate or trichloroacetic acid (Austin *et al.*, 1995; Zanelli *et al.*, 1996; Waxman, 1999). Hepatic microsomal samples from the interim evaluation animals were therefore assayed for lauric acid ω-hydroxylase activity and CYP4A immunoreactive protein. As reported in Appendix E, chloral hydrate did not significantly induce either lauric acid ω-hydroxylase activity or CYP4A immunoreactive protein in any of the dosed groups of *ad libitum*-fed mice. However, 100 mg/kg did significantly induce both lauric acid ω-hydroxylase activity and CYP4A immunoreactive protein in the dietary-controlled mice. Moreover, the induction response profile of CYP4A was similar to the increase in the incidence of liver neoplasms at 2 years in the dietary-controlled mice, with the major effect occurring in the 100 mg/kg group (Figure 10).

The serum enzymes alanine aminotransferase, amylase, aspartate aminotransferase, and lactate dehydrogenase were also assayed at 2 years. There was a significant increase in aspartate aminotransferase activity in the *ad libitum*-fed 50 mg/kg group (Appendix E). There were no other significant effects in any dosed group, but in general the dietary-controlled groups exhibited lower values than the corresponding *ad libitum*-fed groups.

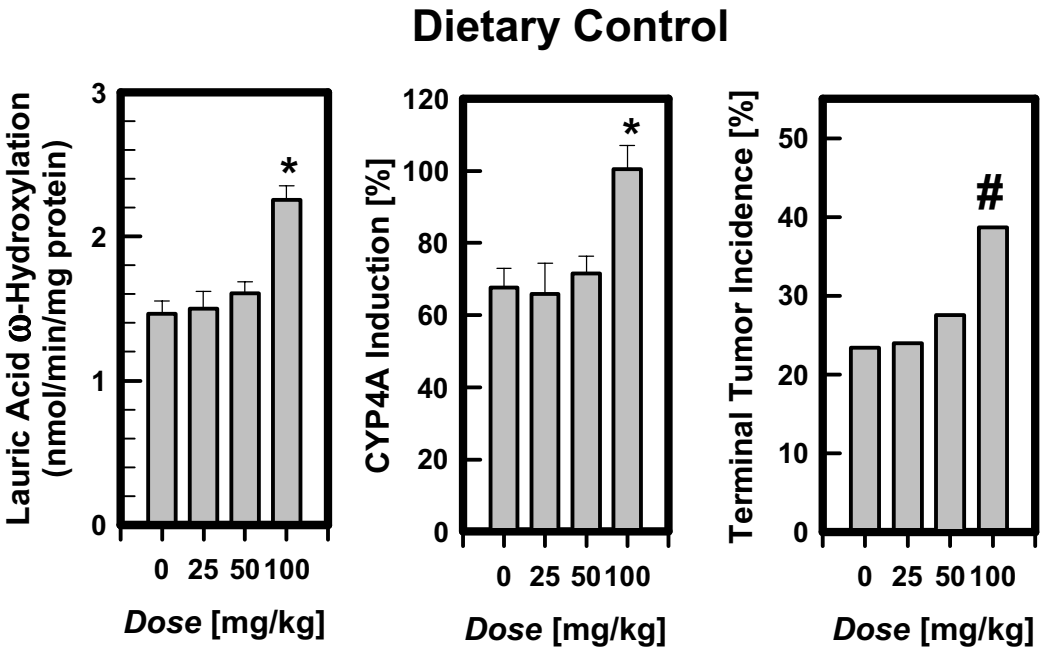


FIGURE 10
Comparison of the Induction of Hepatic CYP4A Immunoreactive Protein after 15 Months of Dosing and Terminally Adjusted Liver Neoplasm Incidence in Male Mice in the 2-Year Gavage Study of Chloral Hydrate
(*=significantly different from the vehicle control and lower dose groups by the SAS GLM-Tukey's test, Appendix E; #=significantly different from the vehicle control group by the Poly-3 test, Table 7)

Organ Weights, Pathology, and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in organ weights and in the incidences of neoplasms and nonneoplastic lesions of the liver. Summaries of the incidences of neoplasms and nonneoplastic lesions and statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group are presented in Appendix A for *ad libitum*-fed male mice and Appendix B for dietary-controlled male mice.

The heart weight of *ad libitum*-fed male mice administered 100 mg/kg and the kidney weights of 50 and 100 mg/kg *ad libitum*-fed mice were significantly less than those of the vehicle controls at 2 years (Table C1).

Liver: The liver weights of dosed groups were greater than those of the vehicle control groups for *ad libitum*-fed and dietary-controlled mice at 2 years (Tables C1 and C2), and the absolute liver weights of dosed groups of mice were generally increased at 15 months. These increases were not statistically significant. However, there was a significant dose response to chloral hydrate of the liver weights relative to body weights in the dietary-controlled mice at 15 months (Table 6). There was no corresponding dose response in the *ad libitum*-fed mice, which showed a much larger variation in liver-to-body-weight ratios. Dietary control clearly reduced the variation in this ratio resulting in standard deviations and standard errors that were from 2- to 17-fold lower for the dietary-controlled groups than for the *ad libitum*-fed groups (Table 6). Thus, dietary control appeared to increase the sensitivity of the bioassay to detect changes in liver-to-body-weight ratios by reducing variability.

At 15 months, hepatocellular adenomas and carcinomas were observed in *ad libitum*-fed mice but not in the dietary-controlled mice (Tables 7, A1, and B1). In the *ad libitum*-fed mice at 2 years, the incidences of hepatocellular adenoma or carcinoma (combined) were greater in the dosed groups than in the vehicle control group, and the increase in the 25 mg/kg group was significant (Tables 7 and A2). The incidences of peripheral or focal peripheral fatty change were increased in all dosed groups of *ad libitum*-fed mice at 15 months (Tables 7 and A4). The incidences of hepatocellular carcinoma and of hepatocellular adenoma or carcinoma (combined) occurred with positive trends in dietary-controlled male mice at 2 years (Tables 7 and B2). The incidence of hepatocellular carcinoma in 100 mg/kg dietary-controlled mice was significantly greater than that in the vehicle controls.

The incidence of hepatocellular neoplasms in the *ad libitum*-fed control group was greater than the NCTR historical control range (Tables 7 and A3). However, previous NCTR studies used mice with smaller body weights than those used in the current study. Traditional methods of reporting historical control data do not account for body weight differences; therefore, comparisons of liver neoplasm incidences are meaningless because these incidences are dependent upon body weight. When historical data was used with an alternative method that adjusted for body weight effects and the effects of dietary restriction (Appendix D), the individual body weight profiles of mice in the *ad libitum*-fed control group predicted a terminal, survival-adjusted rate for the incidences of hepatocellular adenoma or carcinoma (combined) of 34.35% ± 2.16%, whereas the individual body weight profiles of the dietary-controlled vehicle control group predicted a rate of 23.31% ± 1.49% (Table D8).

When dietary-controlled groups were compared to the corresponding *ad libitum*-fed groups, dietary control significantly reduced the incidences of hepatocellular adenoma and hepatocellular adenoma or carcinoma (combined) in the 25 mg/kg group, and hepatocellular adenoma or carcinoma (combined) in the 50 mg/kg group (Table 8). Although all other incidences of hepatocellular neoplasms, except carcinoma in 100 mg/kg mice, were lower in the dietary-controlled groups than in the corresponding *ad libitum*-fed groups, the decreases were not significant.

TABLE 6
Liver-Weight-to-Body-Weight Ratios in Male Mice Evaluated at 15 Months in the 2-Year Gavage Study of Chloral Hydrate

	Ad Libitum-Fed				Dietary-Controlled			
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	12	12	12	12	12	12	12	12
Mean[a]	47.08	46.96	40.87	51.11	35.63	37.46	38.31	39.55
SD[b]	17.59	17.40	4.13	19.58	1.02	1.37	2.09	2.29
SEM[c]	5.08	5.02	1.19	5.65	0.30	0.39	0.60	0.66
Tukey's test[d]	A	A	A	A	A	AB	BC	C
Dunnett's test[e]					0.0001	0.0394	0.0017	0.0000

[a] Ratios are given as mg liver per g body weight.
[b] Standard deviation
[c] Standard error of the mean
[d] Each diet group was treated on a separate ANOVA, and diet/dose groups not sharing the same letter are significantly different from each other (P<0.05).
[e] Beneath the vehicle control group is the P value associated with the trend analysis. Beneath the dosed groups are the P values relative to the vehicle control group.

TABLE 7
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
***Ad Libitum*-Fed**				
15 -Month Interim Evaluation				
Number Examined Microscopically	12	12	12	12
Fatty change, focal, peripheral[a]	2 (1.0)[b]	7* (2.1)	0	0
Fatty change, peripheral	0	0	7** (2.0)	7** (1.6)
Hepatocellular Adenoma	2	2	3	1
Hepatocellular Carcinoma	0	1	0	2
Hepatocellular Adenoma or Carcinoma	2	3	3	3
2-Year Study				
Number Examined Microscopically	48	48	47	48
Hepatocellular Adenoma (includes multiple)	12	19	17	17
Hepatocellular Carcinoma (includes multiple)	4	10	10	7
Hepatocellular Adenoma or Carcinoma (includes multiple)[c]				
Overall rate[d]	16/48 (33%)	25/48 (52%)	23/47 (49%)	22/48 (46%)
Adjusted rate[e]	33.4%	52.6%	50.6%	46.2%
Terminal rate[f]	11/41 (27%)	16/37 (43%)	17/36 (47%)	19/44 (43%)
First incidence (days)	511	639	668	629
Poly-3 test[g]	P=0.2154	P=0.0437	P=0.0684	P=0.1430

TABLE 7
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Dietary-Controlled				
2-Year Study				
Number Examined Microscopically	48	48	48	48
Hepatocellular Adenoma (includes multiple)	9	7	10	10
Hepatocellular Carcinoma (includes multiple)[h]				
Overall rate	2/48 (4%)	5/48 (10%)	4/48 (8%)	8/48 (17%)
Adjusted rate	4.2%	10.9%	8.5%	17.3%
Terminal rate	2/45 (4%)	5/44 (11%)	4/47 (9%)	4/41 (10%)
First incidence (days)	757 (T)	757 (T)	757 (T)	486
Poly-3 test	P=0.0371	P=0.2078	P=0.3382	P=0.0422
Hepatocellular Adenoma or Carcinoma (includes multiple)[c]				
Overall rate	11/48 (23%)	11/48 (23%)	14/48 (29%)	18/48 (38%)
Adjusted rate	23.4%	23.9%	29.7%	38.6%
Terminal rate	11/45 (24%)	11/44 (25%)	14/47 (30%)	13/41 (32%)
First incidence (days)	757 (T)	757 (T)	757 (T)	486
Poly-3 test	P=0.0450	P=0.5728	P=0.3231	P=0.0844

(T) Terminal sacrifice
* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test.
** $P \leq 0.01$
[a] Number of animals with lesion
[b] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked
[c] Historical incidence for control groups in NCTR studies (mean ± standard deviation): 89/374 (24.5% ± 3.8%); range 19%-28%
[d] Number of animals with neoplasm per number of animals with the liver examined microscopically
[e] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[f] Observed incidence at terminal kill
[g] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.
[h] Historical incidence: 36/374 (8.9% ± 1.7%); range 7%-11%

TABLE 8
Comparison of Liver Neoplasms in *Ad Libitum*-Fed and Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Hepatocellular Adenoma				
Ad Libitum-Fed				
Overall rate[a]	12/48 (25%)	19/48 (40%)	17/47 (36%)	17/48 (35%)
Adjusted rate[b]	25.2%	40.8%	37.8%	36.2%
Terminal rate[c]	9/41 (22%)	14/37 (38%)	15/36 (42%)	16/44 (36%)
First incidence (days)	511	639	668	713
Dietary-Controlled				
Overall rate	9/48 (19%)	7/48 (15%)	10/48 (21%)	10/48 (21%)
Adjusted rate	19.1%	15.2%	21.2%	21.8%
Terminal rate	9/45 (20%)	7/44 (16%)	10/47 (21%)	9/41 (22%)
First incidence (days)	757 (T)	757 (T)	757 (T)	625
Poly-3 test[d]	P=0.3238	P=0.0046	P=0.0624	P=0.0951
Hepatocellular Carcinoma				
Ad Libitum-Fed				
Overall rate	4/48 (8%)	10/48 (21%)	10/47 (21%)	7/48 (15%)
Adjusted rate	8.5%	21.4%	22.0%	14.7%
Terminal rate	2/41 (5%)	5/37 (14%)	5/36 (14%)	4/44 (9%)
First incidence (days)	689	666	668	629
Dietary-Controlled				
Overall rate	2/48 (4%)	5/48 (10%)	4/48 (8%)	8/48 (17%)
Adjusted rate	4.2%	10.9%	8.5%	17.3%
Terminal rate	2/45 (4%)	5/44 (11%)	4/47 (9%)	4/41 (10%)
First incidence (days)	757 (T)	757 (T)	757 (T)	486
Poly-3 test	P=0.3356	P=0.1364	P=0.0617	P=0.4740N
Hepatocellular Adenoma or Carcinoma				
Ad Libitum-Fed				
Overall rate	16/48 (33%)	25/48 (52%)	23/47 (49%)	22/48 (46%)
Adjusted rate	33.4%	52.6%	50.6%	46.2%
Terminal rate	11/41 (27%)	16/37 (43%)	17/36 (47%)	19/44 (43%)
First incidence (days)	511	639	668	629
Dietary-Controlled				
Overall rate	11/48 (23%)	11/48 (23%)	14/48 (29%)	18/48 (38%)
Adjusted rate	23.4%	23.9%	29.7%	38.6%
Terminal rate	11/45 (24%)	11/44 (25%)	14/47 (30%)	13/41 (32%)
First incidence (days)	757 (T)	757 (T)	757 (T)	486
Poly-3 test	P=0.1976	P=0.0030	P=0.0309	P=0.2975

(T) Terminal sacrifice
[a] Number of neoplasm-bearing animals/number of animals with tissue examined microscopically.
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the dietary-controlled group incidence is the P value corresponding to pairwise comparisons between the *ad libitum*-fed group and the corresponding dietary-controlled group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A lower incidence in an *ad libitum*-fed group is indicated by **N**.

DISCUSSION AND CONCLUSIONS

Chloral hydrate is widely used as a sedative in pediatric medicine and dentistry. The Food and Drug Administration nominated chloral hydrate for study based upon widespread human exposure and concern about its reported hepatocarcinogenicity in rodents (Smith, 1990; Daniel *et al.*, 1992b). There is some evidence in the literature that chloral hydrate is genotoxic *in vitro* (IARC, 1995; Benson, 2000), and it can also increase oxidative damage in liver microsomes and to DNA (Ni *et al.*, 1995). Two previous limited chronic carcinogenicity studies reported that chloral hydrate increases liver neoplasm incidences in male B6C3F$_1$ mice when administered in drinking water (Daniel *et al.*, 1992b; DeAngelo and George, 1995), and a small-scale study suggested that neonatal exposure to chloral hydrate also increased liver neoplasm incidences in male B6C3F$_1$ mice (Rijsinghani *et al.*, 1986). Trichloroacetic acid, a major metabolite of chloral hydrate, and trichloroethylene, which is converted to chloral hydrate as its primary metabolite, have also been shown to be hepatocarcinogenic in mice (Herren-Freund *et al.*, 1987; NTP, 1990). Chloral, the anhydrous form of chloral hydrate, has been implicated as a possible causative agent in mouse lung carcinoma resulting from inhalation exposure to trichloroethylene (Odum *et al.*, 1992).

Neither chloral hydrate nor trichloroacetic acid was found to be hepatocarcinogenic in rats (DeAngelo *et al.*, 1997; Leuschner and Beuscher, 1998), and in the companion study (NTP, 2002) to the present study, chloral hydrate did not increase the incidences of neoplasms in the livers of 2-year-old male or female B6C3F$_1$ mice treated once during the neonatal period or in female mice dosed for 2 years. Although trichloroethylene has been implicated as a renal carcinogen in humans and rats (Dekant *et al.*, 1986; Henschler *et al.*, 1995), mechanistic studies suggest that the causative agents are metabolites of *S*-1,2-dichlorovinylcysteine, which is not a metabolite of chloral hydrate but is formed from trichloroethylene by a separate pathway (Goeptar *et al.*, 1995).

The current study demonstrates that chronic administration of chloral hydrate by gavage significantly increases the incidence of hepatocellular carcinoma in male B6C3F$_1$ mice. Incidences of hepatocellular adenoma or carcinoma (combined) were significantly increased in the *ad libitum*-fed 25 mg/kg group; incidences of hepatocellular carcinoma were significantly increased in the 100 mg/kg dietary-controlled group. There was no evidence that chloral hydrate affected the incidence of any other neoplasm or nonneoplastic lesion.

The dietary control component of this study was incorporated in response to concerns within the regulatory community relating to increased background neoplasm incidences in rodent strains used for toxicity testing, and to the proposed use of dietary restriction to control background neoplasm incidence in rodent carcinogenicity studies (Roe, 1993; Keenan *et al.*, 1994; Hart *et al.*, 1995; Allaben *et al.*, 1996). Dietary control, as described in this study, involved using variable amounts of dietary restriction or supplementation to maintain each animal at a set body weight. There was concern that the body weight reduction resulting from dietary control might mask any chemical-induced neoplasms and might be too complicated or expensive to maintain on a routine basis. However, dietary control significantly increased survival in some of the dose groups and decreased body weight variability in all groups. Incidences of hepatocellular neoplasms were increased in both the *ad libitum*-fed and dietary-controlled groups, but a significant dose response occurred only in the dietary-controlled groups, suggesting that the sensitivity of the bioassay to detect liver neoplasms had not been reduced by the dietary restriction procedures used in this study. A full discussion of the dietary control component of this study and the relationship between body weight and hepatocarcinogenesis is given in Appendix D.

Data from a supplemental study (Appendix J) suggest that the highest dose used in the 2-year study, 100 mg/kg, was below the minimally toxic dose for chloral hydrate administered by gavage. Doses of 200 or 250 mg/kg probably could have been used without a large negative impact on survival. The dose range used in the 2-year study was chosen for a number of reasons.

Daniel *et al.* (1992b) had demonstrated that chloral hydrate is a mouse hepatocarcinogen, so the present study was designed to examine whether doses in the human therapeutic range (10 mg/kg to 70 mg/kg) would also be hepatocarcinogenic. In addition, the supplemental study was designed to initiate dosing in prepubertal mice to determine if early exposure to chloral hydrate made mice more susceptible to neoplasia than dosing at the conventional time frame. There was concern that doses greater than 100 mg/kg might have been too toxic for the prepubertal mice. Thirdly, at the time the study was initiated, *in vitro* evidence suggested chloral hydrate might be hepatocarcinogenic primarily due to the stimulation of cytochrome P4502E1-mediated lipid peroxidation and free radical damage (Ni *et al.*, 1996). Compounds with similar mechanisms of action have been shown to be more potent hepatocarcinogens when administered by gavage than when administered in drinking water (Pereira, 1994). It was therefore expected that 100 mg/kg chloral hydrate administered by gavage would be at least as potent as the estimated 166 mg/kg daily dose used in the previous drinking water study.

Analysis of the livers of mice examined at the 15-month interim evaluation and of mice in the supplemental study (Appendix J) demonstrated that chloral hydrate induces marker enzymes for peroxisome proliferation. In the supplemental study, chloral hydrate was a more potent inducer in dietary-controlled and calorically restricted mice than in *ad libitum*-fed mice. This did not appear to be due to toxicokinetic effects because plasma concentrations of chloral hydrate, trichloroacetic acid, and trichloroethanol were not significantly different between the three feeding protocols. In the interim evaluation mice, the increased potency may be partially explained by the reduction in individual variation that was achieved by dietary control. The standard errors for the liver-weight-to-body-weight ratios were greater for the *ad libitum*-fed groups than for the dietary-controlled groups, as were the standard errors for the lauric acid ω-hydroxylase activities.

A second factor influencing differences in induction response between the diet groups is the regulation of the nuclear peroxisome proliferator-activated receptor (PPAR) by dietary hormones. Peroxisome proliferators, such as clofibrate, induce CYP4A isoforms by initially binding to the PPAR. The PPAR-ligand complex forms a heterodimer with the retinoic acid receptor, which then binds to specific response element sequences in the regulatory portion of the *cyp4a* gene, triggering induction (Gonzalez *et al.*, 1998; Waxman, 1999). The PPAR exists in three different forms in mammals: PPARα, PPARδ, and PPARγ. PPARα is the predominant form expressed in rodent liver and kidney, and experiments with PPARα knockout mice have shown that it is responsible for the induction of CYP4A, palmitoyl CoA fatty acid hydroxylase, and peroxisome proliferation in rodent liver (Sterchele *et al.*, 1996; Rao and Subbarao, 1997; Waxman, 1999). Cell culture experiments have shown that expression of hepatic PPARα is regulated by insulin and glucocorticoids (Lemberger *et al.*, 1994; Steineger *et al.*, 1994; Sterchele *et al.*, 1996; Rao and Subbarao, 1997). Glucocorticoids induce and insulin represses expression of the PPARα receptor protein (Steineger *et al.*, 1994). Caloric or dietary restriction alters the glucocorticoid-insulin ratio in favor of increased serum glucocorticoid concentrations (Leakey *et al.*, 1998), and dietary restriction and fasting have been shown to increase hepatic PPARα mRNA expression in rats, and to enhance the induction of palmitoyl CoA fatty acid hydroxylase by peroxisome proliferators (Sterchele *et al.*, 1996; Rao and Subbarao, 1997).

The similarity in dose response of CYP4A induction and increases in hepatocellular carcinoma incidences in dietary-controlled male B6C3F$_1$ mice strongly suggests that the hepatocarcinogenic effects of chloral hydrate are mediated through peroxisome proliferation. However, further experimentation, such as carcinogenicity bioassays using PPAR knockout mice, would be necessary to confirm such a mechanism.

Peroxisome proliferators induce hepatocellular neoplasms in rodents by a nongenotoxic mechanism dependent on PPARα. The hepatocarcinogenic response is abolished in PPARα knockout mice deficient in PPARα but which continue to express PPARδ and PPARγ (Peters *et al.*, 1997; Gonzalez *et al.*, 1998; Waxman, 1999). The mechanistic basis for the hepatocarcinogenesis of peroxisome proliferators is not completely understood but is hypothesized to involve several key factors and events. These include (a) the transcriptional activation of lipid-metabolizing enzymes, including CYP4A and palmitoyl CoA fatty acid hydroxylase, leading to the formation of DNA-damaging reduced-oxygen species; and (b)

alteration in the balance between hepatocyte proliferation, which is stimulated by peroxisome proliferators, and hepatocyte apoptosis, which is suppressed. Apoptosis provides a critical mechanism for the elimination of genetically damaged cells prior to their clonal expansion, and its suppression leads to fixation of mutations in initiated cells (Grasl-Kraupp *et al.*, 1994).

Unlike rodents, humans and other primates exhibit very weak liver peroxisome proliferative responses (Makowska *et al.*, 1992; Waxman, 1999). This may be due in part to low expression of PPARα in human liver compared to rodent liver and to the human oligomeric form of PPARα having a low affinity for most xenobiotic peroxisome proliferators (Waxman, 1999).

Constant exposure of inducing chemicals is required to maintain peroxisomes in the proliferated state, and hepatic hyperplasia rapidly subsides following the withdrawal of peroxisome proliferators (Makita, 1995; David *et al.*, 1999). This may explain why the current study obtained a weaker hepatocarcinogenic response to chloral hydrate than the previous drinking water study conducted by DeAngelo *et al.* (1991). The mice received gavage doses 5 days per week, and their livers had the weekend to recover. Trichloroacetic acid is a relatively weak peroxisome proliferator when compared to classic peroxisome proliferators such as clofibrate (Austin *et al.*1995), and relatively high plasma concentrations would be required to maintain peroxisome proliferation. The toxicokinetic data (Appendix J) suggest that daily administration of 100 mg/kg chloral hydrate by gavage to dietary-controlled male $B6C3F_1$ mice results in peak trichloroacetic acid concentrations of approximately 800 µM. The combined area-under-the-curve (AUC) value for five consecutive doses was estimated to be approximately 2,000 µM•hour. According to Gorecki *et al.* (1990), ingestion of 1 g chloral hydrate by a healthy adult male resulted in a peak plasma concentration approximately 160 µM with an AUC value of 1,200 µM•hour. A 50 mg/kg oral dose to a premature human neonate resulted in a peak concentration of approximately 70 µM and a 5-day AUC of 700 µM•hour. It is conceivable, therefore, that human patients receiving very high therapeutic doses of chloral hydrate could acquire plasma trichloroacetate concentrations with AUC values approaching those required to increase the incidence of hepatocarcinoma in dietary-controlled male mice, but it is unlikely that such concentrations would be sufficient to cause a transient induction of hepatic peroxisome proliferation because of the relative insensitivity of the human hepatic PPAR system.

CONCLUSIONS

Under the conditions used in this 2-year gavage study, there was *some evidence of carcinogenic activity** of chloral hydrate in male $B6C3F_1$ mice based on increased incidences of hepatocellular adenoma or carcinoma (combined) in *ad libitum*-fed mice and on increased incidences of hepatocellular carcinoma in dietary-controlled mice. In the dietary controlled mice, induction of enzymes associated with peroxisome proliferation was observed at higher doses.

---

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 10. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 12.

REFERENCES

Abbas, R., and Fisher, J.W. (1997). A physiologically based pharmacokinetic model for trichloroethylene and its metabolites, chloral hydrate, trichloroacetate, dichloroacetate, trichloroethanol, and trichloroethanol glucuronide in B6C3F1 mice. *Toxicol. Appl. Pharmacol.* **147**, 15-30.

Abbas, R.R., Seckel, C.S., Kidney, J.K., and Fisher, J.W. (1996). Pharmacokinetic analysis of chloral hydrate and its metabolism in B6C3F1 mice. *Drug Metab. Dispos.* **24**, 1340-1346.

Adler, I.-D., Kliesch, U., van Hummelen, P., and Kirsch-Volders, M. (1991). Mouse micronucleus tests with known and suspect spindle poisons: Results from two laboratories. *Mutagenesis* **6**, 47-53.

Albertini, S. (1990). Analysis of nine known or suspected spindle poisons for mitotic chromosome malsegregation using *Sacchromyces cerevisiae* D61.M. *Mutagenesis* **5**, 617 (Abstr.).

Allaben, W.T., Turturro, A., Leakey, J.E.A., Seng, J.E., and Hart, R.W. (1996). Points-to-consider documents: The need for dietary control for the reduction of experimental variability within animal assays and the use of dietary restriction to achieve dietary control. *Toxicol. Pathol.* **24**, 776-781.

Allen, J.W., Collins, B.W., and Evansky, P.A. (1994). Spermatid micronucleus analyses of trichloroethylene and chloral hydrate effects in mice. *Mutat. Res.* **323**, 81-88.

Almeyda, J., and Levantine, A. (1972). Drug reactions. XVII. Cutaneous reactions to barbiturates, chloral hydrate and its derivatives. *Br. J. Dermatol.* **86**, 313-316.

American Acadamy of Pediatrics (1993). Use of chloral hydrate for sedation in children. *Pediatrics* **92**, 471-473.

Anonymous (1988). Trichloroethylene. *Rev. Environ. Contam. Toxicol.* **106**, 203-212.

Antonenkov, V.D., Pirozhkov, S.V., and Panchenko, L.F. (1985). Intraparticulate localization and some properties of a clofibrate-induced peroxisomal aldehyde dehydrogenase from rat liver. *Eur. J. Biochem.* **149**, 159-167.

Antonenkov, V.D., Pirozhkov, S.V., Popova, S.V., and Panchenko, L.F. (1988). Effect of clofibrate treatment on lipid peroxidation in rat liver homogenate and subcellular fractions. *Int. J. Biochem.* **20**, 829-836.

Austin, E.W., Okita, J.R., Okita, R.T., Larson, J.L., and Bull, R.J. (1995). Modification of lipoperoxidative effects of dichloroacetate and trichloroacetate is associated with peroxisome proliferation. *Toxicology* **97**, 59-69.

Austin, E.W., Parrish, J.M., Kinder, D.H., and Bull, R.J. (1996). Lipid peroxidation and formation of 8-hydroxydeoxyguanosine from acute doses of halogenated acetic acids. *Fundam. Appl. Toxicol.* **31**, 77-82.

Bailer, A.J., and Portier, C.J. (1988). Effects of treatment-induced mortality and tumor-induced mortality on tests for carcinogenicity in small samples. *Biometrics* **44**, 417-431.

Baumgardner, D.J., and Dewsbury, D.A. (1979). Surgical anesthesia of seven rodent species with chloral hydrate. *Physiol. Behav.* **23**, 609-610.

Beland, F.A., Schmitt, T.C., Fullerton, N.F., and Young, J.F. (1998). Metabolism of chloral hydrate in mice and rats after single and multiple doses. *J. Toxicol. Environ. Health, Part A* **54**, 209-226.

Bennett, L.M., Farnham, P.J., and Drinkwater, N.R. (1995). Strain-dependent differences in DNA synthesis and gene expression in the regenerating livers of CB57BL/6J and C3H/HeJ mice. *Mol. Carcinog.* **14**, 46-52.

Benson, R. (2000). *Concise International Chemical Assessment Document No. 25, Chloral Hydrate*. International Program on Chemical Safety, World Health Organization, Research Triangle Park, NC.

Bergesse, J.R., Domenech, C.E., and Balegno, H.F. (1983). Chloral hydrate inhibition *in vitro* of ATPase in membrane of rat erythrocytes and in microsomes of dog kidney external medulla. *Biochem. Pharmacol.* **32**, 3221-3225.

Bergman, K. (1983). Interactions of trichloroethylene with DNA in vitro and with RNA and DNA of various mouse tissues in vivo. *Arch. Toxicol.* **54**, 181-193.

Bernstine, J.B., Meyer, A.E., and Hayman, H.B. (1954). Maternal and foetal blood estimation following the administration of chloral hydrate during labour. *J. Obstet. Gynaecol. Br. Emp.* **61**, 683-685.

Bernstine, J.B., Meyer, A.E., and Bernstine, R.L. (1956). Maternal blood and breast milk estimation following the administration of chloral hydrate during the puerperium. *J. Obstet. Gynaecol. Br. Emp.* **63**, 228-231.

Bieler, G.S., and Williams, R.L. (1993). Ratio estimates, the delta method, and quantal response tests for increased carcinogenicity. *Biometrics* **49**, 793-801.

Bignami, M., Conti, G., Conti, L., Crebelli, R., Misuraca, F., Puglia, A.M., Randazzo, R., Sciandrello, G., and Carere, A. (1980). Mutagenicity of halogenated aliphatic hydrocarbons in *Salmonella typhimurium*, *Streptomyces coelicolor* and *Aspergillus nidulans*. *Chem. Biol. Interact.* **30**, 9-23.

Blacow, N.W., Ed. (1972). *Martindale: The Extra Pharmacopoeia*, 26th ed. The Pharmaceutical Press. London.

Boorman, G.A., Montgomery, C.A., Jr., Eustis, S.L., Wolfe, M.J., McConnell, E.E., and Hardisty, J.F. (1985). Quality assurance in pathology for rodent carcinogenicity studies. In *Handbook of Carcinogen Testing* (H.A. Milman and E.K. Weisburger, Eds.), pp. 345-357. Noyes Publications, Park Ridge, NJ.

Breimer, D.D. (1977). Clinical pharmacokinetics of hypnotics. *Clin. Pharmacokinet.* **2**, 93-109.

Breimer, D.D., Ketelaars, H.C.J., and Van Rossum, J.M. (1974). Gas chromatographic determination of chloral hydrate, trichloroethanol and trichloracetic acid in blood and urine employing head-space analysis. *J. Chromatogr.* **88**, 55-63.

Bringmann, G., Feineis, D., God, R., Fähr, S., Wesemann, W., Clement, H.-W., Grote, C., Kolasiewicz, W., Sontag, K.-H., Heim, C., Sontag, T.A., Reichmann, H., Janetzky, B., Rausch, W.-D., Abdel-mohsen, M., Koutsilieri, E., Götz, M.E., Gsell, W., Zeilke, B., and Riederer, P. (1996). Neurotoxic effects on the dopaminergic system induced by TaClo (1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline), a potential mammalian alkaloid: *In vivo* and *in vitro* studies. *Biogenic Amines* **12**, 83-102.

Bringmann, G., God, R., Fähr, S., Feineis, D., Fornadi, K., and Fornadi, F. (1999). Identification of the dopaminergic neurotoxin 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline in human blood after intake of the hypnotic chloral hydrate. *Anal. Biochem.* **270**, 167-175.

Bronzetti, G., Galli, A., Corsi, C., Cundari, E., Del Carratore, R., Nieri, R., and Paolini, M. (1984). Genetic and biochemical investigation on chloral hydrate in vitro and in vivo. *Mutat. Res.* **141**, 19-22.

Bruce, W.R., and Heddle, J.A. (1979). The mutagenic activity of 61 agents as determined by the micronucleus, *Salmonella*, and sperm abnormality assays. *Can. J. Genet. Cytol.* **21**, 319-334.

Bruckner, J.V., Davis, B.D., and Blancato, J.N. (1989). Metabolism, toxicity, and carcinogenicity of trichloroethylene. *Crit. Rev. Toxicol.* **20**, 31-50.

Brüning, T., Vamvakas, S., Makropoulos, V., and Birner, G. (1998). Acute intoxication with trichloroethene: Clinical symptoms, toxicokinetics, metabolism, and development of biochemical parameters for renal damage. *Toxicol. Sci.* **41**, 157-165.

Bull, R.J., Sanchez, I.M., Nelson, M.A., Larson, J.L., and Lansing, A.J. (1990). Liver tumor induction in B6C3F1 mice by dichloroacetate and trichloroacetate. *Toxicology* **63**, 341-359.

Bull, R.J., Templin, M., Larson, J.L., and Stevens, D.K. (1993). The role of dichloroacetate in the hepatocarcinogenicity of trichloroethylene. *Toxicol. Lett.* **68**, 203-211.

Burg, J.R., and Gist, G.L. (1999). Health effects of environmental contaminant exposure: An intrafile comparison of the Trichloroethylene Subregistry. *Arch. Environ. Health* **54**, 231-241.

Butler, T.C. (1970). The introduction of chloral hydrate into medical practice. *Bull. Hist. Med.* **44**, 168-172.

Cabana, B.E., and Gessner, P.K. (1970). The kinetics of chloral hydrate metabolism in mice and the effect thereon of ethanol. *J. Pharmacol. Exp. Ther.* **174**, 260-275.

Campbell, R.L., Ross, G.A., Campbell, J.R., and Mourino, A.P. (1998). Comparison of oral chloral hydrate with intramuscular ketamine, meperidine, and promethazine for pediatric sedation – Preliminary report. *Anesth. Prog.* **45**, 46-50.

Canuto, R.A., Ferro, M., Muzio, G., Bassi, A.M., Leonarduzzi, G., Maggiora, M., Adamo, D., Poli, G., and Lindahl, R. (1994). Role of aldehyde metabolizing enzymes in mediating effects of aldehyde products of lipid peroxidation in liver cells. *Carcinogenesis* **15**, 1359-1364.

Carmichael, N.G., Enzmann, H., Pate, I., and Waechter, F. (1997). The significance of mouse liver tumor formation for carcinogenic risk assessment: Results and conclusions from a survey of ten years of testing by the agrochemical industry. *Environ. Health Perspect.* **105**, 1196-1203.

Chakrabarti, A., Schatten, H., Mitchell, K.D., Crosser, M., and Taylor, M. (1998). Chloral hydrate alters the organization of the ciliary basal apparatus and cell organelles in sea urchin embryos. *Cell Tissue Res.* **293**, 453-462.

Chang, L.W., Daniel, F.B., and DeAngelo, A.B. (1992). Analysis of DNA strand breaks induced in rodent liver in vivo, hepatocytes in primary culture, and human cell line by chlorinated acetic acids and chlorinated acetaldehydes. *Environ. Mol. Mutagen.* **20**, 277-288.

Channel, S.R., Latendresse, J.R., Kidney, J.K., Grabau, J.H., Lane, J.W., Steel-Goodwin, L., and Gothaus, M.C. (1998). A subchronic exposure to trichloroethylene causes lipid peroxidation and hepatocellular proliferation in male B6C3F1 mouse liver. *Toxicol. Sci.* **43**, 145-154.

Chaudhary, A.K., Nokubo, M., Reddy, G.R., Yeola, S.N., Morrow, J.D., Blair, I.A., and Marnett, L.J. (1994). Detection of endogenous malondialdehyde-deoxyguanosine adducts in human liver. *Science* **265**, 1580-1582.

Chia, S.E., Ong, C.-N., Tsakok, M.F.H., and Ho, A. (1996). Semen parameters in workers exposed to trichloroethylene. *Reprod. Toxicol.* **10**, 295-299.

Code of Federal Regulations (CFR) **21**, Part 58.

Costa, A.K., and Ivanetich, K.M. (1984). Chlorinated ethylenes: Their metabolism and effect on DNA repair in rat hepatocytes. *Carcinogenesis* **5**, 1629-1636.

Cox, D.R. (1972). Regression models and life-tables. *J. R. Stat. Soc.* **B34**, 187-220.

Crebelli, R., and Carere, A. (1987). Chemical and physical agents assayed in tests for mitotic intergenic and intragenic recombination in *Aspergillus nidulans* diploid strains. *Mutagenesis* **2**, 469-475.

Crebelli, R., and Carere, A. (1989). Genetic toxicology of 1,1,2-trichloroethylene. *Mutat. Res.* **221**, 11-37.

Crebelli, R., Conti, G., Conti, L., and Carere, A. (1991). *In vitro* studies with nine known or suspected spindle poisons: Results in tests for chromosome malsegregation in *Aspergillus nidulans*. *Mutagenesis* **6**, 131-136.

Daniel, F.B., Robinson, M., Stober, J.A., Page, N.P., and Olson, G.R. (1992a). Ninety-day toxicity study of chloral hydrate in the Sprague-Dawley rat. *Drug Chem. Toxicol.* **15**, 217-232.

Daniel, F.B., DeAngelo, A.B., Strober, J.A., Olsen, G.R., and Page, N.P. (1992b). Hepatocarcinogenicity of chloral hydrate, 2-chloroacetaldehyde, and dichloroacetic acid in the male B6C3F1 mouse. *Fundam. Appl. Toxicol.* **19**, 159-168.

David, R.M., Moore, M.R., Cifone, M.A., Finney, D.C., and Guest, D. (1999). Chronic peroxisome proliferation and hepatomegaly associated with the hepatocellular tumorigenesis of di(2-ethylhexyl) phthalate and the effects of recovery. *Toxicol. Sci.* **50**, 195-205.

Davidson, I.W.F., and Beliles, R.P. (1991). Consideration of the target organ toxicity of trichloroethylene in terms of metabolite toxicity and pharmacokinetics. *Drug Metab. Rev.* **23**, 493-599.

Dawson, B.V., Johnson, P.D., Goldberg, S.J., and Ulreich, J.B. (1990). Cardiac teratogenesis of trichloroethylene and dichloroethylene in a mammalian model. *J. Am. Coll. Cardiol.* **16**, 1304-1309.

DeAngelo, A.B., and George, M.H. (1995). Evaluation of the carcinogenicity of chloral hydrate in the male B6C3F1 mouse and F344 rat. *Proc. Am. Assoc. Cancer Res.* **36**, 132. (Abstr.).

DeAngelo, A.B., Daniel, F.B., McMillan, L., Wernsing, P., and Savage, R.E., Jr. (1989). Species and strain sensitivity to the induction of peroxisome proliferation by chloroacetic acids. *Toxicol. Appl. Pharmacol.* **101**, 285-298.

DeAngelo, A.B., Daniel, F.B., Strober, J.A., and Olson, G.R. (1991). The carcinogenicity of dichloroacetic acid in the male B6C3F$_1$ mouse. *Fundam. Appl. Toxicol.* **16**, 337-347.

DeAngelo, A.B., Daniel, F.B., Most, B.M., and Olson, G.R. (1996). The carcinogenicity of dichloroacetic acid in the male fischer 344 rat. *Toxicology* **114**, 207-221.

DeAngelo, A.B., Daniel, F.B., Most, B.M., and Olson, G.R. (1997). Failure of monochloroacetic acid and trichloroacetic acid administered in the drinking water to produce liver cancer in male F344/N rats. *J. Toxicol. Environ. Health* **52**, 425-445.

de Fiebre, C.M., Marley, R.J., Miner, L.L., de Fiebre, N.E., Wehner, J.M., and Collins, A.C. (1992). Classical genetic analyses of responses to sedative-hypnotic drugs in crosses derived from long-sleep and short-sleep mice. *Alcohol. Clin. Exp. Res.* **16**, 511-521.

Degrassi, F., and Tanzarella, C. (1988). Immunofluorescent staining of kinetochores in micronuclei: A new assay for the detection of aneuploidy. *Mutat. Res.* **203**, 339-345.

Dekant, W., Metzler, M., and Henschler, D. (1986). Identification of S-1,2-dichlorovinyl-N-acetyl-cysteine as a urinary metabolite of trichloroethylene: A possible explanation for its nephrocarcinogenicity in male rats. *Biochem. Pharmacol.* **35**, 2455-2458.

Duffy, P.H., Feuers, R.J., Leakey, J.E.A., Nakamura, K.D., Turturro, A. and Hart, R.W. (1989). Effect of chronic caloric restriction on the physiological variables related to energy metabolism in the Fischer 344 rat. *Mech. Ageing Devel.* **48**, 117-133.

Duncan, W.K., De Ball, S., and Perkins, T.M. (1994). Chloral hydrate sedation: A simple technique. *Compend. Contin. Educ. Dent.* **XV**, 884-893.

Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.* **50**, 1096-1121.

Elcombe, C.R., Pratt, I.S., and Green, T. (1987). Species differences in biotransformation of and peroxisome proliferation due to trichloroethylene. *Arch. Toxicol. Suppl.* **10**, 147 (Abstr.).

Feldman, R.G., Chirico-Post, J., and Proctor, S.P. (1988). Blink reflex latency after exposure to trichloroethylene in well water. *Arch. Environ. Health* **43**, 143-148.

Fisher, J. (1997). Erratum. *Drug Metab. Dispos.* **25**, 1449.

Fisher, J.W., and Allen, B.C. (1993). Evaluating the risk of liver cancer in humans exposed to trichloroethylene using physiological models. *Risk Anal.* **13**, 87-95.

Fisher, J.W., Gargas, M.L., Allen, B.C., and Andersen, M.E. (1991). Physiologically based pharmacokinetic modeling with trichloroethylene and its metabolite, trichloroacetic acid, in the rat and mouse. *Toxicol. Appl. Pharmacol.* **109**, 183-195.

Fleischman, R.W., McCracken, D., and Forbes, W. (1977). Adynamic ileus in the rat induced by chloral hydrate. *Lab. Anim. Sci.* **27**, 238-243.

Fu, P.P., Dooley, K.L., Von Tungeln, L.S., Bucci, T., Hart, R.W., and Kadlubar, F.F. (1994). Caloric restriction profoundly inhibits liver tumor formation after initiation by 6-nitrochrysene in male mice. *Carcinogenesis* **15**, 159-161.

Furnus, C.C., Ulrich, M.A., Terreros, M.C., and Dulout, F.N. (1990). The induction of aneuploidy in cultured Chinese hamster cells by propionaldehyde and chloral hydrate. *Mutagenesis* **5**, 323-326.

Gart, J.J., Chu, K.C., and Tarone, R.E. (1979). Statistical issues in interpretation of chronic bioassay tests for carcinogenicity. *JNCI* **62**, 957-974.

Gerlach, M., Xiao, A.-Y., Heim, C., Lan, J., God, R., Feineis, D., Bringmann, G., Riederer, P., and Sontag, K.H. (1998). 1-Trichloromethyl-1,2,3,4-tetrahydro-β-carboline increases extracellular serotonin and stimulates hydroxyl radical production in rats. *Neurosci. Lett.* **257**, 17-20.

Goeptar, A.R., Commandeur, J.N.M., van Ommen, B., van Bladeren, P.J., and Vermeulen, N.P.E. (1995). Metabolism and kinetics of trichloroethylene in relation to toxicity and carcinogenicity. Relevance of the mercapturic acid pathway. *Chem. Res. Toxicol.* **8**, 3-21.

Goldberg, S.J., Lebowitz, M.D., Graver, E.J., and Hicks, S. (1990). An association of human congenital cardiac malformations and drinking water contaminants. *J. Am. Coll. Cardiol.* **16**, 155-164.

Goldenthal, E.I. (1971). A compilation of LD50 values in newborn and adult animals. *Toxicol. Appl. Pharmacol.* **18**, 185-207.

Gonthier, B.P., and Barret, L.G. (1989). In-vitro spin-trapping of free radicals produced during trichloroethylene and diethylether metabolism. *Toxicol. Lett.* **47**, 225-234.

Gonzalez, F.J., Peters, J.M., and Cattley, R.C. (1998). Mechanism of action of the nongenotoxic peroxisome proliferators: Role of the peroxisome proliferator-activator receptor α. *J. Natl. Cancer Inst.* **90**, 1702-1709.

Gorecki, D.K.J., Hindmarsh, K.W., Hall, C.A., Mayers, D.J., and Sankaran, K. (1990). Determination of chloral hydrate metabolism in adult and neonate biological fluids after single-dose administration. *J. Chromatogr.* **528**, 333-341.

Graham, S.R., Day, R.O., Lee, R., and Fulde, G.W.O. (1988). Overdose with chloral hydrate: A pharmacological and therapeutic review. *Med. J. Aust.* **149**, 686-688.

Grasl-Kraupp, B., Bursch, W., Ruttkay-Nedecky, B., Wagner, A., Lauer, B., and Schulte-Hermann, R. (1994). Food restriction eliminates preneoplastic cells through apoptosis and antagonizes carcinogenesis in rat liver. *Proc. Natl. Acad. Sci. USA* **91**, 9995-9999.

Grasso, P., and Hinton, R.H. (1991). Evidence for and possible mechanisms of non-genotoxic carcinogenesis in rodent liver. *Mutation Res.* **248**, 271-290.

Green, T., and Prout, M.S. (1985). Species differences in response to trichloroethylene: II. Biotransformation in rats and mice. *Toxicol. Appl. Pharmacol.* **79**, 401-411.

Greenberg, M.S., Burton, G.A., Jr., and Fisher, J.W. (1999). Physiologically based pharmacokinetic modeling of inhaled trichloroethylene and its oxidative metabolites in $B6C3F_1$ mice. *Toxicol. Appl. Pharmacol.* **154**, 264-278.

Gustafson, A., Svensson, S.-E., and Ugander, L. (1977). Cardiac arrhythmias in chloral hydrate poisoning. *Acta Med. Scand.* **201**, 227-230.

Halmes, N.C., Samokyszyn, V.M., and Pumford, N.R. (1997). Covalent binding and inhibition of cytochrome P4502E1 by trichloroethylene. *Xenobiotica* **27**, 101-110.

Hara, A., Yamamoto, H., Deyashiki, Y., Nakayama, T., Oritani, H., and Sawada, H. (1991). Aldehyde dismutation catalyzed by pulmonary carbonyl reductase: Kinetic studies of chloral hydrate metabolism to trichloroacetic acid and trichloroethanol. *Biochim. Biophys. Acta* **1075**, 61-67.

Hardman, D.J. (1991). Biotransformation of halogenated compounds. *Crit. Rev. Biotechnol.* **11**, 1-40.

Harrington-Brock, K., Doerr, C.L., and Moore, M.M. (1998). Mutagenicity of three disinfection by-products: Di- and trichloroacetic acid and chloral hydrate in $L5178Y/TK^{+/-}$ –3.7.2C mouse lymphoma cells. *Mutat. Res.* **413**, 265-276.

Hart, R.W., Keenan, K., Turturro, A., Abdo, K.M., Leakey, J., and Lyn-Cook, B. (1995). Caloric restriction and toxicity. *Fundam. Appl. Toxicol.* **25**, 184-195.

Haseman, J.K. (1985). Issues in carcinogenicity testing: Dose selection. *Fundam. Appl. Toxicol.* **5**, 66-78.

Haseman, J.K., Bourbina, J., and Eustis, S.L. (1994). Effect of individual housing and other experimental design factors on tumor incidence in $B6C3F_1$ mice. *Fundam. Appl. Toxicol.* **23**, 44-52.

Hathway, D.E. (1980). Consideration of the evidence for mechanisms of 1,1,2- trichloroethylene metabolism, including new identification of its dichloroacetic acid and trichloroacetic acid metabolites in mice. *Cancer Lett.* **8**, 263-269.

Hawkins, J.M., Jones, W.E., Bonner, F.W., and Gibson, G.G. (1987). The effect of peroxisome proliferators on microsomal, peroxisomal, and mitochondrial enzyme activities in the liver and kidney. *Drug Metab. Rev.* **18**, 441-515.

Haworth, S., Lawlor, T., Mortlemans, K., Speck, W., and Zeiger, E. (1983). Salmonella mutagenicity results for 250 chemicals. *Environ. Mutagen*. **5** (Suppl. 1), 1-142.

Heller, C., Constantinou, J.C., VandenBerg, K., Benitz, W., and Fleisher, B.E. (1997). Sedation administered to very low birth weight premature infants. *J. Perinatol.* **17**, 107-112.

Henderson, G.N., Yan, Z., James, M.O., Davydova, N., and Stacpoole, P.W. (1997). Kinetics and metabolism of chloral hydrate in children: Identification of dichloroacetate as a metabolite. *Biochem. Biophys. Res. Commun.* **235**, 695-698.

Henschler, D., Elsässer, H., Romen, W., and Eder, E. (1984). Carcinogenicity study of trichloroethylene, with and without epoxide stabilizers, in mice. *J. Cancer Res. Clin. Oncol.* **107**, 149-156.

Henschler, D., Vamvakas, S., Lammert, M., Dekant, W., Kraus, B., Thomas, B., and Ulm, K. (1995). Increased incidence of renal cell tumours in a cohort of cardboard workers exposed to trichloroethene. *Arch. Toxicol.* **70**, 131-133.

Herren-Freund, S.L., Pereira, M.A., Khoury, M.D., and Olson, G. (1987). The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid, in mouse liver. *Toxicol. Appl. Pharmacol.* **90**, 183-189.

Hindmarsh, K.W., Gorecki, D.K.J., Sankaran, K., and Mayers, D.J. (1991). Chloral hydrate administration in neonates: Potential toxicological implications. *Can. Soc. Forensic Sci. J.* **24**, 239-245.

Hobara, T., Kobayashi, H., Kawamoto, T., Iwamoto, S., and Sakai, T. (1987). Extrahepatic metabolism of chloral hydrate, trichloroethanol and trichloroacetic acid in dogs. *Pharmacol. Toxicol.* **61**, 58-62.

Ikeda, M., Miyake, Y., Ogata, M., and Ohmori, S. (1980). Metabolism of trichloroethylene. *Biochem. Pharmacol.* **29**, 2983-2992.

Ikeda, M., Ezaki, M., Kokegouchi, S., and Ohmori, S. (1981a). Studies on NADPH-dependent chloral hydrate reducing enzymes in rat liver cytosol. *Biochem. Pharmacol.* **30**, 1931-1939.

Ikeda, M., Hayakawa, S., Ezaki, M., and Ohmori, S. (1981b). An NADP-dependent 3α-hydroxysteroid dehydrogenase of rat liver active against C_{19}, C_{20}, C_{23}, C_{24}, C_{25} and C_{26} steroids. *Hoppe-Seylers Z. Physiol. Chem.* **362**, 511-520.

International Agency for Research on Cancer (IARC) (1995). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Chloral and Chloral Hydrate*, Vol. 63, pp. 245-269. IARC, Lyon, France.

Johnson, P.D., Dawson, B.V., and Goldberg, S.J. (1998a). Cardiac teratogenicity of trichloroethylene metabolites. *J. Am. Coll. Cardiol.* **32**, 540-545.

Johnson, P.D., Dawson, B.V., and Goldberg, S.J. (1998b). A review. Trichloroethylene metabolites: Potential cardiac teratogens. *Environ. Health Perspect.* **106**, 995-999.

Juntunen, J. (1986). Occupational toxicology of trichloroethylene with special reference to neurotoxicity. *Toxicol. Lett.* **31** (Suppl.), 16 (Abstr.).

Kallman, M.J., Kaempf, G.L., and Balster, R.L. (1984). Behavioral toxicity of chloral in mice: An approach to evaluation trichloroethylene. *Neurobehav. Toxicol. Teratol.* **6**, 137-146.

Kaplan, E.L., and Meier, P. (1958). Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **53**, 457-481.

Kauffmann, B.M., White, K.L., Jr., Sanders, V.M., Douglas, K.A., Sain, L.E., Borzelleca, J.F., and Munson, A.E. (1982). Humoral and cell-mediated immune status in mice exposed to chloral hydrate. *Environ. Health Perspect.* **44**, 147-151.

Kawamoto, T., Hobara, T., Kobayashi, H., Iwamoto, S., Sakai, T., Takano, T., and Miyazaki, Y. (1987a). The metabolite ratio as a function of chloral hydrate dose and intracellular redox state in the perfused rat liver. *Pharmacol. Toxicol.* **60**, 325-329.

Kawamoto, T., Hobara, T., Kobayashi, H., Iwamoto, S., Sakai, T., Imamura, A., and Koshiro, A. (1987b). The metabolism of trichloroethylene and its metabolites in the perfused liver. *J. Toxicol. Sci.* **12**, 87-96.

Kawamoto, T., Hobara, T., Aoki, T., Kobayashi, H., Iwamoto, S., Sakai, T., Takano, T., Miyazaki, Y., Imamura, A., and Ogino, K. (1988). Metabolism of chloral hydrate in the anoxic perfused liver. *Toxicol. Lett.* **40**, 225-231.

Keenan, K.P., Smith, P.F., Hertzog, P., Soper, K., Ballam, G.C., and Clark, R.L. (1994). The effects of overfeeding and dietary restriction on Sprague-Dawley rat survival and early pathology biomarkers of aging. *Toxicol. Pathol.* **22**, 300-315.

Keenan, K.P., Soper, K.A., Hertzog, P.R., Gumprecht, L.A., Smith, P.F., Mattson, B.A., Ballam, G.C. and Clark, R.L. (1995). Diet, overfeeding, and moderate dietary restriction in control Sprague-Dawley rats: II. Effects on age-related proliferative and degenerative lesions. *Toxicol. Pathol.* **23**, 287-302.

Keenan, K.P., Ballam, G.C., Dixit, R., Soper, K.A., Laroque, P., Mattson, B.A., Adams, S.P., and Coleman, J.B. (1997). The effects of diet, overfeeding and moderate dietary restriction on Sprague-Dawley rat survival, disease and toxicology. *J. Nutr.* **127**, 851S-856S.

Keller, D.A., and Heck, H.d'A (1988). Mechanistic studies on chloral toxicity: Relationship to trichloroethylene carcinogenesis. *Toxicol. Lett.* **42**, 183-191.

Kemp, C.J., and Drinkwater, N.R. (1990). The androgen receptor and liver tumor development in mice. In *Mouse Liver Carcinogenesis: Mechanisms and Species Comparisons*, pp. 203-214 (D.E. Stevenson, J.A. Popp, J.M. Ward, R.M. McClain, T.J. Slaga, and H.C. Pitot, Eds.), Alan R. Liss, New York.

Ketcha, M.M., Stevens, D.K., Warren, D.A., Bishop, C.T., and Brashear, W.T. (1996). Conversion of trichloroacetic acid to dichloroacetic acid in biological samples. *J. Anal. Toxicol.* **20**, 236-241.

Klaunig, J.E., Ruch, R.J., and Lin, E.L.C. (1989). Effects of trichloroethylene and its metabolites on rodent hepatocyte intercellular communication. *Toxicol. Appl. Pharmacol.* **99**, 454-465.

Klinefelter, G.R., Suarez, J.D., Roberts, N.L., and DeAngelo, A.B. (1995). Preliminary screening for the potential of drinking water disinfection byproducts to alter male reproduction. *Reprod. Toxicol.* **9**, 571-578.

Krauss, B., and Zurakowski, D. (1998). Sedation patterns in pediatric and general community hospital emergency departments. *Pediatr. Emerg. Care* **14**, 99-103.

Lambert, G.H., Muraskas, J., Anderson, C.L., and Myers, T.F. (1990). Direct hyperbilirubinemia associated with chloral hydrate administration in the newborn. *Pediatrics* **86**, 277-281.

Larson, J.L., and Bull, R.J. (1992a). Species differences in the metabolism of trichloroethylene to the carcinogenic metabolites trichloroacetate and dichloroacetate. *Toxicol. Appl. Pharmacol.* **115**, 278-285.

Larson, J.L., and Bull, R.J. (1992b). Metabolism and lipoperoxidative activity of trichloroacetate and dichloroacetate in rats and mice. *Toxicol. Appl. Pharmacol.* **115**, 268-277.

Lash, L.H., Lipscomb, J.C., Putt, D.A., and Parker, J.C. (1999a). Glutathione conjugation of trichloroethylene in human liver and kidney: Kinetics and individual variation. *Drug Metab. Dispos.* **27**, 351-359.

Lash, L.H., Putt, D.A., Brashear, W.T., Abbas, R., Parker, J.C., and Fisher, J.W. (1999b). Identification of *S*-(1,2-dichlorovinyl)glutathione in the blood of human volunteers exposed to trichloroethylene. *J. Toxicol. Environ. Health* **56**, 1-21.

Leakey, J.E.A., Seng, J.E., Barnas, C.R., Baker, V.M., and Hart, R.W. (1998). A mechanistic basis for the beneficial effects of dietary restriction on longevity and disease: Consequences for the interpretation of rodent toxicity studies. *Int. J. Toxicol.* **17**, 5-56.

Lee, G.M., Diguiseppi, J., Gawdi, G.M., and Herman, B. (1987). Chloral hydrate disrupts mitosis by increasing intracellular free calcium. *J. Cell Sci.* **88**, 603-612.

Lemberger, T., Staels, B., Saladin, R., Desvergne, B., Auwerx, J., and Wahli, W. (1994). Regulation of the peroxisome proliferator-activated receptor α gene by glucocorticoids. *J. Biol. Chem.* **269**, 24,527-24,530.

Leopardi, P., Zijno, A., Bassani, B., and Pacchierotti, F. (1993). In vivo studies on chemically induced aneuploidy in mouse somatic and germinal cells. *Mutat. Res.* **287**, 119-130.

Leuschner, J., and Beuscher, N. (1998). Studies on the mutagenic and carcinogenic potential of chloral hydrate. *Arzneimittelforschung* **48**, 961-968.

Leuschner, J., and Leuschner, F. (1991). Evaluation of the mutagenicity of chloral hydrate in vitro and in vivo. *Arzneimittelforschung* **41**, 1101-1103.

Leuschner, J., and Zimmermann, T. (1996). Examination of the dependence potential of chloral hydrate by oral administration to normal monkeys. *Arzneimittelforschung* **46**, 751-754.

Leuschner, J., Beuscher, N., Zimmermann, T., Schürer, M., Schulz, H.U., Jeromin, J., and Ritter, H. (1998). Investigation on the plasma levels of the neurotoxin 1-trichloromethyl-1,2,3,4-tetrahydro-β-carboline after oral administration of chloral hydrate in man [in German, English summary]. *Arzneimittelforschung* **48**, 1-5.

Liang, J.C., and Pacchierotti, F. (1988). Cytogenic investigation of chemically-induced aneuploidy in mouse spermatocytes. *Mutat. Res.* **201**, 325-335.

Lipscomb, J.C., Mahle, D.A., Brashear, W.T., and Barton, H.A. (1995). Dichloroacetic acid: Metabolism in cytosol. *Drug Metab. Dispos.* **23**, 1202-1205.

Lipscomb, J.C., Mahle, D.A., Brashear, W.T., and Garrett, C.M. (1996). A species comparison of chloral hydrate metabolism in blood and liver. *Biochem. Biophys. Res. Commun.* **227**, 340-350.

Lipscomb, J.C., Fisher, J.W., Confer, P.D., and Byczkowski, J.Z. (1998). *In vitro* to *in vivo* extrapolation for trichloroethylene metabolism in humans. *Toxicol. Appl. Pharmacol.* **152**, 376-387.

Loeber, C.P., Hendrix, M.J.C., Diez De Pinos, S., and Goldberg, S.J. (1988). Trichloroethylene: A cardiac teratogen in developing chick embryos. *Pediatr. Res.* **24**, 740-744.

Ludwigs, U., Divino Filho, J.C., Magnusson, A., and Berg, A. (1996). Suicidal chloral hydrate poisoning. *Clin. Toxicol.* **34**, 97-99.

McCarver-May, D.G., Kang, J., Aouthmany, M., Elton, R., Mowery, J.L., Slovis, T.L., and Kauffman, R. (1996). Comparison of chloral hydrate and midazolam for sedation of neonates for neuroimaging studies. *J. Pediatr.* **128**, 573-576.

McConnell, E.E., Solleveld, H.A., Swenberg, J.A., and Boorman, G.A. (1986). Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. *JNCI* **76**, 283-289.

Mailhes, J.B., Preston, R.J., Yuan, Z.P., and Payne, H.S. (1988). Analysis of mouse metaphase II oocytes as an assay for chemically induced aneuploidy. *Mutat. Res.* **198**, 145-152.

Mailhes, J.B., Aardema, M.J., and Marchetti, F. (1993). Investigation of aneuploidy induction in mouse oocytes following exposure to vinblastine-sulfate, pyrimethamine, diethylstilbestrol diphosphate, or chloral hydrate. *Environ. Mol. Mutagen.* **22**, 107-114.

Makita, T. (1995). Molecular organization of hepatocyte peroxisomes. *Int. Rev. Cytol.* **160**, 303-352.

Makowska, J.M., Gibson, G.G., and Bonner, F.W. (1992). Species differences in ciprofibrate induction of hepatic cytochrome P4504A1 and peroxisome proliferation. *J. Biochem. Toxicol.* **7**, 183-191.

Maltoni, C., Lefemine, G., Cotti, G., and Perino, G. (1988). Long-term carcinogenicity bioassays on trichloroethylene administered by inhalation to Sprague-Dawley rats and Swiss and B6C3F1 mice. *Ann. N.Y. Acad. Sci.* **534**, 316-342.

Maronpot, R.R., and Boorman, G.A. (1982). Interpretation of rodent hepatocellular proliferative alterations and hepatocellular tumors in chemical safety assessment. *Toxicol. Pathol.* **10**, 71-80.

Marshall, E.K., Jr., and Owens, A.H., Jr. (1954). Absorbtion, excretion and metabolic fate of chloral hydrate and trichloroethanol. *Bull. Johns Hopkins Hosp.* **95**, 1-18.

Marti-Bonmati, L., Casillas, C., Poyatos, C., Torrijo, C., Ronchera-Oms, C.L., and Jimenez, N.V. (1995). Randomised double-blind clinical trial of intermediate-versus high-dose chloral hydrate for neuroimaging of children. *Neuroradiology* **37**, 687-691.

Material Safety Data Sheet (MSDS) (1991). Chloral Hydrate Sheet No. OHS23800. Occupational Health Services, Inc., New York.

Mather, G.G., Exon, J.H., and Koller, L.D. (1990). Subchronic 90 day toxicity of dichloroacetic and trichloroacetic acid in rats. *Toxicology* **64**, 71-80.

Mayers, D.J., Hindmarsh, K.W., Sankaran, K., Gorecki, D.K.J., and Kasian, G.F. (1991). Chloral hydrate disposition following single-dose administration to critically ill neonates and children. *Dev. Pharmacol. Ther.* **16**, 71-77.

Mayers, D.J., Hindmarsh, K.W., Gorecki, D.K.J., and Sankaran, K. (1992). Sedative/hypnotic effects of chloral hydrate in the neonate: Trichloroethanol or parent drug? *Dev. Pharmacol. Ther.* **19**, 141-146.

The Merck Index (1989). 11th ed. (S. Budavari, Ed.), p. 317. Merck and Company, Rahway, NJ.

Merdink, J.L., Gonzalez-Leon, A., Bull, R.J., and Schultz, I.R. (1998). The extent of dichloroacetate formation from trichloroethylene, chloral hydrate, trichloroacetate, and trichloroethanol in B6C3F1 mice. *Toxicol. Sci.* **45**, 33-41.

Merdink, J.L., Stenner, R.D., Stevens, D.K., Parker, J.C., and Bull, R.J. (1999). Effect of enterohepatic circulation on the pharmacokinetics of chloral hydrate and its metabolites in F344 rats. *J. Toxicol. Environ. Health, Part A* **57**, 357-368.

Miller, B.M., and Adler, I.-D. (1992). Aneuploidy induction in mouse spermatocytes. *Mutagenesis* **7**, 69-76.

Miller, R.E., and Guengerich, F.P. (1983). Metabolism of trichloroethylene in isolated hepatocytes, microsomes, and reconstituted enzyme systems containing cytochrome P-450. *Cancer Res.* **43**, 1145-1152.

Miller, R.R., and Greenblatt, D.J. (1979). Clinical effects of chloral hydrate in hospitalized medical patients. *J. Clin. Pharmacol.* **19**, 669-674.

Mitchell, D.Y., and Petersen, D.R. (1987). The oxidation of α-β unsatured aldehydic products of lipid peroxidation by rat liver aldehyde dehydrogenases. *Toxicol. Appl. Pharmacol.* **87**, 403-410.

Moghaddam, A.P., Abbas, R., Fisher, J.W., Stavrou, S., and Lipscomb, J.C. (1996). Formation of dichloroacetic acid by rat and mouse gut microflora, an *in vitro* study. *Biochem. Biophys. Res. Commun.* **228**, 639-645.

Moghaddam, A.P., Abbas, R., Fisher, J.W., and Lipscomb, J.C. (1997). The role of mouse intestinal microflora in the metabolism of trichloroethylene, an *in vivo* study. *Hum. Exp. Toxicol.* **16**, 629-635.

Nakajima, T., Wang, R.-S., Murayama, N., and Sato, A. (1990). Three forms of trichloroethylene-metabolizing enzymes in rat liver induced by ethanol, phenobarbital, and 3-methylcholanthrene. *Toxicol. Appl. Pharmacol.* **102**, 546-552.

Nakajima, T., Wang, R.-S., Elovaara, E., Park, S.S., Gelboin, H.V., and Vainio, H. (1993). Cytochrome P450-related differences between rats and mice in the metabolism of benzene, toluene and trichloroethylene in liver microsomes. *Biochem. Pharmacol.* **45**, 1079-1085.

Natarajan, A.T., Duivenvoorden, W.C.M., Meijers, M., and Zwanenburg, T.S.B. (1993). Induction of mitotic aneuploidy using hamster primary embryonic cells. Test results of 10 chemicals. *Mutat. Res.* **287**, 47-56.

National Cancer Institute (NCI) (1976). Carcinogenesis Bioassay of Trichloroethylene (CAS No. 79-01-6). Technical Report Series No. 2. NIH Publication No. 76-802. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Bethesda, MD.

National Toxicology Program (NTP) (1983). National Toxicology Program draft report abstracts on nine chemical carcinogenesis animal bioassays. *Chem. Regul. Rep.* **6**, 67-768.

National Toxicology Program (NTP) (1988). Toxicology and Carcinogenesis Studies of Trichloroethylene (CAS No. 79-01-6) in Four Strains of Rats (ACI, August, Marshall, Osborne-Mendel) (Gavage Studies). Technical Report Series No. 273. NIH Publication No. 88-2529. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1990). Carcinogenesis Studies of Trichloroethylene (Without Epichlorohydrin) (CAS No. 79-01-6) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 243. NIH Publication No. 90-1799. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1996). Toxicology and Carcinogenesis Studies of 1-trans-delta^9-Tetrahydrocannabinol (CAS No. 1972-08-3) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 446. NIH Publication No. 97-3362. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1997). Effect of Dietary Restriction on Toxicology and Carcinogenesis Studies in F344/N Rats and B6C3F$_1$ Mice. Technical Report Series No. 460. NIH Publication No. 97-3376. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1999). Toxicity and Metabolism Studies of Chloral Hydrate (CAS No. 302-17-0) Administered by Gavage to F344/N Rats and B6C3F$_1$ Mice. Toxicity Report Series No. 59. NIH Publication No. 99-3944. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (2000). Toxicology and Carcinogenesis Studies of Methyleugenol (CAS No. 93-15-12) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 491. NIH Publication No. 00-3950. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (2002). Toxicology and Carcinogenesis Studies of Chloral Hydrate (CAS No. 302-17-0) in B6C3F$_1$ Mice (Gavage Study). Technical Report Series No. 502. NIH Publication No. 02-4436. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

Nelson, M.A., and Bull, R.J. (1988). Induction of strand breaks in DNA by trichloroethylene and metabolites in rat and mouse liver *in vivo*. *Toxicol. Appl. Pharmacol.* **94**, 45-54.

Ni, Y.-C., Wong, T.-Y., Kadlubar, F.F., and Fu, P.P. (1994). Hepatic metabolism of chloral hydrate to free radical(s) and induction of lipid peroxidation. *Biochem. Biophys. Res. Commun.* **204**, 937-943.

Ni, Y.-C., Kadlubar, F.F., and Fu, P.P. (1995). Formation of malondialdehyde-modified 2′-deoxyguanosinyl adduct from metabolism of chloral hydrate by mouse liver microsomes. *Biochem. Biophys. Res. Commun.* **216**, 1110-1117.

Ni, Y.-C., Wong, T.-Y., Lloyd, R.V., Heinze, T.M., Shelton, S., Casciano, D., Kadlubar, F.F., and Fu, P.P. (1996). Mouse liver microsomal metabolism of chloral hydrate, trichloroacetic acid, and trichloroethanol leading to induction of lipid peroxidation *via* a free radical mechanism. *Drug Metab. Dispos.* **24**, 81-90.

Nilsson, R., Beije, B., Préat, V., Erixon, K., and Ramel, C. (1991). On the mechanism of the hepatocarcinogenicity of peroxisome proliferators. *Chem. Biol. Interact.* **78**, 235-250.

Nohynek, G.J., Longeart, L., Geffray, B., Provost, J.P., and Lodola, A. (1993). Fat, frail and dying young: Survival, body weight and pathology of the Charles River Sprague-Dawley-derived rat prior to and since the introduction of the VAF^R variant in 1988. *Hum. Exp. Toxicol.* **12**, 87-98.

Nutley, E.V., Tcheong, A.C., Allen, J.W., Collins, B.W., Ma, M., Lowe, X.R., Bishop, J.B., Moore, D.H., II, and Wyrobek, A.J. (1996). Micronuclei induced in round spermatids of mice after stem-cell treatment with chloral hydrate: Evaluations with centromeric DNA probes and kinetochore antibodies. *Environ. Mol. Mutagen.* **28**, 80-89.

Odum, J., Green, T., Foster, J.R., and Hext, P.M. (1988). The role trichloroacetic acid and peroxisome proliferation in the differences in carcinogenicity of perchloroethylene in the mouse and rat. *Toxicol. Appl. Pharmacol.* **92**, 103-112.

Odum, J., Foster, J.R., and Green, T. (1992). A mechanism for the development of Clara cell lesions in the mouse lung after exposure to trichloroethylene. *Chem. Biol. Interact.* **83**, 135-153.

Ogino, K., Hobara, T., Kobayashi, H., and Iwamoto, S. (1990a). Comparative study of the tissue distribution of NADH and NADPH-dependent chloral hydrate reducing enzymes in the rat. *Bull. Environ. Contam. Toxicol.* **44**, 377-379.

Ogino, K., Hobara, T., Kobayashi, H., and Iwamoto, S. (1990b). Gastric mucosal injury induced by chloral hydrate. *Toxicol. Lett.* **52**, 129-133.

Onks, D.L., Robertson, A.F., and Brodersen, R. (1992). The effect of chloral hydrate and its metabolites, trichloroethanol and trichloroacetic acid, on bilirubin-albumin binding. *Pharmacol. Toxicol.* **71**, 196-197.

Owens, A.H., Jr., and Marshall, E.K., Jr. (1955). Further studies on the metabolic fate of chloral hydrate and trichloroethanol. *Bull. Johns Hopkins Hosp.* **97**, 320-326.

Parnell, M.J., Koller, L.D., Exon, J.H., and Arnzen, J.M. (1986). Trichloroacetic acid effects on rat liver peroxisomes and enzyme-altered foci. *Environ. Health Perspect.* **69**, 72-79.

Parodi, S., Malacarne, D., and Taningher, M. (1992). Non-genotoxic factors in the carcinogenetic process: Problems of detection and hazard evaluation. *Toxicol. Lett.* **64/65**, 621-630.

Parry, J.M., and James, S. (1988). The detection of aneugenic chemicals using yeast strainD_6. *Mutagenesis* **3**, 447 (Abstr.).

Pereira, M.A. (1994). Route of administration determines whether chloroform enhances or inhibits cell proliferation in the liver of B6C3F1 mice. *Fundam. Appl. Toxicol.* **23**, 87-92.

Pereira, M.A. (1996). Carcinogenic activity of dichloroacetic acid and trichloroacetic acid in the liver of female B6C3F1 mice. *Fundam. Appl. Toxicol.* **31**, 192-199.

Perocco, P., and Prodi, G. (1981). DNA damage by haloalkanes in human lymphocytes cultured in vitro. *Cancer Lett.* **13**, 213-218.

Peters, J.M., Cattley, R.C., and Gonzalez, F.J. (1997). Role of PPAR alpha in the mechanism of action of the nongenotoxic carcinogen and peroxisome proliferator Wy-14,643. *Carcinogenesis* **18**, 2029-2033.

Piegorsch, W.W., and Bailer, A.J. (1997). *Statistics for Enviromental Biology and Toxicology*, Section 6.3.2. Chapman and Hall, London.

Portier, C.J., and Bailer, A.J. (1989). Testing for increased carcinogenicity using a survival-adjusted quantal response test. *Fundam. Appl. Toxicol.* **12**, 731-737.

Portier, C.J., Hedges, J.C., and Hoel, D.G. (1986). Age-specific models of mortality and tumor onset for historical control animals in the National Toxicology Program's carcinogenicity experiments. *Cancer Res.* **46**, 4372-4378.

Prout, M.S., Provan, W.M., and Green, T. (1985). Species differences in response to trichloroethylene. I. Pharmacokinetics in rats and mice. *Toxicol. Appl. Pharmacol.* **79**, 389-400.

Rao, G.N., Haseman, J.K., Grumbein, S., Crawford, D.D., and Eustis, S.L. (1990). Growth, body weight, survival, and tumor trends in (C57BL/6 × C3H/HeN) F1 (B6C3F1) mice during a nine-year period. *Toxicol. Pathol.* **18**, 71-77.

Rao, M.S., and Subbarao, V. (1997). Effect of dexamethasone on ciprofibrate-induced cell proliferation and peroxisome proliferation. *Fundam. Appl. Toxicol.* **35**, 78-83.

Reimche, L.D., Sankaran, K., Hindmarsh, K.W., Kasian, G.F., Gorecki, D.K.J, and Tan, L. (1989). Chloral hydrate sedation in neonates and infants – Clinical and pharmacologic considerations. *Dev. Pharmacol. Ther.* **12**, 57-64.

Rijsinghani, K.S., Abrahams, C., Swerdlow, M.A., Rao, K.V.N., and Ghose, T. (1986). Induction of neoplastic lesions in the livers of $C_{57}BL \times C_3HF_1$ mice by chloral hydrate. *Cancer Detect. Prev.* **9**, 279-288.

Roe, F.J.C. (1981). Are nutritionists worried about the epidemic of tumours in laboratory animals? *Proc. Nutr. Soc.* **40**, 57-65.

Roe, F.J.C. (1993). What does carcinogenicity mean and how should we test for it? *Food Chem. Toxicol.* **31**, 225-229.

Roe, F.J.C., and Salaman, M.H. (1955). Further studies on incomplete carcinogenesis: Triethylene melamine (T.E.M.), 1,2-benzanthracene and β-propiolactone, as initiators of skin tumor formation in the mouse. *Br. J. Cancer* **9**, 177-203.

Russo, A., and Levis, A.G. (1992). Detection of aneuploidy in male germ cells of mice by means of a meiotic micronucleus assay. *Mutat. Res.* **281**, 187-191.

Russo, A., Pacchierotti, F., and Metalli, P. (1984). Nondisjunction induced in mouse spermatogenesis by chloral hydrate, a metabolite of trichloethylene. *Environ. Mutagen.* **6**, 695-703.

Saillenfait, A.M., Langonné, I., and Sabaté, J.P. (1995). Developmental toxicity of trichloroethylene, tetrachloroethylene and four of their metabolites in rat whole embryo culture. *Arch. Toxicol.* **70**, 71-82.

Sanders, V.M., Kauffmann, B.M., White, K.L., Jr., Douglas, K.A., Barnes, D.W., Sain, L.E., Bradshaw, T.J., Borzelleca, J.F., and Munson, A.E. (1982). Toxicology of chloral hydrate in the mouse. *Environ. Health Perspect.* **44**, 137-146.

Sbrana, I., Di Sibio, A., Lomi, A., and Scarcelli, V. (1993). C-Mitosis and numerical chromosome aberration analyses in human lymphocytes: 10 known or suspected spindle poisons. *Mutat. Res.* **287**, 57-70.

Seilkop, S.K. (1995). The effect of body weight on tumor incidence and carcinogenicity testing in B6C3F_1 mice and F344 rats. *Fundam. Appl. Toxicol.* **24**, 247-259.

Sheldon, W.G., Bucci, T.J., Hart, R.W., and Turturro, A. (1995). Age-related neoplasia in a lifetime study of *ad libitum*-fed and food-restricted B6C3F1 mice. *Toxicol. Pathol.* **23**, 458-476.

Silverman, J., and Muir, W.W. (1993). A review of laboratory animal anesthesia with chloral hydrate and chloralose. *Lab. Anim. Sci.* **43**, 210-216.

Smith, M.K., Randall, J.L., Read, E.J. and Stober, J.A. (1989). Teratogenic activity of trichloroacetic acid in the rat. *Teratology* **40**, 445-451.

Smith, M.T. (1990). Chloral hydrate warning. *Science* **250**, 359.

Sora, S., and Agostini Carbone, M.L. (1987). Chloral hydrate, methylmercury hydroxide and ethydium bromide affect chromosomal segregation during meiosis of *Saccharomyces cerevisiae*. *Mutat. Res.* **190**, 13-17.

Sourkes, T.L. (1992). Early clinical neurochemistry of CNS-active drugs: Chloral hydrate. *Mol. Chem. Neuropathol.* **17**, 21-30.

Stauber, A.J., and Bull, R.J. (1997). Differences in phenotype and cell replicative behavior of hepatic tumors induced by dichloroacetate (DCA) and trichloroacetate (TCA). *Toxicol. Appl. Pharmacol.* **144**, 235-246.

Steel-Goodwin, L., Pravecek, T.L., and Carmichael, A.J. (1996). Trichloroethylene metabolism *in vitro*: An EPR/SPIN trapping study. *Hum. Exp. Toxicol.* **15**, 878-884.

Steineger, H.H., Sørensen, H.N., Tugwood, J.D., Skrede, S., Spydevold, Ø., and Gautvik, K.M. (1994). Dexamethasone and insulin demonstrate marked and opposite regulation of the steady-state mRNA level of the peroxisomal proliferator-activated receptor (PPAR) in hepatic cells. Hormonal modulation of fatty-acid-induced transcription. *Eur. J. Biochem.* **225**, 967-974.

Stenner, R.D., Merdink, J.L., Stevens, D.K., Springer, D.L., and Bull, R.J. (1997). Enterohepatic recirculation of trichloroethanol glucuronide as a significant source of trichloroacetic acid: Metabolites of trichloroethylene. *Drug Metab. Dispos.* **25**, 529-535.

Stenner, R.D., Merdink, J.L., Fisher, J.W., and Bull, R.J. (1998). Physiologically-based pharmacokinetic model for trichloroethylene considering enterohepatic recirculation of major metabolites. *Risk Anal.* **18**, 261-269.

Sterchele, P.F., Sun, H., Peterson, R.E., and Vanden Heuvel, J.P. (1996). Regulation of peroxisome proliferator-activated receptor-α mRNA in rat liver. *Arch. Biochem. Biophys.* **326**, 281-289.

Stott, W.T., Quast, J.F., and Watanabe, P.G. (1982). The pharmacokinetics and macromolecular interactions of trichloroethylene in mice and rats. *Toxicol. Appl. Pharmacol.* **62**, 137-151.

Tabakoff, B., Vugrincic, C., Anderson, R., and Alivisatos, S.G.A. (1974). Reduction of chloral hydrate to trichloroethanol in brain extracts. *Biochem. Pharmacol.* **23**, 455-460.

Tao, L., Li, K., Kramer, P.M., and Pereira, M.A. (1996). Loss of heterozygosity on chromosome 6 in dichloroacetic acid and trichloroacetic acid-induced liver tumors in female B6C3F1 mice. *Cancer Lett.* **108**, 257-261.

Tarone, R.E. (1975). Tests for trend in life table analysis. *Biometrika* **62**, 679-682.

Toxopeus, C., and Frazier, J.M. (1998). Kinetics of trichloroacetic acid and dichloroacetic acid in the isolated perfused rat liver. *Toxicol. Appl. Pharmacol.* **152**, 90-98.

Turturro, A., Duffy, P.H., and Hart, R.W. (1993). Modulation of toxicity by diet and dietary macronutrient restriction. *Mutat. Res.* **295**, 151-164.

Turturro, A., Duffy, P., Hart, R.W., and Allaben, W.T. (1996). Rational use of dietary control in toxicity studies – B6C3F1 mouse. *Toxicol. Pathol.* **24**, 769-775.

Uehleke, H., and Poplawski-Tabarelli, S. (1977). Irreversible binding of ^{14}C-labelled trichloroethylene to mice liver constituents in vivo and in vitro. *Arch. Toxicol.* **37**, 289-294.

Vagnarelli, P., De Sario, A., and De Carli, L. (1990). Aneuploidy induced by chloral hydrate detected in human lymphocytes ith the Y97 probe. *Mutagenesis* **5**, 591-592.

Waskell, L. (1978). A study of the mutagenicity of anesthetics and their metabolites. *Mutat. Res.* **57**, 141-153.

Waxman, D.J. (1999). P450 gene induction by structurally diverse xenochemicals: Central role of nuclear receptors CAR, PXR, and PPAR. *Arch. Biochem. Biophys.* **369**, 11-23.

Wehner, J.M., Pounder, J.I., Parham, C., and Collins, A.C. (1992). A recombinant inbred strain analysis of sleep-time responses to several sedative-hypnotics. *Alcohol. Clin. Exp. Res.* **16**, 522-528.

Williams, G.M., Iatropoulos, M.J., Wang, C.X., Ali, N., Rivenson, A., Peterson, L.A., Schulz, C., and Gebhardt, R. (1996). Diethylnitrosamine exposure-responses for DNA damage, centrilobular cytotoxicity, cell proliferation and carcinogenesis in rat liver exhibit some non-linearities. *Carcinogenesis* **17**, 2253-2258.

Witt, W.M., Sheldon, W.G., and Thurman, J.D. (1991). Pathological endpoints in dietary restricted rodents – Fischer 344 rats and B6C3F$_1$ mice. In *Biological Effects of Dietary Restriction* (L. Fishbein, Ed.), pp. 73-86. Springer-Verlag, New York.

Wolff, G.L., Leakey, J.E.A., Bazare, J.J., Harmon, J.R., Webb, P.J., and Law, M.G. (1991). Susceptibility to phenobarbital promotion of hepatotumorigenesis: Correlation with differential expression and induction of hepatic drug metabolizing enzymes in heavy or light male (C3H × VY) F1 hybrid mice. *Carcinogenesis* **12**, 911-915.

Xu, W., and Adler, I.-D. (1990). Clastogenic effects of known and suspect spindle poisons studied by chromosome analysis in mouse bone marrow cells. *Mutagenesis* **5**, 371-374.

Yoon, J.S., Mason, J.M., Valencia, R., Woodruff, R.C., and Zimmering, S. (1985). Chemical mutagenesis testing in *Drosophila*. IV. Results of 45 coded compounds tested for the National Toxicology Program. *Environ. Mutagen.* **7**, 349-367.

Zanelli, U., Puccini, P., Acerbi, D., Ventura, P., and Gervasi, P.G. (1996). Induction of peroxisomal β-oxidation and P-450 4A-dependent activities by pivalic and trichloroacetic acid in rat liver and kidney. *Arch. Toxicol.* **70**, 145-149.

Zimmermann, T., Wehling, M., and Schulz, H.-U. (1998). Untersuchungen zur relativen Bioverfügbarkeit und Pharmakokinetik von Chloralhydrat und seinen Metaboliten. *Arzneimittelforschung* **48**, 5-12.

APPENDIX A
SUMMARY OF LESIONS
IN *AD LIBITUM*-FED MALE MICE
IN THE 2-YEAR GAVAGE STUDY
OF CHLORAL HYDRATE

TABLE A1
Summary of the Incidence of Neoplasms in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
15-Month interim evaluation	12	12	12	12
Early deaths				
Moribund		4	2	1
Natural deaths	6	7	10	3
Survivors				
Terminal sacrifice	42	37	36	44
Animals examined microscopically	60	60	60	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(12)	(12)	(12)	(12)
Hepatocellular adenoma, single	2 (17%)	2 (17%)	2 (17%)	1 (8%)
Hepatocellular adenoma, multiple			1 (8%)	
Hepatocellular carcinoma, single		1 (8%)		2 (17%)
Lymphoma malignant	1 (8%)			
2-Year Study				
Alimentary System				
Gallbladder	(43)	(10)	(10)	(44)
Hepatocellular carcinoma, metastatic, liver				1 (2%)
Lymphoma malignant	2 (5%)			
Intestine large, cecum	(41)	(5)	(4)	(46)
Lymphoma malignant				1 (2%)
Intestine small	(42)	(7)	(5)	(46)
Lymphoma malignant				1 (2%)
Intestine small, duodenum	(41)	(7)	(4)	(46)
Lymphoma malignant	2 (5%)	1 (14%)		
Intestine small, ileum	(42)	(5)	(5)	(46)
Adenocarcinoma				1 (2%)
Histiocytic sarcoma		1 (20%)		
Lymphoma malignant	2 (5%)		1 (20%)	2 (4%)
Intestine small, jejunum	(42)	(5)	(4)	(46)
Lymphoma malignant	2 (5%)			
Liver	(48)	(48)	(47)	(48)
Carcinoma, bile duct			1 (2%)	
Hemangioma		1 (2%)		
Hepatocellular adenoma, multiple	2 (4%)	8 (17%)	8 (17%)	7 (15%)
Hepatocellular adenoma, single	10 (21%)	11 (23%)	9 (19%)	10 (21%)
Hepatocellular carcinoma		1 (2%)		
Hepatocellular carcinoma, multiple	1 (2%)		3 (6%)	1 (2%)
Hepatocellular carcinoma, single	3 (6%)	9 (19%)	7 (15%)	6 (13%)
Histiocytic sarcoma				1 (2%)
Lymphoma malignant	2 (4%)		2 (4%)	

TABLE A1
Summary of the Incidence of Neoplasms in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Alimentary System (continued)				
Mesentery			(1)	
Lymphoma malignant			1 (100%)	
Pancreas	(46)	(10)	(9)	(48)
Carcinoma, duct		1 (10%)		
Histiocytic sarcoma		1 (10%)		
Lymphoma malignant	1 (2%)		1 (11%)	
Neoplasm NOS			1 (11%)	
Salivary glands	(48)	(10)	(10)	(48)
Lymphoma malignant	1 (2%)	1 (10%)		1 (2%)
Stomach, glandular	(43)	(10)	(10)	(46)
Carcinoma, metastatic, liver			1 (10%)	
Cardiovascular System				
Blood vessel	(48)	(11)	(11)	(48)
Carcinoma, metastatic, pancreas		1 (9%)		
Hepatocellular carcinoma, metastatic, aorta, liver				1 (2%)
Lymphoma malignant	1 (2%)		1 (9%)	
Heart	(48)	(11)	(11)	(48)
Carcinoma, metastatic, liver			1 (9%)	
Carcinoma, metastatic, pancreas		1 (9%)		
Hepatocellular carcinoma, metastatic, liver				2 (4%)
Lymphoma malignant	1 (2%)		1 (9%)	
Endocrine System				
Adrenal gland	(45)	(11)	(10)	(46)
Carcinoma, metastatic, liver			1 (10%)	
Adrenal gland, cortex	(45)	(11)	(10)	(46)
Lymphoma malignant	2 (4%)	1 (9%)		
Adrenal gland, medulla	(45)	(11)	(10)	(45)
Lymphoma malignant	1 (2%)			
Islets, pancreatic	(48)	(10)	(11)	(48)
Carcinoma	1 (2%)			
Parathyroid gland	(47)	(6)	(6)	(42)
Lymphoma malignant	1 (2%)			
Pituitary gland	(42)	(10)	(9)	(46)
Adenoma, pars distalis			1 (11%)	
Thyroid gland	(47)	(11)	(10)	(48)
Adenoma, follicular cell	1 (2%)			
Lymphoma malignant	1 (2%)			
General Body System				
Tissue NOS				(2)
Hemangiosarcoma				1 (50%)
Hepatocellular carcinoma, metastatic, mediastinum, liver				1 (50%)

TABLE A1
Summary of the Incidence of Neoplasms in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Genital System				
Coagulating gland	(46)	(10)	(10)	(48)
Carcinoma, metastatic, liver			1 (10%)	
Lymphoma malignant		1 (10%)	1 (10%)	
Epididymis	(46)	(10)	(11)	(48)
Carcinoma, metastatic, liver			1 (9%)	
Histiocytic sarcoma			1 (9%)	
Lymphoma malignant	1 (2%)	1 (10%)	2 (18%)	
Lymphoma malignant, unilateral				1 (2%)
Schwannoma benign				1 (2%)
Preputial gland	(46)	(10)	(11)	(48)
Lymphoma malignant	1 (2%)			
Prostate	(46)	(10)	(11)	(48)
Hepatocellular carcinoma, metastatic, liver				1 (2%)
Lymphoma malignant	1 (2%)	1 (10%)	2 (18%)	
Seminal vesicle	(46)	(11)	(14)	(48)
Carcinoma, metastatic, liver			1 (7%)	
Lymphoma malignant	1 (2%)	1 (9%)	2 (14%)	
Testes	(45)	(11)	(10)	(48)
Adenoma, interstitial cell	1 (2%)			1 (2%)
Lymphoma malignant			1 (10%)	
Hematopoietic System				
Bone marrow	(47)	(10)	(10)	(48)
Hemangiosarcoma, metastatic, spleen	1 (2%)			
Lymphoma malignant	1 (2%)			
Lymph node	(48)	(15)	(17)	(48)
Histiocytic sarcoma, bronchial		1 (7%)		
Histiocytic sarcoma, renal		1 (7%)		
Lymphoma malignant, axillary	1 (2%)			
Lymphoma malignant, inguinal				1 (2%)
Lymphoma malignant, lumbar	1 (2%)	1 (7%)		
Lymphoma malignant, renal	2 (4%)			
Sarcoma, metastatic, uncertain primary site			1 (6%)	
Lymph node, mandibular	(48)	(10)	(9)	(45)
Lymphoma malignant	2 (4%)		3 (33%)	3 (7%)
Lymph node, mesenteric	(47)	(13)	(16)	(48)
Carcinoma, metastatic, liver			1 (6%)	
Carcinoma, metastatic, pancreas		1 (8%)		
Hemangiosarcoma				1 (2%)
Hemangiosarcoma, metastatic, spleen	1 (2%)			
Histiocytic sarcoma		2 (15%)	1 (6%)	
Lymphoma malignant	4 (9%)	2 (15%)	4 (25%)	4 (8%)
Spleen	(47)	(16)	(17)	(48)
Hemangiosarcoma	1 (2%)		2 (12%)	2 (4%)
Histiocytic sarcoma		1 (6%)		
Lymphoma malignant	5 (11%)	2 (13%)	3 (18%)	3 (6%)
Thymus	(29)	(6)	(4)	(35)
Carcinoma, metastatic, liver			1 (25%)	
Hepatocellular carcinoma, metastatic, liver				1 (3%)
Lymphoma malignant	2 (7%)	1 (17%)	2 (50%)	2 (6%)

TABLE A1
Summary of the Incidence of Neoplasms in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Integumentary System				
Mammary gland	(5)			(2)
Lymphoma malignant	1 (20%)			
Skin	(48)	(10)	(10)	(48)
Fibroma	1 (2%)			
Keratoacanthoma				1 (2%)
Musculoskeletal System				
Bone, femur	(46)	(11)	(12)	(48)
Lymphoma malignant	1 (2%)			
Bone, sternum	(47)	(11)	(12)	(48)
Hemangiosarcoma				1 (2%)
Lymphoma malignant	1 (2%)			
Skeletal muscle	(47)	(10)	(11)	(48)
Carcinoma, metastatic, liver			1 (9%)	
Lymphoma malignant			1 (9%)	1 (2%)
Rhabdomyosarcoma			1 (9%)	
Nervous System				
Brain, cerebrum	(47)	(10)	(9)	(48)
Lymphoma malignant			1 (11%)	
Respiratory System				
Larynx	(46)	(11)	(11)	(47)
Lymphoma malignant	1 (2%)			
Lung	(48)	(20)	(16)	(48)
Alveolar/bronchiolar adenoma	12 (25%)	6 (30%)	3 (19%)	9 (19%)
Alveolar/bronchiolar adenoma, multiple	1 (2%)	2 (10%)	1 (6%)	2 (4%)
Alveolar/bronchiolar carcinoma	2 (4%)	4 (20%)	1 (6%)	3 (6%)
Carcinoma, metastatic, liver			1 (6%)	
Carcinoma, metastatic, pancreas		1 (5%)		
Hepatocellular carcinoma, metastatic, liver	1 (2%)			2 (4%)
Hepatocellular carcinoma, metastatic, multiple, liver			2 (13%)	
Lymphoma malignant	2 (4%)	1 (5%)	2 (13%)	2 (4%)
Nose	(47)	(11)	(12)	(48)
Carcinoma			1 (8%)	
Lymphoma malignant	1 (2%)			
Special Senses System				
Eye	(46)	(11)	(11)	(48)
Lymphoma malignant, unilateral				1 (2%)
Harderian gland	(47)	(16)	(12)	(47)
Adenoma	5 (11%)	6 (38%)	2 (17%)	5 (11%)
Carcinoma		1 (6%)		
Lymphoma malignant	2 (4%)			
Lymphoma malignant, bilateral			1 (8%)	
Lacrimal gland	(47)	(10)	(10)	(46)
Lymphoma malignant		1 (10%)		

TABLE A1
Summary of the Incidence of Neoplasms in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Urinary System				
Kidney	(46)	(10)	(10)	(48)
Carcinoma, metastatic, pancreas		1 (10%)		
Lymphoma malignant	2 (4%)		2 (20%)	1 (2%)
Urinary bladder	(46)	(10)	(11)	(48)
Lymphoma malignant	2 (4%)		1 (9%)	1 (2%)
Neoplasm Summary				
Total animals with primary neoplasms[b]				
15-Month interim evaluation	3	3	3	3
2-Year study	34	36	33	35
Total primary neoplasms				
15-Month interim evaluation	3	3	3	3
2-Year study	95	72	77	78
Total animals with benign neoplasms				
15-Month interim evaluation	2	2	3	1
2-Year study	27	27	19	25
Total benign neoplasms				
15-Month interim evaluation	2	2	3	1
2-Year study	33	34	24	36
Total animals with malignant neoplasms				
15-Month interim evaluation	1	1		2
2-Year study	12	19	20	17
Total malignant neoplasms				
15-Month interim evaluation	1	1		2
2-Year study	62	38	53	42
Total animals with metastatic neoplasms				
2-Year study	2	1	4	2
Total metastatic neoplasms				
2-Year study	3	5	13	9
Total animals with uncertain neoplasms-benign or malignant				
2-Year study			1	
Total uncertain neoplasms				
2-Year study			1	

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A2
Statistical Analysis of Primary Neoplasms in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Harderian Gland: Adenoma				
Overall rate[a]	5/47 (11%)	6/16 (38%)	2/12 (17%)	5/47 (11%)
Adjusted rate[b]	10.9%	42.4%	20.8%	10.9%
Terminal rate[c]	5/41 (12%)	5/6 (83%)	1/1 (100%)	5/44 (11%)
First incidence (days)	757 (T)	666	738	757 (T)
Poly-3 test[d]	NA	—[e]	—	P=0.6264N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	5/47 (11%)	7/16 (44%)	2/12 (17%)	5/47 (11%)
Adjusted rate	10.9%	49.5%	20.8%	10.9%
Terminal rate	5/41 (12%)	6/6 (100%)	1/1 (100%)	5/44 (11%)
First incidence (days)	757 (T)	666	738	757 (T)
Poly-3 test	NA	—	—	P=0.6264N
Liver: Hepatocellular Adenoma				
Overall rate	12/48 (25%)	19/48 (40%)	17/47 (36%)	17/48 (35%)
Adjusted rate	25.2%	40.8%	37.8%	36.2%
Terminal rate	9/41 (22%)	14/37 (38%)	15/36 (42%)	16/44 (36%)
First incidence (days)	511	639	668	713
Poly-3 test	P=0.2362	P=0.0792	P=0.1373	P=0.1722
Liver: Hepatocellular Carcinoma				
Overall rate	4/48 (8%)	10/48 (21%)	10/47 (21%)	7/48 (15%)
Adjusted rate	8.5%	21.4%	22.0%	14.7%
Terminal rate	2/41 (5%)	5/37 (14%)	5/36 (14%)	4/44 (9%)
First incidence (days)	689	666	668	629
Poly-3 test	P=0.3737	P=0.0716	P=0.0631	P=0.2713
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	16/48 (33%)	25/48 (52%)	23/47 (49%)	22/48 (46%)
Adjusted rate	33.4%	52.6%	50.6%	46.2%
Terminal rate	11/41 (27%)	16/37 (43%)	17/36 (47%)	19/44 (43%)
First incidence (days)	511	639	668	629
Poly-3 test	P=0.2154	P=0.0437	P=0.0684	P=0.1430
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	13/48 (27%)	8/20 (40%)	4/16 (25%)	11/48 (23%)
Adjusted rate	27.8%	45.0%	29.4%	23.4%
Terminal rate	12/41 (29%)	7/9 (78%)	3/5 (60%)	10/44 (23%)
First incidence (days)	709	736	738	662
Poly-3 test	NA	—	—	P=0.4012N
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	2/48 (4%)	4/20 (20%)	1/16 (6%)	3/48 (6%)
Adjusted rate	4.3%	21.7%	7.4%	6.4%
Terminal rate	2/41 (5%)	2/9 (22%)	1/5 (20%)	3/44 (7%)
First incidence (days)	757 (T)	639	757 (T)	757 (T)
Poly-3 test	NA	—	—	P=0.5010

TABLE A2
Statistical Analysis of Primary Neoplasms in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	15/48 (31%)	11/20 (55%)	5/16 (31%)	14/48 (29%)
Adjusted rate	32.0%	59.3%	36.7%	29.7%
Terminal rate	14/41 (34%)	8/9 (89%)	4/5 (80%)	13/44 (30%)
First incidence (days)	709	639	738	662
Poly-3 test	NA	—	—	P=0.4930N
All Organs: Malignant Lymphoma				
Overall rate	5/48 (10%)	3/48 (6%)	5/48 (10.4%)	5/48 (10%)
Adjusted rate	10.7%	6.6%	10.6%	10.7%
Terminal rate	3/41 (7%)	2/37 (5%)	2/36 (5.6%)	4/44 (9.1%)
First incidence (days)	744	736	353	713
Poly-3 test	P=0.4761	P=0.3674N	P=0.6262N	P=0.6269N
All Organs: Benign Neoplasms				
Overall rate	27/48 (56%)	27/48 (56%)	20/48 (42%)[f]	25/48 (52%)
Adjusted rate	56.6%	57.6%	43.6%	52.9%
Terminal rate	24/41 (59%)	21/37 (57%)	17/36 (47%)	23/44 (52%)
First incidence (days)	511	639	668	662
Poly-3 test	P=0.3184N	P=0.5417	P=0.1461N	P=0.4394N
All Organs: Malignant Neoplasms				
Overall rate	12/48 (25%)	19/48 (40%)	20/48 (42%)	17/48 (35%)
Adjusted rate	25.5%	39.7%	41.7%	35.4%
Terminal rate	7/41 (17%)	9/37 (24%)	9/36 (25%)	13/44 (30%)
First incidence (days)	689	639	353	629
Poly-3 test	P=0.2596	P=0.1034	P=0.0715	P=0.2037
All Organs: Benign or Malignant Neoplasms				
Overall rate	34/48 (71%)	36/48 (75%)	33/48 (69%)	35/48 (73%)
Adjusted rate	70.8%	75.0%	68.8%	72.9%
Terminal rate	27/41 (66%)	25/37 (68%)	22/36 (61%)	31/44 (71%)
First incidence (days)	511	639	353	629
Poly-3 test	P=0.5213	P=0.4100	P=0.5012N	P=0.5000

(T) Terminal sacrifice
NA Not applicable
[a] Number of neoplasm-bearing animals/number of animals with tissue examined microscopically
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in a dose group is indicated by **N**.
[e] Tissue was examined microscopically only when it was observed to be abnormal at necropsy; thus, statistical comparisons with the vehicle controls are not appropriate.
[f] Includes one animal with a neoplasm of uncertain malignancy

TABLE A3
Historical Incidence of Hepatocellular Neoplasms in Control Male B6C3F$_1$/Nctr BR Mice[a]

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Doxylamine	6/48	4/48	9/48
Fumonisin B_1	9/47	4/47	12/47
Pyrilamine	10/46	3/46	13/46
Sulfamethazine	25/185	20/185	42/185
Triprolidine	9/48	5/48	13/48
Total (%)	59/374 (15.8%)	36/374 (9.6%)	89/374 (23.8%)
Mean ± standard deviation	17.1% ± 4.0%	8.9% ± 1.7%	24.5% ± 3.8%
Range	13%-22%	7%-11%	19%-28%

[a] Data as of September 1999. Studies conducted at the National Center for Toxicological Research in animals given NIH-31 diet

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
15-Month interim evaluation	12	12	12	12
Early deaths				
Moribund		4	2	1
Natural deaths	6	7	10	3
Survivors				
Terminal sacrifice	42	37	36	44
Animals examined microscopically	60	60	60	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(12)	(12)	(12)	(12)
Fatty change, focal, peripheral	2 (17%)	7 (58%)		
Fatty change, peripheral			7 (58%)	7 (58%)
Granuloma, multiple	3 (25%)			
Vacuolization cytoplasmic, centrilobular	9 (75%)	5 (42%)	6 (50%)	4 (33%)
2-Year Study				
Alimentary System				
Esophagus	(47)	(11)	(11)	(48)
Autolysis	4 (9%)		1 (9%)	
Polyarteritis				1 (2%)
Gallbladder	(43)	(10)	(10)	(44)
Autolysis	2 (5%)	4 (40%)	6 (60%)	1 (2%)
Crystals, epithelium	1 (2%)			
Cyst			1 (10%)	
Fibrosis				2 (5%)
Inflammation, chronic, serosa				1 (2%)
Intestine large, cecum	(41)	(5)	(4)	(46)
Autolysis				1 (2%)
Hyperplasia, lymphoid				1 (2%)
Polyarteritis				1 (2%)
Intestine large, colon	(41)	(5)	(4)	(46)
Autolysis				1 (2%)
Polyarteritis				1 (2%)
Intestine large, rectum	(41)	(5)	(4)	(43)
Granuloma	1 (2%)			
Polyarteritis				1 (2%)
Intestine small, duodenum	(41)	(7)	(4)	(46)
Autolysis		1 (14%)		1 (2%)
Intestine small, ileum	(42)	(5)	(5)	(46)
Autolysis				1 (2%)
Intestine small, jejunum	(42)	(5)	(4)	(46)
Autolysis				1 (2%)

[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 *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Alimentary System (continued)				
Liver	(48)	(48)	(47)	(48)
Autolysis	3 (6%)	2 (4%)		1 (2%)
Basophilic focus	3 (6%)	3 (6%)	1 (2%)	5 (10%)
Clear cell focus	1 (2%)	1 (2%)	3 (6%)	2 (4%)
Clear cell focus, multiple	1 (2%)		2 (4%)	1 (2%)
Congestion			1 (2%)	
Eosinophilic focus		1 (2%)	1 (2%)	1 (2%)
Fatty change, focal		1 (2%)		
Fibrosis, focal			1 (2%)	
Granuloma, multiple	1 (2%)		1 (2%)	
Hyperplasia, bile duct		1 (2%)		
Infarct		2 (4%)		2 (4%)
Infiltration cellular, lymphocytic	2 (4%)		1 (2%)	2 (4%)
Mixed cell focus		1 (2%)		
Necrosis, focal, hepatocyte			1 (2%)	
Necrosis, hepatocyte	1 (2%)	1 (2%)		
Necrosis, multifocal, hepatocyte	1 (2%)			
Regeneration, focal		1 (2%)		
Vacuolization cytoplasmic, centrilobular	2 (4%)	1 (2%)	2 (4%)	4 (8%)
Pancreas	(46)	(10)	(9)	(48)
Atrophy, acinar cell		1 (11%)		
Autolysis	4 (9%)		2 (22%)	1 (2%)
Cyst, duct		1 (10%)		
Fibrosis		1 (10%)		
Polyarteritis				1 (2%)
Salivary glands	(48)	(10)	(10)	(48)
Autolysis	5 (10%)			
Degeneration	1 (2%)			
Infiltration cellular, lymphocytic	9 (19%)	2 (20%)		10 (21%)
Stomach, forestomach	(43)	(10)	(10)	(46)
Autolysis	1 (2%)		1 (10%)	
Hyperkeratosis, focal	1 (2%)			
Hyperplasia, squamous	1 (2%)			
Stomach, glandular	(43)	(10)	(10)	(46)
Autolysis	2 (5%)	4 (40%)	5 (50%)	
Inflammation, acute, submucosa	1 (2%)			
Inflammation, chronic, focal				1 (2%)
Polyarteritis				1 (2%)
Tongue	(47)	(10)	(11)	(47)
Autolysis	4 (9%)		1 (9%)	
Polyarteritis				1 (2%)
Cardiovascular System				
Blood vessel	(48)	(11)	(11)	(48)
Autolysis, aorta	3 (6%)			

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Cardiovascular System (continued)				
Heart	(48)	(11)	(11)	(48)
Autolysis	3 (6%)		1 (9%)	
Cardiomyopathy	1 (2%)			1 (2%)
Congestion			1 (9%)	
Hemorrhage, myocardium		1 (9%)		
Polyarteritis				1 (2%)
Endocrine System				
Adrenal gland, cortex	(45)	(11)	(10)	(46)
Autolysis	4 (9%)	2 (18%)	2 (20%)	1 (2%)
Cyst, unilateral	1 (2%)			1 (2%)
Cytologic alterations	1 (2%)			
Cytoplasmic alteration	4 (9%)	2 (18%)		5 (11%)
Degeneration, focal, unilateral				1 (2%)
Hyperplasia	6 (13%)		1 (10%)	4 (9%)
Hyperplasia, spindle cell	24 (53%)	7 (64%)	3 (30%)	27 (59%)
Hypertrophy	2 (4%)			
Adrenal gland, medulla	(45)	(11)	(10)	(45)
Autolysis	3 (7%)		1 (10%)	1 (2%)
Hyperplasia				1 (2%)
Parathyroid gland	(47)	(6)	(6)	(42)
Autolysis	4 (9%)		1 (17%)	
Cyst	1 (2%)			
Pituitary gland	(42)	(10)	(9)	(46)
Autolysis	3 (7%)	2 (20%)	1 (11%)	1 (2%)
Cyst	2 (5%)			1 (2%)
Hyperplasia, focal, pars distalis	1 (2%)			
Thyroid gland	(47)	(11)	(10)	(48)
Autolysis	4 (9%)		3 (30%)	
Cyst, follicle	5 (11%)	1 (9%)		3 (6%)
Polyarteritis				1 (2%)
General Body System				
None				
Genital System				
Coagulating gland	(46)	(10)	(10)	(48)
Autolysis	3 (7%)	1 (10%)	2 (20%)	
Distended			1 (10%)	1 (2%)
Inflammation, chronic active			1 (10%)	
Epididymis	(46)	(10)	(11)	(48)
Autolysis	3 (7%)	1 (10%)	2 (18%)	1 (2%)
Granuloma sperm	1 (2%)			2 (4%)
Granuloma, unilateral				1 (2%)
Infiltration cellular, lymphocytic				1 (2%)
Inflammation, chronic active			1 (9%)	
Inflammation, chronic	1 (2%)			
Polyarteritis				1 (2%)

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Genital System (continued)				
Penis				(1)
Concretion				1 (100%)
Inflammation, acute				1 (100%)
Preputial gland	(46)	(10)	(11)	(48)
Abscess		1 (10%)		
Atrophy	15 (33%)	4 (40%)	6 (55%)	21 (44%)
Autolysis	3 (7%)		1 (9%)	
Cyst	1 (2%)		1 (9%)	3 (6%)
Dilatation	1 (2%)			1 (2%)
Ectasia, duct		2 (20%)	5 (45%)	2 (4%)
Inflammation, acute, unilateral				1 (2%)
Inflammation, chronic, unilateral	1 (2%)			
Inflammation	1 (2%)			
Prostate	(46)	(10)	(11)	(48)
Atrophy	2 (4%)			3 (6%)
Autolysis	3 (7%)		3 (27%)	
Congestion			1 (9%)	
Ectasia, focal			1 (9%)	
Inflammation, acute				1 (2%)
Inflammation, focal				1 (2%)
Polyarteritis				1 (2%)
Seminal vesicle	(46)	(11)	(14)	(48)
Atrophy	1 (2%)			2 (4%)
Autolysis	3 (7%)	1 (9%)	2 (14%)	1 (2%)
Depletion			1 (7%)	
Dilatation	1 (2%)			1 (2%)
Distended		1 (9%)	3 (21%)	
Inflammation, chronic active			1 (7%)	
Inflammation, chronic				1 (2%)
Polyarteritis, unilateral				1 (2%)
Testes	(45)	(11)	(10)	(48)
Atrophy, unilateral	1 (2%)			
Autolysis	3 (7%)	1 (9%)	3 (30%)	1 (2%)
Degeneration, bilateral	1 (2%)			
Hyperplasia, unilateral, interstitial cell				1 (2%)
Hematopoietic System				
Bone marrow	(47)	(10)	(10)	(48)
Autolysis	3 (6%)			
Hyperplasia	1 (2%)	2 (20%)	2 (20%)	4 (8%)
Lymph node	(48)	(15)	(17)	(48)
Granuloma, inguinal	1 (2%)			
Lymph node, mandibular	(48)	(10)	(9)	(45)
Autolysis	4 (8%)			
Hyperplasia				1 (2%)
Inflammation, chronic		1 (10%)		

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Hematopoietic System (continued)				
Lymph node, mesenteric	(47)	(13)	(16)	(48)
Angiectasis, focal			1 (6%)	
Autolysis	5 (11%)			
Congestion			1 (6%)	1 (2%)
Degeneration	4 (9%)	4 (31%)	5 (31%)	5 (10%)
Granuloma			1 (6%)	
Hematopoietic cell proliferation	1 (2%)			
Hemorrhage	1 (2%)			1 (2%)
Hyperplasia	1 (2%)		1 (6%)	4 (8%)
Polyarteritis, artery				1 (2%)
Spleen	(47)	(16)	(17)	(48)
Angiectasis, focal			1 (6%)	
Apoptosis, lymphocyte		1 (6%)		
Atrophy, lymphocyte		3 (19%)	1 (6%)	2 (4%)
Autolysis	4 (9%)		2 (12%)	
Congestion		1 (6%)		
Hematopoietic cell proliferation	2 (4%)	7 (44%)	2 (12%)	7 (15%)
Hyperplasia, lymphoid	3 (6%)		3 (18%)	1 (2%)
Thymus	(29)	(6)	(4)	(35)
Autolysis	3 (10%)			1 (3%)
Ectopic parathyroid gland		1 (17%)		
Integumentary System				
Mammary gland	(5)			(2)
Autolysis	2 (40%)			
Skin	(48)	(10)	(10)	(48)
Autolysis	4 (8%)		1 (10%)	
Granuloma	1 (2%)			
Musculoskeletal System				
Bone, femur	(46)	(11)	(12)	(48)
Autolysis	3 (7%)			
Bone, sternum	(47)	(11)	(12)	(48)
Autolysis	3 (6%)			
Fibrous osteodystrophy	4 (9%)			2 (4%)
Polyarteritis				1 (2%)
Skeletal muscle	(47)	(10)	(11)	(48)
Autolysis	4 (9%)		1 (9%)	
Infiltration cellular, lymphocytic				1 (2%)
Mineralization, focal	1 (2%)			
Polyarteritis				2 (4%)
Nervous System				
Brain, cerebellum	(47)	(10)	(9)	(48)
Autolysis	4 (9%)	1 (10%)		1 (2%)
Brain, cerebrum	(47)	(10)	(9)	(48)
Autolysis	4 (9%)	1 (10%)		1 (2%)
Hydrocephalus				1 (2%)
Mineralization, thalamus	25 (53%)	2 (20%)	1 (11%)	14 (29%)

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Nervous System (continued)				
Peripheral nerve	(47)	(10)	(11)	(48)
Autolysis	4 (9%)		1 (9%)	
Degeneration	2 (4%)			1 (2%)
Demyelination	1 (2%)			
Infiltration cellular, lymphocytic	1 (2%)			
Spinal cord	(47)	(10)	(9)	(48)
Autolysis	4 (9%)	1 (10%)		1 (2%)
Respiratory System				
Larynx	(46)	(11)	(11)	(47)
Autolysis	4 (9%)		1 (9%)	
Polyarteritis				1 (2%)
Lung	(48)	(20)	(16)	(48)
Atelectasis			1 (6%)	
Autolysis	3 (6%)		1 (6%)	
Congestion			1 (6%)	
Dilatation, bronchiole, glands	1 (2%)			1 (2%)
Hemorrhage, focal, alveolus			1 (6%)	
Hyperplasia, alveolar epithelium				2 (4%)
Infiltration cellular, lymphocytic	4 (8%)	1 (5%)		2 (4%)
Inflammation, chronic active		1 (5%)		
Polyarteritis				1 (2%)
Nose	(47)	(11)	(12)	(48)
Autolysis	4 (9%)		1 (8%)	
Trachea	(47)	(10)	(11)	(48)
Autolysis	4 (9%)		1 (9%)	
Dilatation, glands	1 (2%)			
Special Senses System				
Eye	(46)	(11)	(11)	(48)
Autolysis	3 (7%)		2 (18%)	
Cataract, lens		1 (9%)		
Polyarteritis				1 (2%)
Harderian gland	(47)	(16)	(12)	(47)
Atrophy, focal, unilateral	1 (2%)			
Autolysis	4 (9%)		1 (8%)	
Hyperplasia, diffuse, unilateral				1 (2%)
Hyperplasia, focal, bilateral				1 (2%)
Hyperplasia, focal, unilateral	1 (2%)			1 (2%)
Hyperplasia, unilateral	1 (2%)			1 (2%)
Hyperplasia, papillary, unilateral				1 (2%)
Infiltration cellular, lymphocytic	5 (11%)	1 (6%)	2 (17%)	
Lacrimal gland	(47)	(10)	(10)	(46)
Atrophy, focal	1 (2%)			
Autolysis	5 (11%)			
Cytoplasmic alteration				1 (2%)
Infiltration cellular, lymphocytic				2 (4%)
Zymbal's gland	(45)	(9)	(7)	(48)
Autolysis	2 (4%)			

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Urinary System				
Kidney	(46)	(10)	(10)	(48)
Accumulation hyaline droplet, renal tubule		2 (20%)		
Angiectasis				1 (2%)
Autolysis	5 (11%)	1 (10%)	4 (40%)	1 (2%)
Congestion			1 (10%)	
Cyst, renal tubule	5 (11%)			3 (6%)
Degeneration, renal tubule	5 (11%)			3 (6%)
Glomerulosclerosis	2 (4%)	4 (40%)	1 (10%)	10 (21%)
Hydronephrosis			2 (20%)	
Infarct, unilateral	2 (4%)	1 (10%)		1 (2%)
Infiltration cellular, lymphocytic			1 (10%)	3 (6%)
Inflammation, chronic	1 (2%)			
Mineralization, renal tubule	4 (9%)	1 (10%)		3 (6%)
Nephropathy	6 (13%)		1 (10%)	3 (6%)
Regeneration, renal tubule	11 (24%)			7 (15%)
Vacuolization cytoplasmic, bilateral, renal tubule	1 (2%)			
Urinary bladder	(46)	(10)	(11)	(48)
Autolysis	3 (7%)		3 (9%)	
Infiltration cellular, lymphocytic	4 (9%)			
Inflammation, acute				1 (2%)

APPENDIX B
SUMMARY OF LESIONS
IN DIETARY-CONTROLLED MALE MICE
IN THE 2-YEAR GAVAGE STUDY
OF CHLORAL HYDRATE

TABLE B1
Summary of the Incidence of Neoplasms in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
15-Month interim evaluation	12	12	12	12
Early deaths				
Moribund	1	1	1	
Natural deaths	2	3		7
Survivors				
Terminal sacrifice	45	44	47	41
Animals examined microscopically	60	60	60	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(12)	(12)	(12)	(12)
2-Year Study				
Alimentary System				
Intestine large, colon	(47)	(4)	(1)	(45)
Histiocytic sarcoma		1 (25%)		
Intestine small, duodenum	(47)	(4)	(1)	(44)
Adenoma	1 (2%)			
Hemangiosarcoma		1 (25%)		
Intestine small, ileum	(48)	(4)	(1)	(44)
Histiocytic sarcoma		1 (25%)		
Lymphoma malignant	1 (2%)			
Intestine small, jejunum	(47)	(5)	(1)	(44)
Lymphoma malignant	1 (2%)	2 (40%)		
Liver	(48)	(48)	(48)	(48)
Hemangiosarcoma				2 (4%)
Hepatocellular adenoma, single	7 (15%)	7 (15%)	10 (21%)	9 (19%)
Hepatocellular adenoma, multiple	2 (4%)			1 (2%)
Hepatocellular carcinoma, single	2 (4%)	3 (6%)	3 (6%)	7 (15%)
Hepatocellular carcinoma, multiple		2 (4%)	1 (2%)	1 (2%)
Histiocytic sarcoma	2 (4%)	1 (2%)		2 (4%)
Leukemia granulocytic		1 (2%)		
Lymphoma malignant	1 (2%)	1 (2%)		2 (4%)
Pancreas	(48)	(4)	(1)	(48)
Lymphoma malignant	1 (2%)			
Salivary glands	(48)	(4)	(1)	(48)
Lymphoma malignant				1 (2%)
Cardiovascular System				
Blood vessel	(48)	(4)	(1)	(48)
Histiocytic sarcoma				1 (2%)
Lymphoma malignant		1 (25%)		1 (2%)
Heart	(48)	(4)	(1)	(48)
Histiocytic sarcoma				1 (2%)
Lymphoma malignant				1 (2%)

TABLE B1
Summary of the Incidence of Neoplasms in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Endocrine System				
Pituitary gland	(43)	(3)	(1)	(38)
Adenoma, pars distalis		1 (33%)		
General Body System				
None				
Genital System				
Epididymis	(48)	(4)	(1)	(48)
Fibroma				1 (2%)
Lymphoma malignant				1 (2%)
Prostate	(47)	(4)	(1)	(46)
Histiocytic sarcoma		1 (25%)		
Lymphoma malignant				1 (2%)
Hematopoietic System				
Bone marrow	(48)	(4)	(1)	(48)
Lymphoma malignant				1 (2%)
Lymph node	(47)	(8)	(3)	(48)
Histiocytic sarcoma, renal		1 (13%)		
Lymphoma malignant, axillary				1 (2%)
Lymphoma malignant, inguinal				1 (2%)
Lymphoma malignant, lumbar	1 (2%)			1 (2%)
Lymphoma malignant, popliteal				1 (2%)
Lymphoma malignant, renal		1 (13%)		1 (2%)
Lymph node, mandibular	(46)	(3)	(1)	(48)
Lymphoma malignant				1 (2%)
Lymph node, mesenteric	(44)	(8)	(3)	(48)
Histiocytic sarcoma		1 (13%)		3 (6%)
Lymphoma malignant	3 (7%)	4 (50%)	1 (33%)	3 (6%)
Spleen	(48)	(8)	(4)	(48)
Hemangiosarcoma	1 (2%)		1 (25%)	1 (2%)
Histiocytic sarcoma				2 (4%)
Leukemia granulocytic		1 (13%)		
Lymphoma malignant	6 (13%)	2 (25%)	1 (25%)	2 (4%)
Thymus	(39)	(2)	(1)	(30)
Lymphoma malignant	1 (3%)	1 (50%)		1 (3%)
Integumentary System				
Skin	(48)	(4)	(1)	(48)
Hemangiosarcoma	1 (2%)			
Lymphoma malignant				1 (2%)
Musculoskeletal System				
Skeletal muscle	(48)	(4)	(1)	(48)
Lymphoma malignant				1 (2%)

TABLE B1
Summary of the Incidence of Neoplasms in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Nervous System				
None				
Respiratory System				
Lung	(48)	(6)	(5)	(48)
Alveolar/bronchiolar adenoma	9 (19%)	1 (17%)	3 (60%)	5 (10%)
Alveolar/bronchiolar carcinoma	2 (4%)			
Carcinoma, metastatic, harderian gland		1 (17%)		1 (2%)
Hepatocellular carcinoma, metastatic, liver				1 (2%)
Histiocytic sarcoma	1 (2%)			1 (2%)
Lymphoma malignant	2 (4%)	1 (17%)		1 (2%)
Special Senses System				
Ear		(1)		
Fibrous histiocytoma		1 (100%)		
Harderian gland	(48)	(8)	(2)	(48)
Adenoma	4 (8%)	3 (38%)	1 (50%)	4 (8%)
Carcinoma		1 (13%)		1 (2%)
Lacrimal gland	(47)	(4)	(1)	(46)
Lymphoma malignant				1 (2%)
Urinary System				
None				
Total animals with primary neoplasms[b]				
2-Year study	27	24	16	29
Total primary neoplasms				
2-Year study	49	41	21	66
Total animals with benign neoplasms				
2-Year study	20	12	12	17
Total benign neoplasms				
2-Year study	23	12	14	20
Total animals with malignant neoplasms				
2-Year study	12	14	5	16
Total malignant neoplasms				
2-Year study	26	29	7	46
Total animals with metastatic neoplasms				
2-Year study		1		
Total metastatic neoplasms				
2-Year study		1		

[a] Number of animals examined microscopically at the site and the number of animals with neoplasm
[b] Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE B2
Statistical Analysis of Primary Neoplasms in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Harderian Gland: Adenoma				
Overall rate[a]	4/48 (8%)	3/8 (38%)	1/2 (50%)	4/48 (8%)
Adjusted rate[b]	8.5%	49.5%	87.1%	8.8%
Terminal rate[c]	4/45 (9%)	3/4 (75%)	1/1 (100%)	3/41 (7%)
First incidence (days)	757 (T)	757 (T)	757 (T)	739
Poly-3 test[d]	NA	—[e]	—	P=0.6243
Harderian Gland: Adenoma or Carcinoma				
Overall rate	4/48 (8%)	4/8 (50%)	1/2 (50%)	5/48 (10%)
Adjusted rate	8.5%	65.9%	87.1%	11.0%
Terminal rate	4/45 (9%)	4/4 (100%)	1/1 (100%)	4/41 (10%)
First incidence (days)	757 (T)	757 (T)	757 (T)	739
Poly-3 test	NA	—	—	P=0.4790
Liver: Hepatocellular Adenoma				
Overall rate	9/48 (19%)	7/48 (15%)	10/48 (21%)	10/48 (21%)
Adjusted rate	19.1%	15.2%	21.2%	21.8%
Terminal rate	9/45 (20%)	7/44 (16%)	10/47 (21%)	9/41 (22%)
First incidence (days)	757 (T)	757 (T)	757 (T)	625
Poly-3 test	P=0.3381	P=0.4111N	P=0.5013	P=0.4753
Liver: Hepatocellular Carcinoma				
Overall rate	2/48 (4%)	5/48 (10%)	4/48 (8%)	8/48 (17%)
Adjusted rate	4.2%	10.9%	8.5%	17.3%
Terminal rate	2/45 (4%)	5/44 (11%)	4/47 (9%)	4/41 (10%)
First incidence (days)	757 (T)	757 (T)	757 (T)	486
Poly-3 test	P=0.0371	P=0.2078	P=0.3382	P=0.0422
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	11/48 (23%)	11/48 (23%)	14/48 (29%)	18/48 (38%)
Adjusted rate	23.4%	23.9%	29.7%	38.6%
Terminal rate	11/45 (24%)	11/44 (25%)	14/47 (30%)	13/41 (32%)
First incidence (days)	757 (T)	757 (T)	757 (T)	486
Poly-3 test	P=0.0450	P=0.5728	P=0.3231	P=0.0844
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	9/48 (19%)	1/6 (17%)	3/5 (60%)	5/48 (10%)
Adjusted rate	19.1%	24.6%	72.3%	11.0%
Terminal rate	9/45 (20%)	1/2 (50%)	3/4 (75%)	5/41 (12%)
First incidence (days)	757 (T)	757 (T)	757 (T)	757 (T)
Poly-3 test	NA	—	—	P=0.2130N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	11/48 (23%)	1/6 (17%)	3/5 (60%)	5/48 (10%)
Adjusted rate	23.4%	24.6%	72.3%	11.0%
Terminal rate	11/45 (24%)	1/2 (50%)	3/4 (75%)	5/41 (12%)
First incidence (days)	757 (T)	757 (T)	757 (T)	757 (T)
Poly-3 test	NA	—	—	P=0.0966N

TABLE B2
Statistical Analysis of Primary Neoplasms in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
All Organs: Histiocytic Sarcoma				
Overall rate	2/48 (4%)	1/48 (2%)	0/48 (0%)	3/48 (6%)
Adjusted rate	4.2%	2.1%	0.0%	6.6%
Terminal rate	1/45 (2%)	0/44 (0%)	0/47 (0%)	3/41 (7%)
First incidence (days)	754	612	—	757 (T)
Poly-3 test	P=0.3319	P=0.5042N	P=0.2371N	P=0.4839
All Organs: Malignant Lymphoma				
Overall rate	6/48 (13%)	4/48 (8%)	1/48 (2%)	4/48 (8%)
Adjusted rate	12.5%	8.6%	2.1%	8.8%
Terminal rate	4/45 (9%)	3/44 (7%)	1/47 (2%)	4/41 (10%)
First incidence (days)	250	645	757 (T)	757 (T)
Poly-3 test	P=0.2895N	P=0.3907N	P=0.0592N	P=0.4044N
All Organs: Benign Neoplasms				
Overall rate	20/48 (42%)	12/48 (25%)	12/48 (25%)	17/48 (35%)
Adjusted rate	42.5%	26.0%	25.5%	37.0%
Terminal rate	20/45 (44%)	12/44 (27%)	12/47 (26%)	15/41 (37%)
First incidence (days)	757 (T)	757 (T)	757 (T)	625
Poly-3 test	P=0.4418N	P=0.0722N	P=0.0622N	P=0.3719N
All Organs: Malignant Neoplasms				
Overall rate	12/48 (25%)	14/48 (29%)	5/48 (10%)	16/48 (33%)
Adjusted rate	25.0%	29.8%	10.6%	34.3%
Terminal rate	9/45 (20%)	11/44 (25%)	5/47 (11%)	11/41 (27%)
First incidence (days)	250	612	757 (T)	486
Poly-3 test	P=0.2713	P=0.3861	P=0.0574N	P=0.2227
All Organs: Benign or Malignant Neoplasms				
Overall rate	27/48 (56%)	24/48 (50%)	16/48 (33%)	29/48 (60%)
Adjusted rate	56.3%	51.0%	33.9%	61.6%
Terminal rate	24/45 (53%)	21/44 (48%)	16/47 (34%)	23/41 (56%)
First incidence (days)	250	612	757 (T)	486
Poly-3 test	P=0.3573	P=0.3821N	P=0.0221N	P=0.3745

(T) Terminal sacrifice
NA Not applicable
[a] Number of neoplasm-bearing animals/number of animals with tissue examined microscopically
[b] Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
[c] Observed incidence at terminal kill
[d] Beneath the vehicle control incidence is the P value associated with the trend test. Beneath the dosed group incidence are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in a dose group is indicated by **N**.
[e] Tissue was examined microscopically only when it was observed to be abnormal at necropsy; thus statistical comparisons with the vehicle controls are not appropriate.

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Disposition Summary				
Animals initially in study	60	60	60	60
15-Month interim evaluation	12	12	12	12
Early deaths				
Moribund	1	1	1	
Natural deaths	2	3		7
Survivors				
Terminal sacrifice	45	44	47	41
Animals examined microscopically	60	60	60	60
15-Month Interim Evaluation				
Alimentary System				
Liver	(12)	(12)	(12)	(12)
Fatty change, peripheral	1 (8%)	2 (17%)	2 (17%)	
Granuloma, multiple		1 (8%)		
Vacuolization cytoplasmic, centrilobular	1 (8%)	2 (17%)	2 (17%)	
Integumentary System				
Skin			(1)	
Atrophy, focal, hair follicle			1 (100%)	
2-Year Study				
Alimentary System				
Esophagus	(47)	(4)	(1)	(48)
Autolysis				2 (4%)
Gallbladder	(46)	(4)	(1)	(43)
Autolysis				1 (2%)
Hemorrhage				1 (2%)
Infiltration cellular, lymphocytic				1 (2%)
Intestine large, cecum	(45)	(4)	(1)	(44)
Autolysis		1 (25%)		2 (5%)
Intestine large, colon	(47)	(4)	(1)	(45)
Autolysis		1 (25%)		2 (4%)
Intestine large, rectum	(47)	(4)	(1)	(46)
Autolysis		1 (25%)		1 (2%)
Intestine small, duodenum	(47)	(4)	(1)	(44)
Autolysis		1 (25%)		2 (5%)
Ulcer		1 (25%)		
Intestine small, ileum	(48)	(4)	(1)	(44)
Autolysis		1 (25%)		2 (5%)
Intestine small, jejunum	(47)	(5)	(1)	(44)
Autolysis				2 (5%)

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

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Alimentary System (continued)				
Liver	(48)	(48)	(48)	(48)
Autolysis				1 (2%)
Basophilic focus		1 (2%)	2 (4%)	2 (4%)
Clear cell focus	1 (2%)	1 (2%)	2 (4%)	
Cyst			1 (2%)	1 (2%)
Eosinophilic focus		2 (4%)		
Hyperplasia, focal, ito cell			1 (2%)	
Infarct	1 (2%)			
Infiltration cellular, lymphocytic	1 (2%)	2 (4%)		
Mixed cell focus	1 (2%)			
Necrosis, hepatocyte	1 (2%)			2 (4%)
Necrosis, hepatocyte, midzonal		1 (2%)		
Thrombus				1 (2%)
Vacuolization cytoplasmic, centrilobular		1 (2%)		
Pancreas	(48)	(4)	(1)	(48)
Atrophy, acinar cell	2 (4%)			1 (2%)
Autolysis				2 (4%)
Cyst, duct	1 (2%)			
Dilatation, duct	1 (2%)			
Hyperplasia, lymphoid	1 (2%)			
Salivary glands	(48)	(4)	(1)	(48)
Atrophy				1 (2%)
Autolysis				2 (4%)
Infiltration cellular, lymphocytic	5 (10%)			5 (10%)
Stomach, forestomach	(47)	(5)	(1)	(47)
Autolysis				1 (2%)
Cyst epithelial inclusion				1 (2%)
Hyperplasia, focal		1 (20%)		
Metaplasia, squamous		1 (20%)		
Stomach, glandular	(47)	(5)	(1)	(47)
Autolysis		1 (20%)		2 (4%)
Cyst	1 (2%)			
Hyperkeratosis				1 (2%)
Hyperplasia				1 (2%)
Tongue	(48)	(4)	(1)	(48)
Autolysis				2 (4%)
Cardiovascular System				
Blood vessel	(48)	(4)	(1)	(48)
Arteriosclerosis, artery	2 (4%)			
Autolysis, aorta				1 (2%)
Mineralization, aorta	1 (2%)			
Heart	(48)	(4)	(1)	(48)
Autolysis				1 (2%)
Cardiomyopathy	3 (6%)			2 (4%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Endocrine System				
Adrenal gland, cortex	(45)	(4)	(1)	(47)
Autolysis				3 (6%)
Cytoplasmic alteration	1 (2%)			4 (9%)
Hyperplasia	4 (9%)			3 (6%)
Hyperplasia, spindle cell	33 (73%)			24 (51%)
Adrenal gland, medulla	(45)	(4)	(1)	(47)
Autolysis				3 (6%)
Parathyroid gland	(44)	(3)		(47)
Autolysis				4 (9%)
Cyst				1 (2%)
Thyroid gland	(47)	(4)		(48)
Autolysis				5 (10%)
Cyst, follicle	3 (6%)			1 (2%)
General Body System				
None				
Genital System				
Coagulating gland	(48)	(4)	(1)	(48)
Autolysis		1 (25%)		3 (6%)
Epididymis	(48)	(4)	(1)	(48)
Autolysis		1 (25%)		4 (8%)
Granuloma sperm				1 (2%)
Inflammation, chronic				1 (2%)
Spermatocele				1 (2%)
Preputial gland	(48)	(4)	(1)	(47)
Atrophy	13 (27%)	2 (50%)		13 (28%)
Autolysis		1 (25%)		3 (6%)
Ectasia, duct	1 (2%)	1 (25%)		1 (2%)
Hyperplasia				1 (2%)
Prostate	(47)	(4)	(1)	(46)
Atrophy				1 (2%)
Autolysis				4 (9%)
Inflammation, acute				1 (2%)
Seminal vesicle	(48)	(4)	(1)	(48)
Autolysis		1 (25%)		4 (8%)
Inflammation, acute				1 (2%)
Testes	(48)	(4)	(1)	(46)
Autolysis		1 (25%)		3 (7%)
Degeneration				1 (2%)
Degeneration, unilateral, seminiferous tubule	1 (2%)			
Mineralization, unilateral, seminiferous tubule	1 (2%)			

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Hematopoietic System				
Bone marrow	(48)	(4)	(1)	(48)
Autolysis				1 (2%)
Hyperplasia	1 (2%)	2 (50%)		2 (4%)
Lymph node, mandibular	(46)	(3)	(1)	(48)
Angiectasis	1 (2%)			
Apoptosis, lymphocyte		1 (33%)		
Atrophy, lymphocyte				1 (2%)
Autolysis				2 (4%)
Lymph node, mesenteric	(44)	(8)	(3)	(48)
Apoptosis, lymphocyte		1 (13%)		
Atrophy	1 (2%)			
Atrophy, lymphocyte				1 (2%)
Autolysis				2 (4%)
Degeneration	4 (9%)	1 (13%)	1 (33%)	3 (6%)
Hyperplasia	2 (5%)			3 (6%)
Pigmentation				1 (2%)
Spleen	(48)	(8)	(4)	(48)
Apoptosis, lymphocyte		1 (13%)		
Atrophy, lymphocyte	1 (2%)			2 (4%)
Autolysis				2 (4%)
Hematopoietic cell proliferation	2 (4%)	4 (50%)	1 (25%)	6 (13%)
Hyperplasia, lymphoid	2 (4%)			1 (2%)
Thymus	(39)	(2)	(1)	(30)
Apoptosis			1 (100%)	
Autolysis				1 (3%)
Cyst				1 (3%)
Integumentary System				
Mammary gland				(2)
Autolysis				1 (50%)
Skin	(48)	(4)	(1)	(48)
Autolysis				1 (2%)
Musculoskeletal System				
Bone, femur	(48)	(4)	(1)	(48)
Autolysis				1 (2%)
Fibrous osteodystrophy				1 (2%)
Bone, sternum	(48)	(4)	(1)	(48)
Autolysis				1 (2%)
Fibrous osteodystrophy	2 (4%)			2 (4%)
Osteopetrosis	1 (2%)			
Skeletal muscle	(48)	(4)	(1)	(48)
Autolysis				2 (4%)
Infiltration cellular, lymphocytic				1 (2%)
Polyarteritis				1 (2%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Nervous System				
Brain, cerebellum	(48)	(4)	(1)	(48)
Autolysis				1 (2%)
Brain, cerebrum	(48)	(4)	(1)	(47)
Hydrocephalus		1 (25%)		
Infiltration cellular, lymphocytic, lateral ventricle	1 (2%)			
Mineralization, thalamus	19 (40%)	2 (50%)		19 (40%)
Peripheral nerve	(48)	(4)	(1)	(47)
Autolysis				2 (4%)
Degeneration				1 (2%)
Spinal cord	(48)	(3)	(1)	(48)
Autolysis				1 (2%)
Respiratory System				
Larynx	(46)	(4)	(1)	(48)
Autolysis				2 (2%)
Lung	(48)	(6)	(5)	(48)
Atelectasis	1 (2%)			
Autolysis				1 (2%)
Granuloma, focal	1 (2%)			
Hemorrhage, focal, right, apical lobe, sub pleura			1 (20%)	
Hemorrhage	1 (2%)			
Hyperplasia, alveolar epithelium	1 (2%)			5 (10%)
Infiltration cellular, lymphocytic	3 (6%)			2 (4%)
Nose	(48)	(4)	(1)	(48)
Autolysis				1 (2%)
Granuloma				1 (2%)
Trachea	(47)	(4)	(1)	(48)
Autolysis				2 (4%)
Special Senses System				
Eye	(48)	(4)	(1)	(47)
Autolysis				1 (2%)
Harderian gland	(48)	(8)	(2)	(48)
Autolysis				3 (6%)
Hyperplasia, unilateral	1 (2%)			2 (4%)
Infiltration cellular, lymphocytic				2 (4%)
Lacrimal gland	(47)	(4)	(1)	(46)
Atrophy, focal				1 (2%)
Autolysis				2 (4%)
Infiltration cellular, lymphocytic	1 (2%)			
Zymbal's gland	(47)	(3)	(1)	(47)
Autolysis				2 (4%)

TABLE B3
Summary of the Incidence of Nonneoplastic Lesions in Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
2-Year Study (continued)				
Urinary System				
Kidney	(48)	(4)	(1)	(47)
Accumulation hyaline droplet, renal tubule	1 (2%)			2 (4%)
Autolysis				2 (4%)
Cyst, renal tubule	1 (2%)			
Degeneration, renal tubule	1 (2%)			4 (9%)
Glomerulosclerosis	2 (4%)			3 (6%)
Hyperplasia, focal, unilateral, renal tubule	1 (2%)			
Infarct, unilateral		1 (25%)		
Infiltration cellular, lymphocytic	1 (2%)			1 (2%)
Mineralization, renal tubule	1 (2%)			
Nephropathy	1 (2%)			
Regeneration, renal tubule	2 (4%)			4 (9%)
Urinary bladder	(48)	(4)	(1)	(48)
Autolysis				4 (8%)
Infiltration cellular, lymphocytic	1 (2%)			

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

TABLE C1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
15-Month Interim Evaluation				
n	12	12	12	12
Necropsy body wt	41.63 ± 0.859	42.34 ± 1.062	42.26 ± 1.191	41.36 ± 1.486
Brain				
Absolute	0.470 ± 0.007	0.460 ± 0.005	0.470 ± 0.008	0.470 ± 0.005
Liver				
Absolute	1.960 ± 0.212	1.990 ± 0.213	1.740 ± 0.098	2.060 ± 0.170
Relative to brain wt	4.210 ± 0.450	4.300 ± 0.470	3.690 ± 0.220	4.390 ± 0.340
Relative to body wt	47.08 ± 5.08	46.96 ± 5.02	40.87 ± 1.19	51.11 ± 5.65
2-Year Study				
n	41	37	36	44
Necropsy body wt	41.60 ± 0.847	40.89 ± 1.088	41.46 ± 1.181	40.74 ± 0.875
Heart				
Absolute	0.240 ± 0.006	0.230 ± 0.006	0.230 ± 0.006	0.220 ± 0.004***
Relative	5.900 ± 0.100	5.800 ± 0.200	5.700 ± 0.200	5.400 ± 0.100**
R. Kidney				
Absolute	0.380 ± 0.007[b]	0.360 ± 0.008	0.350 ± 0.008**	0.340 ± 0.007***
Relative	9.100 ± 0.100[b]	9.000 ± 0.100	8.600 ± 0.100**	8.400 ± 0.100***
Liver				
Absolute	2.200 ± 0.110	2.390 ± 0.125	2.550 ± 0.157	2.380 ± 0.120
Relative	53.50 ± 3.100	60.30 ± 4.000	63.80 ± 5.400	60.00 ± 3.800
Lungs				
Absolute	0.280 ± 0.011	0.280 ± 0.012	0.280 ± 0.009	0.280 ± 0.014
Relative	6.800 ± 0.300	7.100 ± 0.400	6.800 ± 0.300	7.200 ± 0.500
R. Testis				
Absolute	0.100 ± 0.002	0.100 ± 0.002	0.100 ± 0.002	0.100 ± 0.002
Relative	2.500 ± 0.100	2.600 ± 0.100	2.500 ± 0.100	2.500 ± 0.100
Thymus				
Absolute	0.040 ± 0.004	0.050 ± 0.006	0.040 ± 0.004	0.040 ± 0.004
Relative	1.000 ± 0.100	1.100 ± 0.100	1.100 ± 0.100	1.000 ± 0.100

** Significantly different ($P \leq 0.01$) from the vehicle control group by Dunnett's test
*** $P \leq 0.001$
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-brain-weight ratios (relative weights) are given as g organ weight/g brain weight (mean ± standard error); organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n=40

TABLE C2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
15-Month Interim Evaluation				
n	12	12	12	12
Necropsy body wt	37.78 ± 0.430	36.71 ± 0.669	36.71 ± 0.825	35.68 ± 0.839
Brain				
Absolute	0.470 ± 0.007	0.460 ± 0.005	0.470 ± 0.008	0.460 ± 0.005
Liver				
Absolute	1.350 ± 0.020	1.380 ± 0.030	1.410 ± 0.040	1.410 ± 0.040
Relative to brain wt	2.880 ± 0.050	3.000 ± 0.080	2.980 ± 0.070	3.070 ± 0.090
Relative to body wt	35.63 ± 0.30	37.46 ± 0.39*	38.31 ± 0.60**	39.55 ± 0.66***
2-Year Study				
n	45	44	47	41
Necropsy body wt	35.38 ± 0.473	35.29 ± 0.459	35.50 ± 0.369	34.88 ± 0.454
Heart				
Absolute	0.200 ± 0.004	0.210 ± 0.006	0.210 ± 0.005	0.200 ± 0.004
Relative	5.800 ± 0.100	6.000 ± 0.200	6.000 ± 0.100	5.600 ± 0.100
R. Kidney				
Absolute	0.310 ± 0.005	0.310 ± 0.005	0.310 ± 0.005	0.290 ± 0.005
Relative	8.700 ± 0.100	8.700 ± 0.100	8.600 ± 0.100	8.400 ± 0.100
Liver				
Absolute	1.660 ± 0.064	1.850 ± 0.126	1.740 ± 0.107	1.860 ± 0.143
Relative	46.70 ± 1.700	53.40 ± 4.100	49.20 ± 3.100	53.90 ± 4.500
Lungs				
Absolute	0.240 ± 0.006	0.250 ± 0.006	0.260 ± 0.005	0.250 ± 0.007
Relative	6.900 ± 0.200	7.100 ± 0.200	7.300 ± 0.100	7.300 ± 0.300
R. Testis				
Absolute	0.100 ± 0.001	0.100 ± 0.002	0.100 ± 0.001	0.100 ± 0.002
Relative	3.000 ± 0.000	2.900 ± 0.100	2.900 ± 0.000	2.900 ± 0.000
Thymus				
Absolute	0.040 ± 0.003	0.040 ± 0.004	0.040 ± 0.003	0.030 ± 0.003
Relative	1.000 ± 0.100	1.200 ± 0.100	1.000 ± 0.100	1.000 ± 0.100

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunnett's test
** ($P \leq 0.01$)
*** ($P \leq 0.001$)
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-brain-weight ratios (relative weights) are given as g organ weight/g brain weight (mean ± standard error); organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX D
BODY WEIGHT CONSIDERATIONS AND DIETARY CONTROL TECHNIQUES

BODY WEIGHT CONSIDERATIONS AND DIETARY CONTROL TECHNIQUES

INTRODUCTION

Over the past three decades, improvements in diet formulations and animal husbandry techniques and commercial breeding considerations have resulted in a general drift towards heavier animals for all the major rodent strains used in toxicity testing (Nohynek *et al.*, 1993; Roe, 1993; Hart *et al.*, 1995; Seilkop, 1995). Body weight increases in these strains are frequently associated with decreased survival and increased susceptibility to neoplastic and degenerative diseases (Nohynek *et al.*, 1993; Roe, 1993; Keenan *et al.*, 1995). These effects have compromised 2-year bioassays because of insufficient animals surviving until the termination of the study and the excessively high background neoplasm incidences preventing the demonstration of positive dose response relationships. Dietary restriction, which increases survival and lowers background neoplasm incidence, has been proposed as a mechanism for solving this problem (Roe, 1981, 1993; Keenan *et al.,* 1997).

Upward drifts in body weight and background neoplasm incidence have been observed in B6C3F$_1$ mice used in recent NTP bioassays (Seilkop, 1995). For example, in control groups of male mice, the incidence of background liver neoplasms has been reported to have increased at a rate of 3.9% per year during the 1980s; whereas for females, this increase was 7.3% (Turturro *et al.*, 1996).

Data from NTP studies using the mean body weights of control groups from individual studies (Turturro *et al.*, 1993, 1996, Seilkop, 1995), or weights of individual mice from groups of studies (Seilkop, 1995; Leakey *et al.*, 1998) have demonstrated a proportional relationship between body weight during mid-life (i.e., 20 to 60 weeks of age) and terminal liver neoplasm incidence in female B6C3F$_1$ mice and in "heavy" (i.e., mice that reach body weights of more than 36 g) male mice. This proportionality is lost in lighter male B6C3F$_1$ mice, giving rise to a J-curve in the body weight/neoplasm incidence profile (Seilkop 1995; Leakey *et al.*, 1998). This results in a larger sex difference in liver neoplasm incidence in smaller mice than in larger mice (Figure D1).

Castration studies with the parent strains of B6C3F$_1$ mice, which also show sex differences in liver neoplasm risk, suggested that the increased incidence of liver neoplasms in male mice was partly due to testicular androgens (Poole and Drinkwater, 1995).

Although dietary and caloric restriction have been shown to reduce body weight and spontaneous neoplasm incidence in rodents including the B6C3F$_1$ mouse (see Leakey *et al.,* 1998 for a comprehensive review), they can also inhibit chemical-induced hepatocarcinogenesis in rodents, and in the case of the relatively lean NCTR B6C3F$_1$ mice, 40% caloric restriction completely inhibited the hepatocarcinogenicity of known genotoxic carcinogens when tested in the newborn mouse assay (Fu *et al.,* 1994). Moreover, dietary restriction is generally implemented by limiting feed consumption to a set percentage of *ad libitum* feed consumption, and this may vary between rodent populations of the same strain in different laboratories (Turturro *et al.*, 1993, 1996).

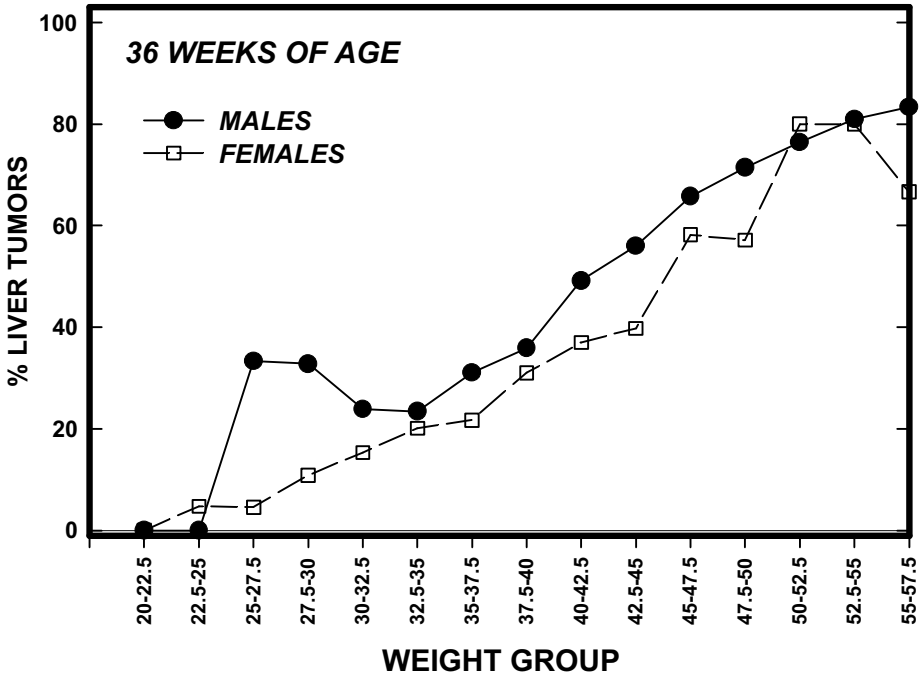


FIGURE D1
Sex Differences in Neoplasm Risk in 36-Week-Old B6C3F$_1$ Mice

Further difficulties arise with this approach when exposure to the test chemical causes additional body weight decreases. This frequently occurs due to toxic responses to the test chemical, and a 10% reduction in body weight has been used as a criterion for achieving a minimally toxic dose (Haseman, 1985). Chemical-induced body weight reductions can arise for several reasons, including decreased food consumption due to palatability problems in feed studies, anorexia due to toxic stress, disrupted intestinal absorption, or toxic wasting syndromes resulting from disruption of metabolism or endocrine systems (Leakey *et al.*, 1998). This occurred in a study of *O*-nitroanisole in female mice, where a treatment-related 38% decrease in body weight appeared to mask an increase in liver neoplasms in the high dose group (Haseman, 1998).

This also occurred in a recent NTP study that examined the effect of a 10% dietary restriction on the hepatocarcinogeneity of salicylazosulfapyridine (SASP) in male B6C3F$_1$ mice (NTP, 1997a). SASP-treatment caused an additional body weight decrease in both the *ad libitum*-fed and dietary restricted mice (Table D1).

TABLE D1
Liver Neoplasm Incidence in the 2-year Study of Salicylazosulfapyridine using Dietary Restriction[a]

Experimental Group	Liver Neoplasms[b] (%)	Body Weight at 30 Weeks[c] (g)
Ad libitum, Control	54.3	45.2
Ad libitum, SASP-Treated	91.7	39.2
Weight-Matched, Control	30.3	37.5
Dietary Restriction Control	42.2	40.7
Dietary Restriction, SASP-Treated	22.7	32.6

[a] NTP, 1997a
[b] Reported neoplasm rate is the Kaplan-Meier estimated incidence of hepatocellular adenoma or carcinoma after adjustment for incremental survival.
[c] Refers to weeks on study and is equivalent to 37 weeks of age. SASP was administered at a dose of 2,700 mg/kg in corn oil by gavage.

When body weight effects were ignored, salicylazosulfapyridine appeared to increase the terminal-adjusted rate of liver adenomas and carcinomas by 69% in the *ad libitum*-fed mice, but decreased the rate by 57% in the dietary-restricted mice. A weight-matched control was incorporated into the study which used dietary restriction to adjust the body weights of an additional control group to match the *ad libitum*-fed SASP-treated group. The neoplasm rate in this group was 30.3%, suggesting that SASP administration had caused a threefold increase in liver neoplasm incidence when body weight effects were taken into consideration. However, there was no weight-matched control available for the dietary restriction group. It is therefore not possible to determine from the study alone whether the negative effect on liver neoplasm incidence is a result of the use of dietary restriction or whether a positive neoplasm response is simply masked by the SASP-induced body weight reduction.

Very large body weight reductions, such as those due to the combined action of dietary restriction and SASP administration, have occasionally occurred in other NTP studies. The NTP study of 1-trans-delta^9-tetrahydrocannabinol (THC) (NTP, 1996a) is a rare example of the test chemical producing a body weight decrease very similar to that in the SASP study, with no evidence of treatment-induced hepatocarcinogenicity (Figure D2). The survival-adjusted liver neoplasm incidence was 54.3% for the control groups of both the SASP and THC studies, but the THC-treated group had a survival-adjusted tumor incidence of 8.4% as compared to 22.7% for the dietary restricted SASP group. This suggests that SASP had actually increased liver neoplasm incidence in the dietary restricted mice by a percentage increment approaching that observed in the *ad libitum*-fed mice. However, THC treatment also markedly decreased survival; only 58% of the mice survived to terminal sacrifice as compared to 80% for the dietary restricted SASP-treated group (Table D2). It is therefore possible that the low liver neoplasm rate in the THC-treated group is an artifact of low survival due to the THC treatment preferentially killing the mice that were destined to develop liver tumors. Survival adjustment procedures do not compensate for such situations. Although such a situation is conceivable, it appears unlikely because the high dose (500 mg/kg) THC treatment group exhibited a delayed first neoplasm incidence relative to the controls and other dose groups and a decreased incidence of liver foci relative to the control group (Table D2). If the THC was preferentially killing mice destined to develop liver neoplasms rather than inhibiting or delaying liver neoplasm onset, at least some of the mice which died prematurely would be expected to have developed liver neoplasms or foci.

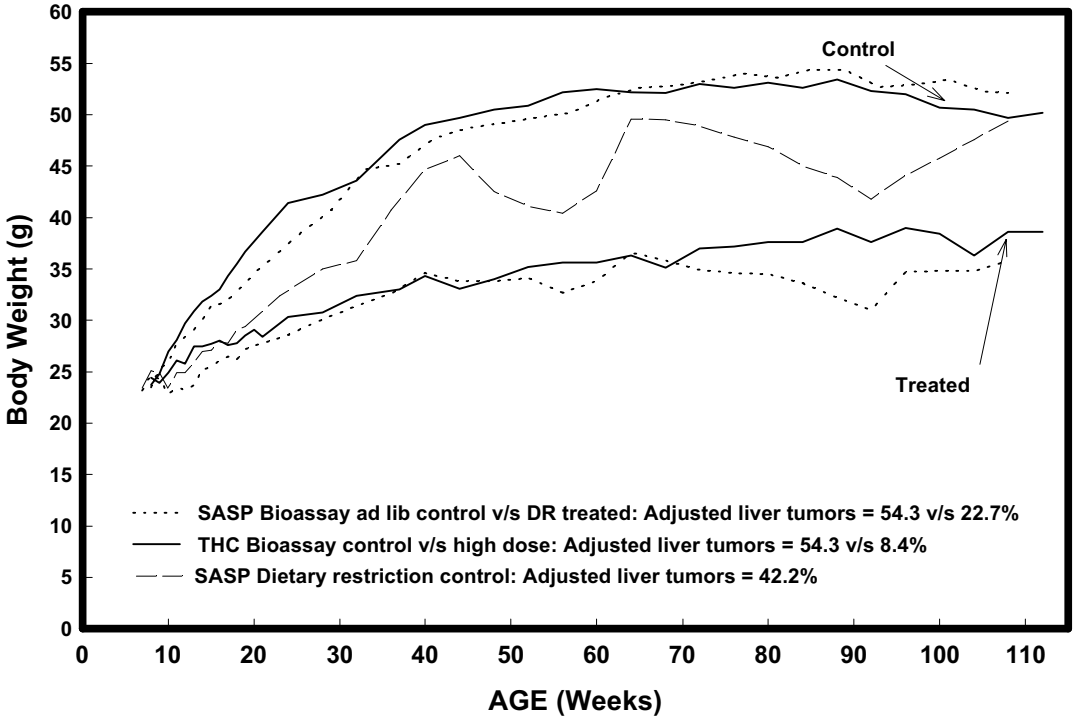


FIGURE D2
NTP 2-Year Studies in which the Test Chemical Caused Large Decreases in Body Weight
The weight curves for the *ad libitum*-fed control groups from the 2-year bioassays of 1-trans-delta^9- tetrahydrocannabinol (THC) and salicylazosulfapyridine (SASP) studies are very similar, as are their adjusted liver neoplasm rates. The high dose THC group (500 mg/kg) experienced a large decrease in body weight similar to the combined dietary restriction and high dose effect in the SASP study. The weight curve plotted for the SASP study is the 2-year dietary restriction high dose (2,700 mg/kg) group, and it is either approximately the same or slightly lower than the weight curve from the THC study. The adjusted tumor incidence is 22.7% as compared to 8.4% for the THC group. The forced weight reduction is due partly to dietary restriction and partly to SASP administration. The dietary restricted control weight curve is also plotted. It is apparent that more of the weight reduction is due to the SASP than is due to the dietary restriction. This is consistent with the *ad libitum* study where SASP also caused a large decrease in body weight (Table D1).

TABLE D2
Survival and Incidences of Liver Neoplasms and Foci in Male Mice in the 2-Year Gavage Study of 1-trans-delta^9-Tetrahydrocannabinol[a]

	Vehicle Control	125 mg/kg	250 mg/kg	500 mg/kg
Hepatocellular Adenoma or Carcinoma				
Overall Rate	31/62 (50%)	13/60 (22%)	9/61 (15%)	3/57 (5%)
Adjusted Rate	54.3%	23.0%	18.7%	8.4%
Terminal Rate	24/50 (48%)	10/53 (19%)	6/45 (13%)	2/34 (6%)
First Incidence	554 days	563 days	574 days	611 days
Foci				
Basophilic Foci	2/62 (3%)	2/60 (3%)	1/61 (2%)	1/57 (2%)
Clear Cell Foci	7/62 (11%)	1/60 (2%)	0	0
Eosinophilic Foci	18/62 (29%)	1/60 (2%)	0	0
Terminal Survival	80.6%	88.3%	76.3%	57.6%

[a] NTP, 1996a

An alternative explanation is that stress due to dietary restriction or chemical exposure reduces not only the body weight-related liver tumors in male mice, but also the sex-dependent liver tumors that occur independently of body weight in male B6C3F$_1$ mice weighing between 27 and 33 g at 36 weeks of age (Figure D1). Short-term caloric restriction has been reported to reduce the testosterone/estradiol ratios and impair male reproductive function in rodents (Leakey *et al.*, 1998), and restraint stress or food depression suppresses LH secretion in male mice (Jeong *et al.*, 1999). It is likely therefore that when male mice are exposed to severe food restriction or chemical toxic stress, the incidence of sex-specific liver tumors is decreased in addition to the incidence of body weight-associated tumors. This is supported by the NTP study of scopolamine in mice (NTP, 1997b), where the effects of dietary restriction and/or chemical exposure resulted in male mice with reduced body weights and liver neoplasm incidences below what would be expected for control, *ad libitum*-fed mice of similar weight. When a database of mice comprising those from the NTP and NCTR dietary restriction studies and the NTP THC study was used to plot liver neoplasm incidence as a function of body weight as outlined below, the J-curve profile seen with *ad libitum*-fed control mice disappeared (Figures D5, D6, and D7). Instead there was a more linear relationship reminiscent of the profile seen in female mice (Figure D1).

Thus, these studies suggest that body weight decreases resulting from exposure to the test chemical will, in certain cases, mask a positive liver tumor response and, as illustrated in Figure D3, this effect will be greater for experimental groups of small mice than in groups of heavy mice. Although the use of simple dietary restriction, or selective breeding of smaller mouse stocks, may solve the problem of survival and high background incidence of liver neoplasms, it will not address the issue of altered sensitivity due to chemical-induced body weight changes.

An alternative approach involves using dietary control to manipulate body growth in all B6C3F$_1$ mice used in bioassays to fit a standardized weight curve. Such standardized or idealized weight curves could be created for male and female B6C3F$_1$ mice, as well as for other rodent strains used for chronic bioassays. These idealized growth curves could then be used throughout industry and the regulatory community to standardize background tumor incidence between laboratories. The mice used for both control and treatment groups in

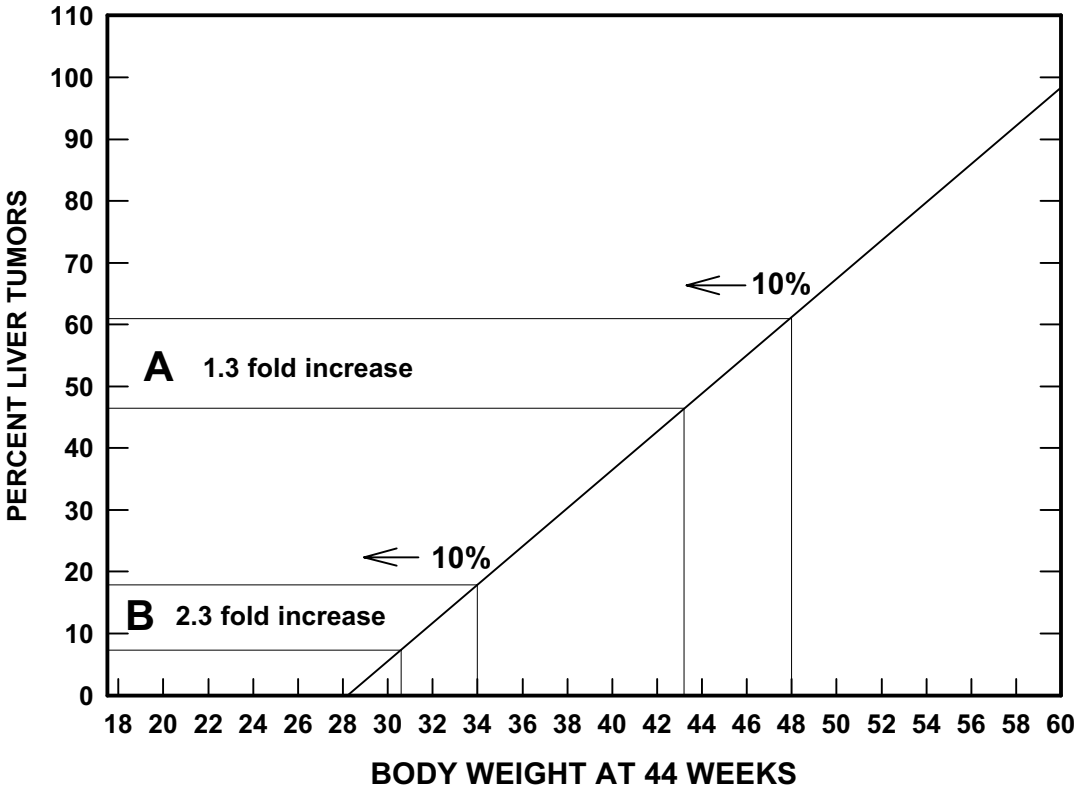


FIGURE D3
Predicted Impact of Chemically Induced Body Weight Reduction on Detection of Hepatocarcinogeneity in Male B6C3F$_1$ Mice
This figure uses the same body weight-neoplasm rate relationship as was used to calculate the idealized weight curve and illustrates two scenarios: (A) mice which would have weighed 48 g at 44 weeks, were treated with a chemical that caused a 10% decrease in mean body weight; (B) mice which would have weighed 34 grams at 44 weeks, were treated with the same chemical that also caused a 10% decrease in mean body weight. For population A, only a 1.3-fold increase in liver tumor incidence is required to reach the predicted control rate, but for group B, a 2.3-fold increase is required. This suggests that the hepatocarcinogenic effect of the chemical would be more easily masked by the body weight decrease in the smaller mice.

future bioassays could be manipulated to fit these growth curves by moderate food restriction or dietary supplementation. For B6C3F$_1$ mice, the growth curves should produce a spontaneous liver tumor incidence of approximately 15% to 20% at 26 months of age. A 15% to 20% liver tumor incidence is sufficiently high to guarantee that the sensitivity of the mouse to chemical carcinogenesis has not been compromised, but is low enough to ensure that the spontaneous neoplasms will not obscure any chemical-induced liver neoplasms, and sufficient mice will survive to the termination of the bioassay. As outlined below, we have utilized data from mice used in NTP and NCTR chronic bioassays and aging studies to construct idealized weight curves for male and female B6C3F$_1$ mice that predict a liver tumor risk of 15% to 20%.

CONSTRUCTION OF IDEALIZED BODY WEIGHT CURVES

Individual body weight data and neoplasm incidences for male and female $B6C3F_1$ mice were obtained from 47 recent NTP studies (Table D3). These were supplemented by data from the NCTR $B6C3F_1$ mouse colony that was obtained from three chronic cancer bioassays conducted at the NCTR in the mid 1980s and data from the NCTR Project on Caloric Restriction. Data on mice that survived less than 500 days or with grossly abnormal body weight values were eliminated. The body weight values were correlated to the actual age of the mice. The body weight values were then adjusted to estimate body weight values at set weekly, biweekly or 4-week intervals so that they could be directly compared. This was done by assuming a linear extrapolation between the body weight values of the consecutive age points that were available for each study. The data from all the studies could then be combined.

Starting with the initial 9-week age point, the mice from all the studies were assigned to one of 17 consecutive weight groups. These ranged from 20 to 57.5 g in 2.5-g intervals with extra groups for high and low outliers. The mice in each weight group were then sorted according to whether they developed a liver neoplasm so the relative neoplasm risk could be calculated for each group. Mice that were reported to have either a hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma were designated positive; mice with no reported liver neoplasms were designated negative. The process was repeated for each age point. Once the neoplasm risk for all the weight ranges at each age had been calculated, the data was plotted graphically to determine the idealized weight curve. Figure D4 illustrates the relationship between body weight and neoplasm risk for 36-week-old male and female mice used in 18 recent NTP single-housing studies. For the male mice, there is a "J"-shaped curve, due to a population of small animals with a relatively high risk for liver neoplasms, rather than a linear relationship between body weight and neoplasm incidence as occurs in the females. These figures are similar to those reported by Seilkop (1995) using a similar set of NTP studies.

As outlined in the introduction, the J-curve weight profile may not accurately describe the body weight curves of male mice subjected to forced body weight reduction due to dietary restriction or toxic effects of the test chemical. An alternative population, which would be more applicable to studies using controlled feeding, was therefore used to determine the body weight-tumor incidence relationship in male $B6C3F_1$ mice. This population consisted of singly-housed mice weighing over 36 g at 52 weeks of age from 18 NTP bioassays. It also included mice from two NTP studies which used dietary restriction (NTP, 1997c), an NTP study where the test chemical caused large decreases in body weight and liver neoplasm incidence and did not appear to be carcinogenic (NTP, 1996a), and data from a large NCTR caloric restriction study which used 40% caloric restriction in male $B6C3F_1$ mice (Witt *et al.*, 1991; Sheldon *et al.*, 1995). This population exhibited a body weight-liver neoplasm relationship which was more linear throughout the entire weight range (Figures D4, D5, D6, and D7) and it was therefore used to determine the idealized weight curve in male mice.

This was done by determining which weight group best corresponded to a 15% to 20% liver neoplasm risk at each age point. These weight groups were then plotted against their respective age points to produce a crude idealized body weight curve. This was then smoothed and extrapolated back to 4 weeks of age by comparing with the body weight curves of actual mice of similar weight. The final idealized body weight curve is also shown in Figure D8. The curve also predicted a background lymphoma rate of approximately 4% as judged by the correlation between body weight at 10 to 20 weeks and lymphoma risk.

TABLE D3
NTP Studies Used to Construct Idealized Body Weight Curve for the 2-Year Gavage Study of Chloral Hydrate[a]

Technical Report Number	Chemical	Route
Studies with Group Housing		
352	N-Methyloacrylamide	Gavage
354	Dimethoxane	Gavage
365	Pentaerythritol Tetranitrate	Feed
366	Hydroquinone	Gavage
378	Benzaldehyde	Gavage
381	*d*-Carvone	Gavage
382	Furfural	Gavage
383	1-Amino-2,4-dibromoanthraquinone	Feed
387	*dl*-Amphetamine Sulfate	Feed
391	Tris(2-Chloroethyl) Phosphate	Gavage
394	Acetaminophen	Feed
395	Probenecid	Gavage
396	Monochloroacetic Acid	Gavage
401	2,4-Diaminophenol Dihydrochloride	Gavage
402	Furan	Gavage
403	Resorcinol	Gavage
406	γ-Butyrolactone	Gavage
407	C.I. Pigment Red 3	Feed
411	C.I. Pigment Red 23	Feed
412	4,4′-Diamino-2,2′-stilbenedisulfonic Acid, Disodium Salt	Feed
413	Ethylene Glycol	Feed
415	Polysorbate 80	Feed
428	Manganese (II) Sulfate Monohydrate	Feed
Studies with Single Housing		
362	4-Vinyl-1-cyclohexene Diepoxide	Dermal
371	Toluene	Inhalation
385	Methyl Bromide	Inhalation
414	Pentachloroanisole	Gavage
416	*o*-Nitroanisole	Feed
418	*p*-Nitroaniline	Gavage
420	Triamterene	Feed
421	Talc	Inhalation
422	Coumarin	Gavage
423	3,4-Dihydrocoumarin	Gavage
424	*o*-Benzyl-*p*-chlorophenol	Gavage
425	Promethazine Hydrochloride	Gavage
427	Turmeric Oleoresin	Feed
431	Benzyl Acetate	Feed
432	Barium Chloride Dihydrate	Drinking Water
433	Tricresyl Phosphate	Feed
434	1,3-Butadiene	Inhalation
435	4,4′-Thiobis(6-*t*-butyl-*m*-cresol)	Drinking Water
436	*t*-Butyl Alcohol	Drinking Water
437	Hexachlorocyclopentadiene	Inhalation
439	Methylphenidate Hydrochloride	Feed
442	*p*-Nitrobenzoic Acid	Feed
484	2-Butoxyethanol	Inhalation

TABLE D3
NTP Studies Used to Construct Idealized Body Weight Curve for the 2-Year Gavage Study of Chloral Hydrate

Technical Report Number	Chemical	Route
Studies with Weight Loss		
445	Scopolamine Hydrobromide Trihydrate	Gavage
446	1-trans-delta^9-Tetrahydrocannabinol	Gavage
457	Salicylsulfapyridine	Gavage
460	Effect of Dietary Restriction:	
	Butyl Benzyl Phthalate	Feed
	t-Butylhydroquinone	Feed
	Salicylazosulfapyridine	Gavage
	Scopolamine Hydrobromide Trihydrate	Gavage
Water Gavage Studies		
366	Hydroquinone	Gavage
396	Monochloroacetic Acid	Gavage
401	2,4-Diaminophenol Dihydrochloride	Gavage
403	Resorcinol	Gavage
408	Mercuric Chloride	Gavage
418	*p*-Nitroaniline	Gavage
425	Promethazine Hydrochloride	Gavage
457	Salicylazosulfapyridine	Gavage
491	Methyleugenol	Gavage
National Center for Toxicological Research Studies		
	Approximately 150 mice from the Project on Caloric Restriction	
	Approximately 150 mice from each of the doxylamine, pyrilamine, and triprolidine bioassays	

[a] NTP 1989a-e, 1990a-d, 1991a-c, 1992a-j, 1993a-o, 1994a-f, 1995a-b, 1996a-b, 1997a-c, 2000a-b; Witt *et al.*, 1991; Sheldon *et al.*, 1995

Origin of Historical Mouse Body Weight and Neoplasm Incidence Data

Data from 47 NTP single and group housing studies, which were available from the NTP *INLIFE* database in 1995 when the initial body weight analysis was performed, were used to calculate the liver neoplasm risk plots. The studies that were used are listed in Table D3. Data from all studies listed as NTP single housing and group housing were used to calculate the neoplasm risks shown in Figure D4. The data from Figure D4 are also plotted in Figure D1. The six inhalation studies were not included in the population used to calculate neoplasm risks for the idealized weight curve as shown in Figures D5, D6, and D7. The idealized weight population of approximately 1,600 mice comprised the NTP studies with weight loss, the calorically restricted mice from the NCTR Project on Caloric Restriction, and mice weighing over 36 g at 52 weeks of age from the non-inhalation NTP single-housing studies listed in Table D3. This population was also used for the neoplasm risk plots shown in Figure D3 and in the dietary control groups of Figures D12 through D18. The neoplasm risk plots for *ad libitum*-fed $B6C3F_1$ mice, used in Figures D9 and D12 through D18, were calculated from a population (approximately 2,250 mice) that incorporated all NTP single and groups housing studies listed in Table D3, *ad libitum*-fed mice from the NCTR Project on Caloric Restriction (Witt *et al.*, 1991; Sheldon *et al.*, 1995), the NTP dietary restriction studies (NTP, 1995e, 1997a), and the mice from the three NCTR Bioassays. Figure D9 utilized control mice from NTP studies conducted in the early 1980s (NTP, 1989a,b,c,d, 1990a) and late 1980s (NTP, 1995b,c, 1996a, 1997a) for the bar graph data in panels A and C, respectively. The water gavage studies listed in Table D3 were used for Figure 3 in the Introduction of this Technical Report. Data from all studies listed as single housing were used to calculate the neoplasm risks shown in Figure 4 in the main Introduction. Unless stated otherwise, all ages given in this appendix are actual ages of the mice and not weeks on study.

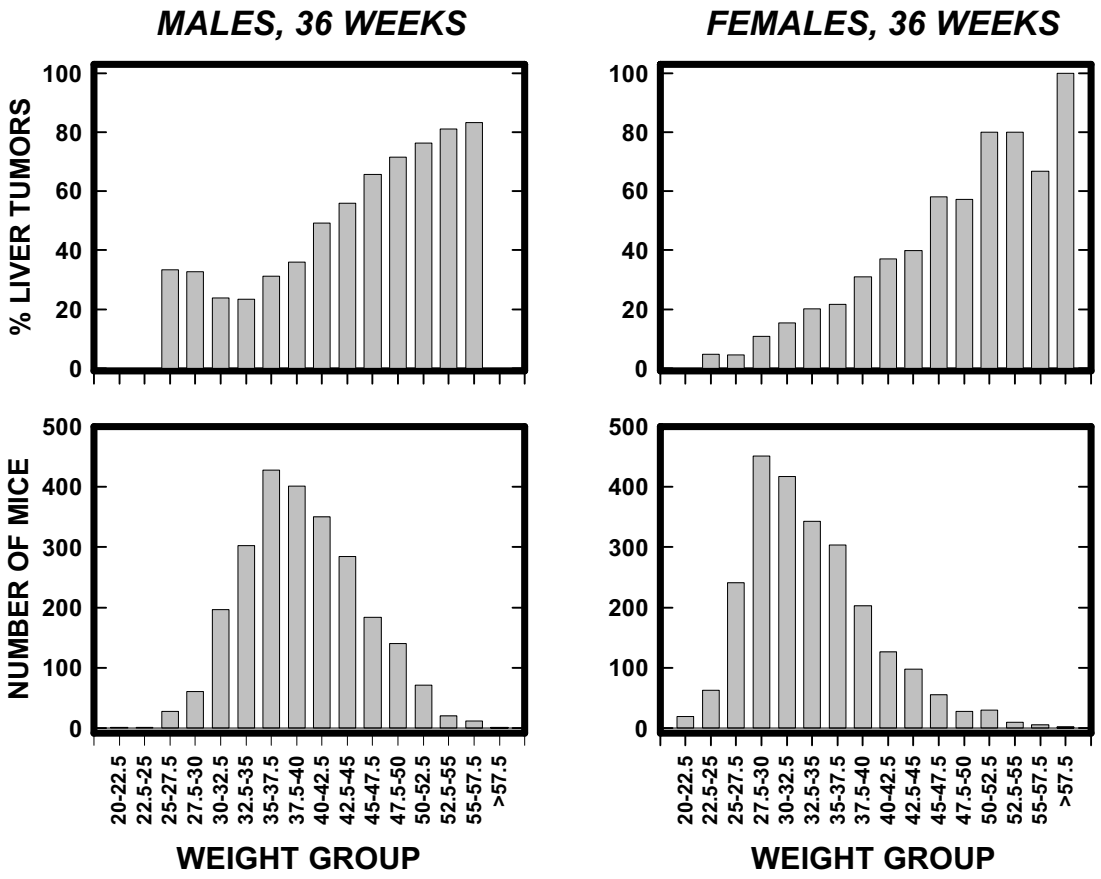


FIGURE D4
Relationship Between Body Weight and Liver Neoplasm Incidence in *Ad Libitum*-Fed B6C3F$_1$ Mice
The figure shows the effect of body weight at 36 weeks of age on liver neoplasm (adenoma, carcinoma, or hepatoblastoma) incidence in B6C3F$_1$ mice from recent NTP chronic studies. A list of the studies used is given in Table D3. The upper graphs show the percentage of mice falling within each weight group that developed liver neoplasms. The lower graphs show the total number of mice falling in each weight group.

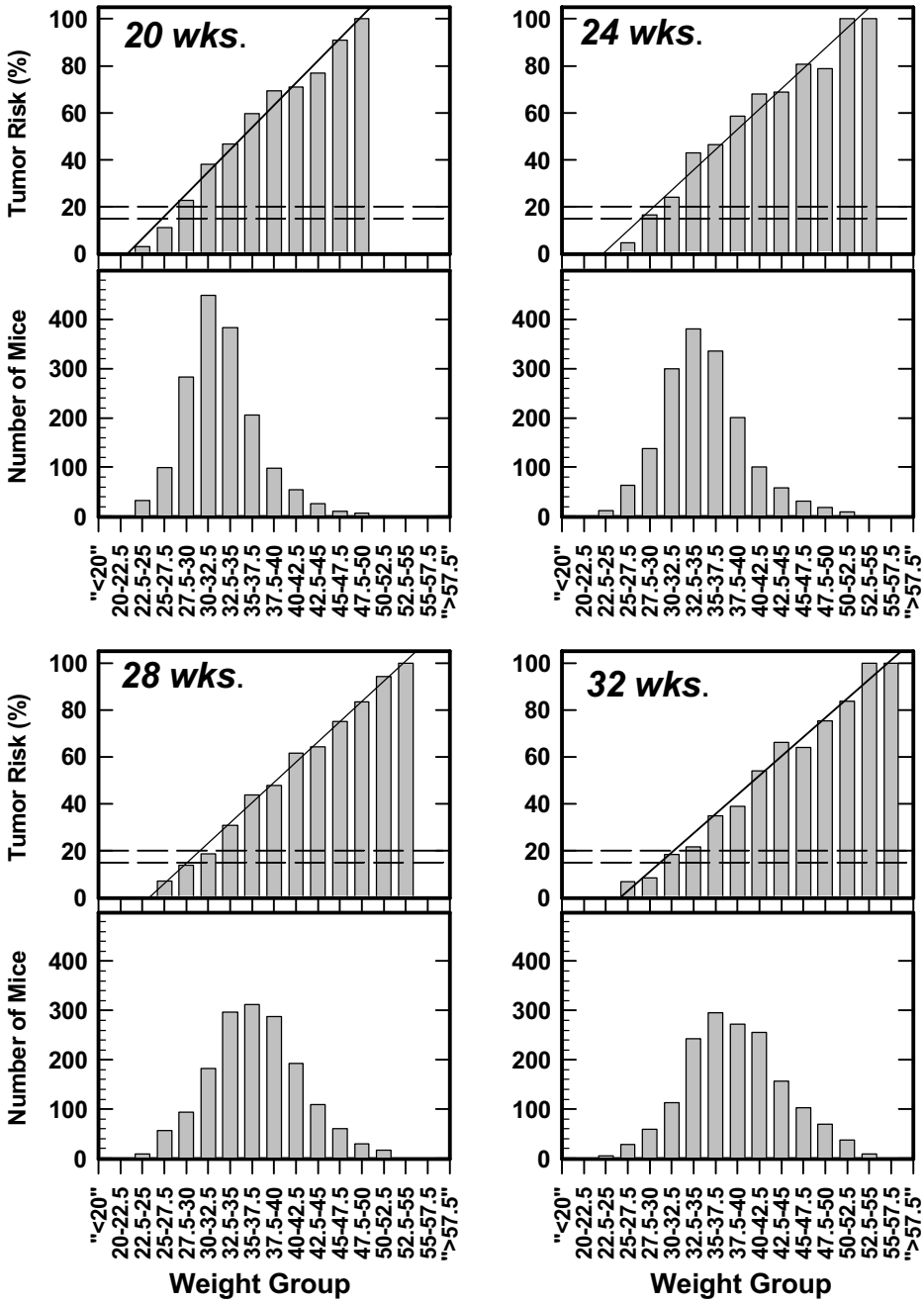


FIGURE D5
Correlation Between Adjusted Liver Neoplasm Incidence and Body Weight at Ages 20 to 32 Weeks in Male B6C3F$_1$ Mice with Diet- or Test Compound-Induced Weight Reduction

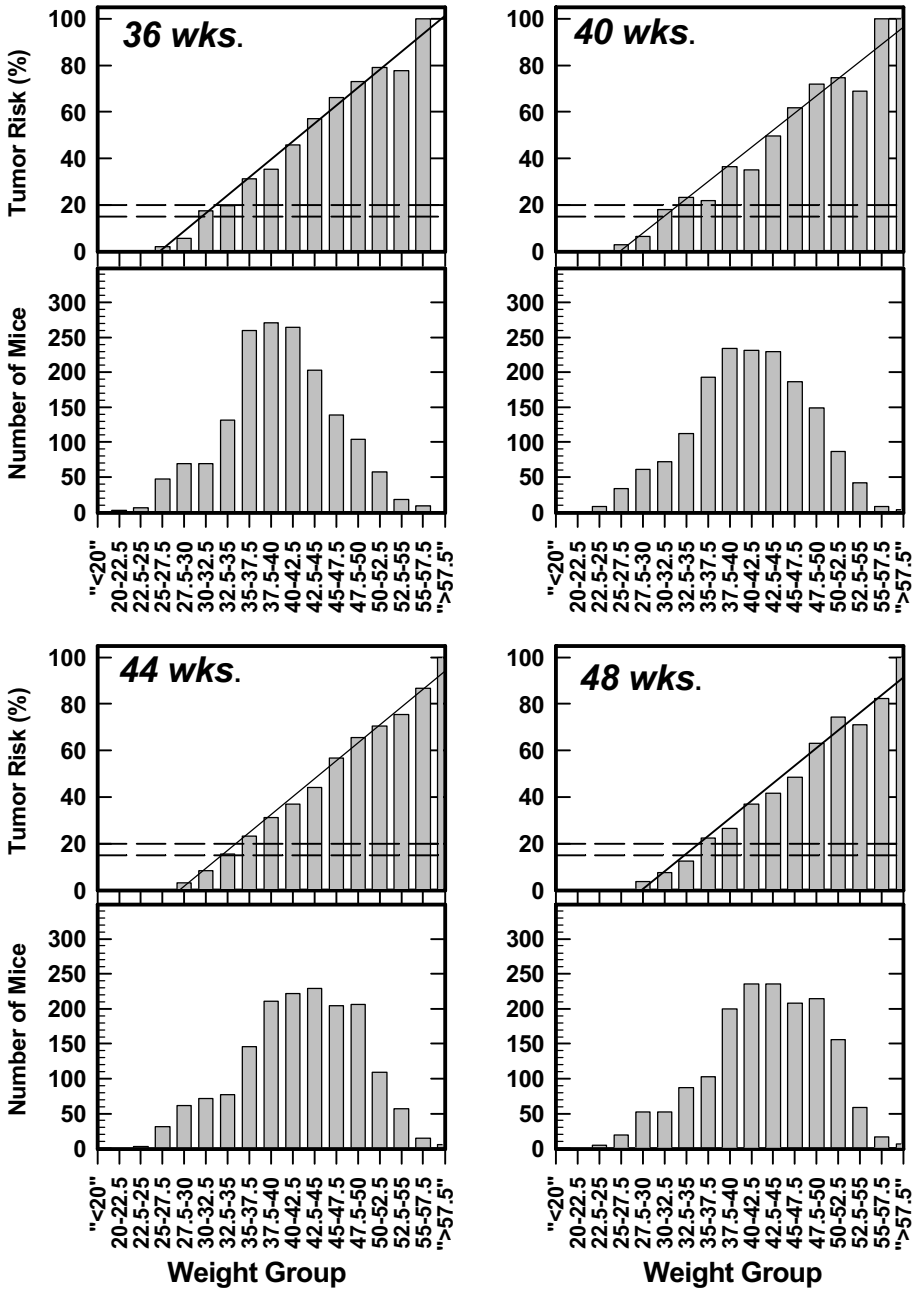


FIGURE D6
Correlation Between Adjusted Liver Neoplasm Incidence and Body Weight at Ages 36 to 48 Weeks in Male B6C3F$_1$ Mice with Diet- or Test Compound-Induced Weight Reduction

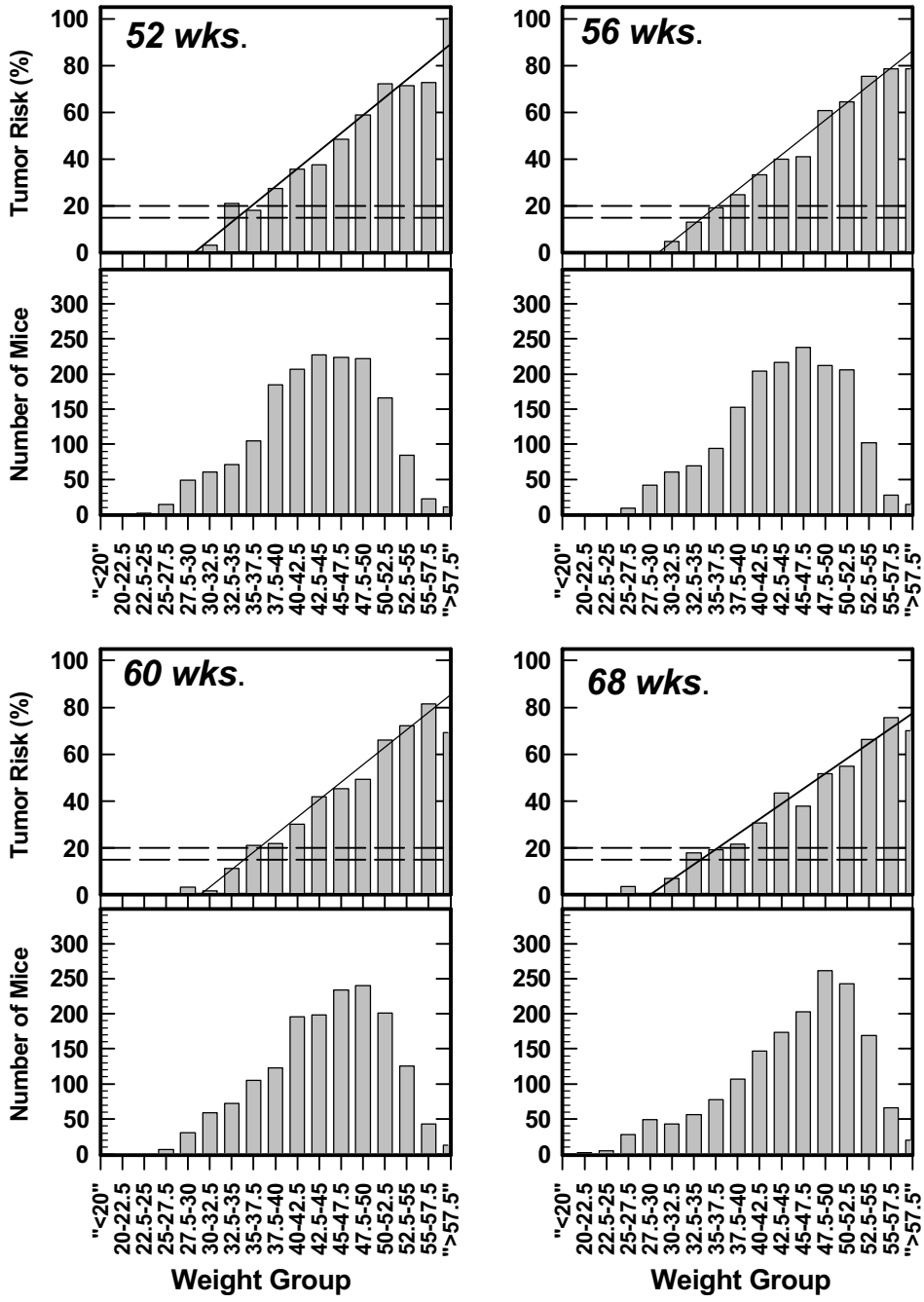


FIGURE D7
Correlation Between Adjusted Liver Neoplasm Incidence and Body Weight at Ages 52 to 68 Weeks in Male B6C3F$_1$ Mice with Diet- or Test Compound-Induced Weight Reduction

NOTES FOR FIGURES D5, D6, AND D7 AND TABLE D4

Figures D5, D6 and D7 show the relationship between body weight at specific ages and terminally adjusted liver neoplasm (adenoma, carcinoma, or hepatoblastoma) incidence. The male $B6C3F_1$ mouse population used here consisted of heavy *ad libitum*-fed mice and light mice which were light because of body weight reduction due to either caloric restriction or exposure to a noncarcinogenic test chemical. Data from approximately 1,600 mice were used. The upper portion of each graph shows the tumor risk for each weight group and the lower portion shows the number of mice in each weight group. The dashed lines on the neoplasm risk graphs show the 15% to 20% risk region that was used to estimate the idealized weight. The values for the idealized weight curve are shown in Table D4, and are compared with historical control values for *ad libitum*-fed male $B6C3F_1$ mice from the NCTR colony. The 5% and 12% confidence intervals are also shown in the table and it can be seen that the *ad libitum* values are within the +12% confidence interval at most ages. The feed allocation required to maintain mice at their idealized weight was calculated from the relative feed consumption of *ad libitum*-fed and 40% calorically restricted mice as outlined in the methods section. The values were rounded to the nearest 0.5 g because the food pellets were sorted in 0.5-g increments.

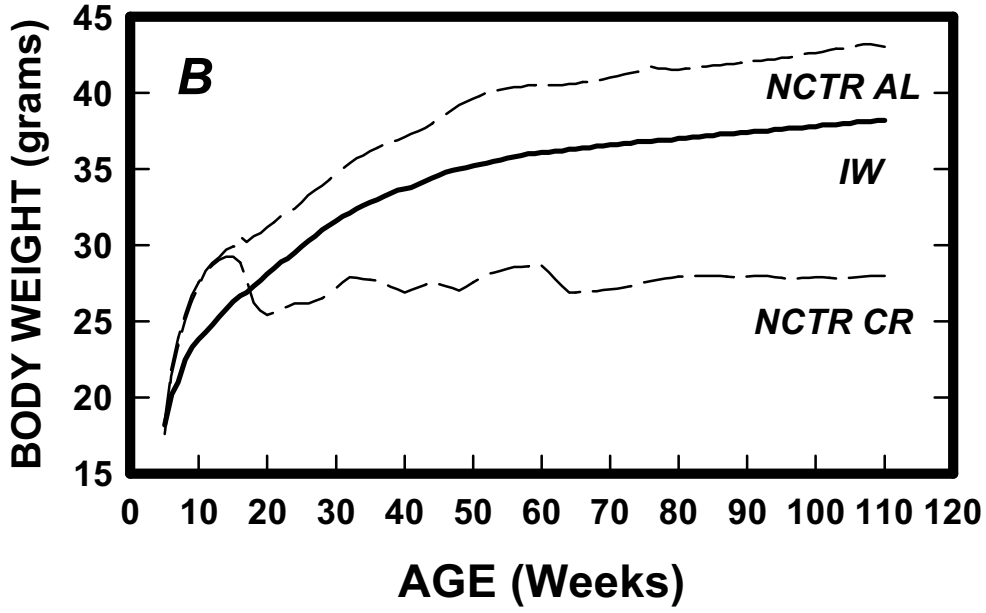


FIGURE D8
Idealized Body Weight Curve for Male B6C3F$_1$ Mice
The idealized body weight curve is shown by the solid line. *Ad libitum*-fed and 40% calorically restricted mice (dashed lines) are shown for comparison. Feed consumption data from these mice were used to construct a feeding schedule for manipulating the mice to fit the idealized body weight curve.

TABLE D4
Weekly Body Weight and Feed Allocation Values for Dietary Control

Weeks of Age	Weights		Confidence Limits for Idealized Weight Values				Feed Allocation		
	Ad Libitum[a]	Idealized Weight[b]	+5%	-5%	+12%	-12%	*Ad Libitum*[c]	Idealized Weight[d]	Idealized Weight[e]
5	17.6	18.2	19.1	17.3	20.4	16.0	4.0	3.9	4.0
6	21.4	20.2	21.2	19.2	22.6	17.8	4.0	3.4	3.5
7	23.4	21.0	22.1	20.0	23.5	18.5	4.3	3.2	3.0
8	25.1	22.5	23.6	21.4	25.2	19.8	4.6	3.4	3.5
9	26.3	23.3	24.5	22.1	26.1	20.5	4.8	3.4	3.5
10	27.4	23.8	25.0	22.6	26.7	20.9	5.1	3.4	3.5
11	28.3	24.3	25.5	23.1	27.2	21.4	5.4	3.5	3.5
12	28.8	24.8	26.0	23.6	27.8	21.8	5.9	3.9	3.5
13	29.2	25.3	26.6	24.0	28.3	22.3	5.6	3.7	3.5
14	29.7	25.8	27.1	24.5	28.9	22.7	5.8	3.9	4.0
15	29.9	26.3	27.6	25.0	29.5	23.1	5.5	3.8	4.0
16	30.6	26.7	28.0	25.4	29.9	23.5	5.7	3.9	4.0
17	30.2	26.9	28.2	25.6	30.1	23.7	5.7	4.1	4.0
18	30.6	27.3	28.7	25.9	30.6	24.0	6.1	4.3	4.5
19	30.8	27.7	29.1	26.3	31.0	24.4	6.1	4.6	4.5
20	31.2	28.1	29.5	26.7	31.5	24.7	6.0	4.7	4.5
21	31.5	28.5	29.9	27.1	31.9	25.1	5.9	4.7	4.5
22	31.9	28.9	30.3	27.5	32.4	25.4	6.0	4.8	5.0
23	32.1	29.1	30.6	27.6	32.6	25.6	5.8	4.7	4.5
24	32.4	29.5	31.0	28.0	33.0	26.0	5.8	4.7	4.5
25	32.8	29.9	31.4	28.4	33.5	26.3	5.8	4.8	5.0
26	33.3	30.3	31.8	28.8	33.9	26.7	5.8	4.8	5.0
27	33.6	30.6	32.1	29.1	34.3	26.9	5.8	4.8	5.0
28	33.9	31.0	32.6	29.5	34.7	27.3	5.8	4.9	5.0
29	34.3	31.3	32.9	29.7	35.1	27.5	5.8	4.9	5.0
30	34.6	31.6	33.2	30.0	35.4	27.8	5.8	4.9	5.0
31	35.0	31.9	33.5	30.3	35.7	28.1	6.1	5.1	5.0
32	35.4	32.1	33.7	30.5	36.0	28.2	6.1	5.0	5.0
33	35.7	32.4	34.0	30.8	36.3	28.5	6.1	5.1	5.0
34	35.9	32.6	34.2	31.0	36.5	28.7	6.1	5.1	5.0
35	36.2	32.8	34.4	31.2	36.7	28.9	6.0	5.0	5.0
36	36.4	33.0	34.7	31.4	37.0	29.0	6.0	5.1	5.0
37	36.6	33.2	34.9	31.5	37.2	29.2	6.0	5.1	5.0
38	36.7	33.4	35.1	31.7	37.4	29.4	6.0	5.2	5.0
39	36.9	33.6	35.3	31.9	37.6	29.6	5.8	5.0	5.0
40	37.1	33.7	35.4	32.0	37.7	29.7	5.8	5.0	5.0
41	37.3	33.8	35.5	32.1	37.9	29.7	5.8	5.0	5.0
42	37.5	34.0	35.7	32.3	38.1	29.9	5.8	5.0	5.0
43	37.7	34.2	35.9	32.5	38.3	30.1	5.8	5.0	5.0
44	38.0	34.4	36.1	32.7	38.5	30.3	5.8	5.0	5.0
45	38.3	34.6	36.3	32.9	38.8	30.4	5.8	5.0	5.0
46	38.6	34.8	36.5	33.1	39.0	30.6	5.8	5.0	5.0
47	38.9	34.9	36.6	33.2	39.1	30.7	5.9	5.1	5.0
48	39.2	35.0	36.8	33.3	39.2	30.8	5.9	5.1	5.0
49	39.4	35.1	36.9	33.3	39.3	30.9	5.9	5.1	5.0
50	39.6	35.2	37.0	33.4	39.4	31.0	6.5	5.6	5.0
51	39.8	35.3	37.1	33.5	39.5	31.1	5.7	4.8	5.0
52	40.0	35.4	37.2	33.6	39.6	31.2	5.7	4.8	5.0

TABLE D4
Weekly Body Weight and Feed Allocation Values for Dietary Control

Weeks of Age	Weights		Confidence Limits for Idealized Weight Values				Feed Allocation		
	Ad Libitum	Idealized Weight	+5%	-5%	+12%	-12%	*Ad Libitum*	Idealized Weight	Idealized Weight
53	40.1	35.5	37.3	33.7	39.8	31.2	5.7	4.8	5.0
54	40.2	35.6	37.4	33.8	39.9	31.3	5.7	4.8	5.0
55	40.3	35.7	37.5	33.9	40.0	31.4	6.1	5.2	5.0
56	40.4	35.8	37.6	34.0	40.1	31.5	6.1	5.2	5.0
57	40.4	35.9	37.7	34.1	40.2	31.6	6.1	5.2	5.0
58	40.5	36.0	37.8	34.2	40.3	31.7	6.1	5.2	5.0
59	40.5	36.0	37.8	34.2	40.3	31.7	5.9	5.0	5.0
60	40.5	36.1	37.9	34.3	40.4	31.8	5.9	5.0	5.0
61	40.5	36.1	37.9	34.3	40.4	31.8	5.9	5.1	5.0
62	40.5	36.2	38.0	34.4	40.5	31.9	5.9	5.1	5.0
63	40.5	36.2	38.0	34.4	40.5	31.9	5.7	5.0	5.0
64	40.6	36.3	38.1	34.5	40.7	31.9	5.7	5.0	5.0
65	40.6	36.3	38.1	34.5	40.7	31.9	5.7	5.0	5.0
66	40.7	36.4	38.2	34.6	40.8	32.0	5.7	5.0	5.0
67	40.7	36.4	38.2	34.6	40.8	32.0	5.8	5.1	5.0
68	40.8	36.5	38.3	34.7	40.9	32.1	5.8	5.1	5.0
69	40.9	36.5	38.3	34.7	40.9	32.1	5.8	5.1	5.0
70	41.0	36.6	38.4	34.8	41.0	32.2	5.8	5.1	5.0
71	41.1	36.6	38.4	34.8	41.0	32.2	5.4	4.7	4.5
72	41.2	36.7	38.5	34.9	41.1	32.3	5.4	4.7	4.5
73	41.3	36.7	38.5	34.9	41.1	32.3	5.4	4.7	4.5
74	41.4	36.8	38.6	35.0	41.2	32.4	5.4	4.7	4.5
75	41.5	36.8	38.6	35.0	41.2	32.4	6.0	5.2	5.0
76	41.7	36.8	38.6	35.0	41.2	32.4	6.0	5.2	5.0
77	41.6	36.9	38.7	35.1	41.3	32.5	6.0	5.2	5.0
78	41.6	36.9	38.7	35.1	41.3	32.5	6.0	5.2	5.0
79	41.5	36.9	38.7	35.1	41.3	32.5	6.0	5.2	5.0
80	41.5	37.0	38.9	35.2	41.4	32.6	6.0	5.2	5.0
81	41.6	37.0	38.9	35.2	41.4	32.6	6.1	5.3	5.5
82	41.6	37.1	39.0	35.2	41.6	32.6	6.1	5.3	5.0
83	41.7	37.1	39.0	35.2	41.6	32.6	6.2	5.4	5.5
84	41.7	37.2	39.1	35.3	41.7	32.7	6.2	5.4	5.0
85	41.8	37.2	39.1	35.3	41.7	32.7	6.2	5.4	5.5
86	41.8	37.3	39.2	35.4	41.8	32.8	6.2	5.4	5.5
87	41.9	37.3	39.2	35.4	41.8	32.8	6.1	5.3	5.0
88	41.9	37.3	39.2	35.4	41.8	32.8	6.1	5.3	5.5
89	42.0	37.4	39.3	35.5	41.9	32.9	6.1	5.3	5.0
90	42.0	37.4	39.3	35.5	41.9	32.9	6.1	5.3	5.5
91	42.1	37.5	39.4	35.6	42.0	33.0	6.2	5.4	5.0
92	42.1	37.5	39.4	35.6	42.0	33.0	6.2	5.4	5.5
93	42.2	37.5	39.4	35.6	42.0	33.0	6.2	5.4	5.5
94	42.2	37.6	39.5	35.7	42.1	33.1	6.2	5.4	5.5
95	42.3	37.6	39.5	35.7	42.1	33.1	5.6	4.9	5.0
96	42.3	37.7	39.6	35.8	42.2	33.2	5.6	4.9	5.0
97	42.4	37.7	39.6	35.8	42.2	33.2	5.6	4.9	5.0
98	42.5	37.7	39.6	35.8	42.2	33.2	5.6	4.9	5.0
99	42.6	37.8	39.7	35.9	42.3	33.3	6.0	5.2	5.0
100	42.6	37.8	39.7	35.9	42.3	33.3	6.0	5.2	5.0
101	42.7	37.9	39.8	36.0	42.4	33.4	6.0	5.2	5.0
102	42.8	37.9	39.8	36.0	42.4	33.4	6.0	5.2	5.5

TABLE D4
Weekly Body Weight and Feed Allocation Values for Dietary Control

Weeks of Age	Weights		Confidence Limits for Idealized Weight Values				Feed Allocation		
	Ad Libitum	Idealized Weight	+5%	-5%	+12%	-12%	*Ad Libitum*	Idealized Weight	Idealized Weight
103	42.9	37.9	39.8	36.0	42.4	33.4	6.0	5.2	5.0
104	42.9	38.0	39.9	36.1	42.6	33.4	6.0	5.2	5.0
105	43.0	38.0	39.9	36.1	42.6	33.4	6.0	5.2	5.5
106	43.1	38.1	40.0	36.2	42.7	33.5	6.0	5.2	5.0
107	43.2	38.1	40.0	36.2	42.7	33.5	5.4	4.7	4.5
108	43.2	38.1	40.0	36.2	42.7	33.5	5.4	4.7	4.5
109	43.1	38.2	40.1	36.3	42.8	33.6	5.4	4.7	4.5
110	43.0	38.2	40.1	36.3	42.8	33.6	5.4	4.7	4.5

[a] Historical body weight from *ad libitum*-fed male mice from the NCTR colony
[b] Target idealized body weight
[c] Feed consumption of *ad libitum*-fed male mice
[d] Feed allocation calculated to produce target idealized body weights
[e] Feed allocation rounded to the nearest 0.5 g

RESULTS

One of the objectives of this study was to investigate the feasibility of using dietary control to reduce body weight variation and increase survival in routine 2-year bioassays and to further study the relationship between body weight and liver neoplasms in mice.

Feasibility

The dietary control procedures involved daily feeding of a set number of feed pellets. Since the mice were being dosed by gavage, they were routinely weighed on each dosing day and their body weights entered into the NCTR *INLIFE* computer system. A program was written as part of this system to calculate and print a weekly feeding schedule for the dietary-controlled mice. This program used the Monday morning weights to identify mice that weighed outside the 5% and 12% confidence limits of the weight curve and to adjust their feed allocation. This program was run weekly by the NCTR Computer Center staff to provide the animal care technicians with a weekly feeding schedule by Wednesday of each week. Each new feeding schedule was started on Thursday. Each week, the Computer Center staff also provided the principal investigator with an ASCII file of the body weights via electronic mail. This file was used to construct the body weight profiles on a spreadsheet program. This macro-driven program just took a few minutes to run and provided a remote mechanism to closely monitor the study. For example, early in the study an uncharacteristic drop in body weight was observed in many of the mice in one of the two animal rooms (because the study used staggered loading this drop was spread across three different ages and is not apparent on the final weight curve). An investigation was immediately initiated and it was found that the gavage needle had been replaced on the gavaging instrument the week before with a larger one according to a schedule designed for *ad libitum*-fed mice. The appropriate size needle had not been available and a slightly larger one had been substituted. This, coupled with the small size of many of the dietary-controlled mice, had probably resulted in throat irritation for the affected mice. The needle was replaced with a smaller one and the mice recovered their body weights by the following week.

The NIH-31 feed pellets were sorted into pellets of different weights in 0.5-g increments from 2 to 5 grams by a mechanical pellet sorter available at the NCTR. The sorted pellets were stored in labeled containers in the animal rooms so that each afternoon the animal care technicians could rapidly provide the required feed aliquot for each mouse as part of the daily observation schedule. Any food remaining from the previous day was removed and the fact that food remained was recorded for each mouse. These procedures took less than 30 minutes per animal room (i.e., 120 dietary-controlled mice per room).

TABLE D5
Body Weights of *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

Weeks of Age	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg	
	Body Weight	n	Body Weight	n	Body Weight	n	Body Weight	n
4	14.7 ± 2.5	60	14.5 ± 2.4	60	14.6 ± 1.8	60	14.8 ± 2.2	60
5	19.2 ± 1.9	60	19.5 ± 2.2	60	19.6 ± 1.9	60	19.4 ± 1.8	60
6	21.8 ± 1.6	60	21.8 ± 1.6	60	21.8 ± 1.5	60	21.7 ± 1.3	60
7	23.6 ± 1.6	60	23.4 ± 1.6	60	23.4 ± 1.2	60	23.1 ± 1.4	60
8	24.9 ± 1.6	60	24.7 ± 1.6	60	24.6 ± 1.3	60	24.4 ± 1.4	60
9	26.0 ± 1.6	60	25.8 ± 1.6	60	25.8 ± 1.4	60	25.4 ± 1.4	60
10	27.0 ± 1.6	60	26.8 ± 1.6	60	26.6 ± 1.3	60	26.3 ± 1.4	60
11	27.8 ± 1.6	60	27.6 ± 1.6	60	27.5 ± 1.5	60	27.0 ± 1.4	60
12	28.4 ± 1.6	60	28.1 ± 1.7	60	27.9 ± 1.7	60	27.4 ± 1.5	60
13	28.8 ± 1.8	60	28.5 ± 1.8	60	28.3 ± 1.7	60	27.9 ± 1.6	60
14	29.3 ± 2.0	60	29.1 ± 1.8	60	28.8 ± 1.9	60	28.2 ± 1.7	60
15	29.6 ± 1.8	60	29.3 ± 2.1	60	29.1 ± 2.1	60	28.6 ± 1.9	60
16	29.9 ± 1.9	60	29.7 ± 2.2	60	29.4 ± 2.2	60	28.9 ± 1.8	60
17	30.4 ± 2.0	60	30.2 ± 2.4	60	29.9 ± 2.4	60	29.4 ± 2.1	60
18	30.9 ± 2.0	60	30.8 ± 2.5	60	30.4 ± 2.3	60	30.0 ± 2.1	60
19	31.3 ± 2.2	60	31.2 ± 2.4	60	30.7 ± 2.6	60	30.4 ± 2.2	60
20	31.6 ± 2.3	60	31.7 ± 2.5	60	31.3 ± 2.6	60	30.8 ± 2.2	60
21	32.0 ± 2.5	60	32.1 ± 2.8	60	31.6 ± 2.8	60	31.2 ± 2.3	60
22	32.4 ± 2.4	60	32.5 ± 2.9	60	32.3 ± 3.0	60	31.7 ± 2.4	60
23	33.0 ± 2.5	60	33.0 ± 3.0	60	32.7 ± 3.0	60	32.2 ± 2.6	60
24	33.5 ± 2.8	60	33.5 ± 3.0	60	33.1 ± 3.2	60	32.6 ± 2.8	60
25	33.7 ± 3.0	60	33.8 ± 3.1	60	33.6 ± 3.3	60	33.1 ± 2.8	60
26	34.2 ± 3.1	60	34.3 ± 3.2	60	34.0 ± 3.5	60	33.5 ± 2.9	60
27	34.7 ± 3.2	60	34.8 ± 3.5	60	34.6 ± 3.6	60	34.0 ± 2.9	60
28	35.1 ± 3.4	60	35.2 ± 3.4	60	35.1 ± 3.6	60	34.8 ± 3.0	60
29	35.6 ± 3.3	60	35.7 ± 3.6	60	35.8 ± 3.8	60	35.2 ± 3.1	60
30	35.9 ± 3.4	60	36.2 ± 3.5	60	35.9 ± 3.7	60	35.7 ± 3.1	60
31	36.3 ± 3.4	60	36.7 ± 3.6	60	36.5 ± 3.8	60	36.1 ± 3.1	60
32	36.7 ± 3.2	60	37.0 ± 3.4	60	36.7 ± 3.6	60	36.4 ± 3.1	60
33	37.0 ± 3.5	60	37.2 ± 3.6	60	37.0 ± 3.9	60	36.5 ± 3.1	60
34	37.1 ± 3.5	60	37.4 ± 3.7	60	37.3 ± 3.9	60	36.8 ± 3.2	60
35	37.4 ± 3.5	60	37.7 ± 3.8	60	37.5 ± 3.9	60	37.2 ± 3.2	60
36	37.4 ± 3.5	60	37.8 ± 3.8	60	37.6 ± 4.1	60	37.2 ± 3.2	60
37	37.6 ± 3.6	60	38.0 ± 3.7	60	37.8 ± 3.9	60	37.5 ± 3.3	60
38	37.9 ± 3.7	60	38.3 ± 3.8	60	38.0 ± 4.1	60	37.9 ± 3.2	60
39	37.9 ± 3.5	60	38.5 ± 3.9	60	38.3 ± 4.1	60	38.1 ± 3.3	60
40	37.9 ± 3.6	60	38.5 ± 3.8	60	38.3 ± 4.1	60	38.2 ± 3.2	60
41	37.9 ± 3.5	60	38.4 ± 3.6	60	38.4 ± 4.2	60	38.2 ± 3.3	60
42	38.1 ± 3.6	60	38.6 ± 3.8	60	38.3 ± 4.3	60	38.2 ± 3.3	60
43	38.2 ± 3.6	60	38.7 ± 3.9	60	38.4 ± 4.5	60	38.3 ± 3.4	60
44	38.7 ± 3.5	60	38.9 ± 4.0	60	38.6 ± 4.1	60	38.4 ± 3.6	60
45	38.6 ± 3.6	60	38.9 ± 3.9	60	38.8 ± 4.1	60	38.5 ± 3.7	60
46	38.7 ± 3.6	60	39.0 ± 4.1	60	39.1 ± 4.1	60	38.8 ± 3.6	60
47	39.1 ± 3.7	60	39.2 ± 4.1	60	39.3 ± 4.2	60	39.0 ± 3.5	60
48	39.6 ± 3.7	60	39.6 ± 3.9	60	39.7 ± 4.3	60	39.5 ± 3.5	60
49	39.9 ± 3.6	60	40.0 ± 3.9	60	40.0 ± 4.2	60	39.6 ± 3.4	60
50	40.0 ± 3.6	60	40.1 ± 3.8	60	40.2 ± 4.2	60	39.8 ± 3.4	60
51	40.1 ± 3.8	60	40.2 ± 3.8	60	40.5 ± 4.4	59	40.0 ± 3.3	60
52	40.3 ± 3.7	60	40.5 ± 4.0	60	40.9 ± 4.3	59	40.4 ± 3.4	60
53	40.3 ± 3.8	60	40.7 ± 3.9	60	41.1 ± 4.3	59	40.8 ± 3.5	60
54	40.9 ± 3.8	60	40.9 ± 3.8	60	41.4 ± 4.3	59	40.8 ± 3.4	60
55	41.2 ± 3.8	60	41.1 ± 3.8	60	41.4 ± 4.1	59	40.9 ± 3.4	60
56	41.3 ± 3.8	60	41.4 ± 3.9	60	41.6 ± 4.1	59	41.1 ± 3.3	60
57	41.4 ± 3.7	60	41.4 ± 3.9	60	41.7 ± 4.1	59	41.2 ± 3.2	60
58	41.3 ± 3.7	60	41.6 ± 3.9	60	41.6 ± 4.1	59	41.3 ± 3.2	60

TABLE D5
Body Weights of *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

Weeks of Age	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg	
	Body Weight	n	Body Weight	n	Body Weight	n	Body Weight	n
59	41.2 ± 3.7	60	41.3 ± 3.9	60	41.5 ± 4.2	59	41.1 ± 3.3	60
60	41.1 ± 3.7	60	41.4 ± 4.0	60	41.5 ± 4.1	59	41.0 ± 3.2	60
61	41.1 ± 3.6	60	41.2 ± 3.8	60	41.5 ± 4.1	59	40.9 ± 3.2	60
62	41.1 ± 3.6	60	41.3 ± 3.9	60	41.5 ± 4.0	59	41.0 ± 3.4	60
63	41.2 ± 3.7	60	41.3 ± 4.0	60	41.7 ± 4.1	59	41.1 ± 3.4	60
64	41.3 ± 3.7	60	41.5 ± 4.1	60	41.6 ± 4.2	59	41.3 ± 3.3	60
65	41.4 ± 3.7	60	41.7 ± 4.0	60	41.9 ± 4.2	59	41.5 ± 3.3	60
66	41.6 ± 3.7	60	41.8 ± 3.9	60	42.0 ± 4.1	59	41.5 ± 3.2	60
67	41.7 ± 3.6	60	42.0 ± 3.9	60	42.0 ± 4.2	59	41.6 ± 3.4	60
68	41.7 ± 3.8	60	42.1 ± 3.9	60	42.2 ± 4.3	59	41.5 ± 3.4	60
69	41.6 ± 3.7	60	42.1 ± 4.0	60	42.0 ± 4.2	59	41.5 ± 3.5	60
70	41.8 ± 3.7	60	42.3 ± 3.9	60	42.2 ± 4.4	59	41.7 ± 3.6	60
71[b]	41.7 ± 3.9	60	42.1 ± 3.9	60	42.4 ± 4.6	59	41.7 ± 3.1	60
72	41.9 ± 4.0	48	42.5 ± 4.1	48	42.5 ± 4.7	47	42.0 ± 3.2	48
73	42.0 ± 4.1	47	42.4 ± 4.0	48	42.5 ± 4.5	47	41.7 ± 3.3	48
74	42.2 ± 4.2	47	42.7 ± 4.1	48	42.5 ± 4.6	47	41.9 ± 3.3	48
75	42.4 ± 4.1	47	42.8 ± 4.1	48	42.6 ± 4.4	47	42.0 ± 3.4	48
76	42.3 ± 3.9	47	42.7 ± 4.2	48	42.6 ± 4.3	47	42.0 ± 3.4	48
77	42.5 ± 3.8	47	42.9 ± 4.2	48	42.9 ± 4.6	47	42.1 ± 3.6	48
78	42.6 ± 4.1	47	43.0 ± 4.4	48	43.0 ± 4.6	47	42.2 ± 3.8	48
79	42.8 ± 3.8	47	43.2 ± 4.3	48	43.2 ± 4.8	47	42.4 ± 3.8	48
80	43.0 ± 3.9	47	43.1 ± 4.3	48	43.4 ± 4.8	47	42.6 ± 4.1	48
81	42.9 ± 3.9	47	43.1 ± 4.2	48	43.3 ± 4.7	47	42.4 ± 4.1	48
82	43.3 ± 3.8	47	43.2 ± 4.2	48	43.3 ± 4.8	47	42.6 ± 4.3	48
83	43.2 ± 3.8	47	43.1 ± 4.3	48	43.3 ± 4.9	47	42.5 ± 4.3	48
84	43.4 ± 4.0	47	43.3 ± 4.4	48	43.3 ± 4.9	47	42.7 ± 4.5	48
85	43.4 ± 4.0	47	43.3 ± 4.7	48	43.2 ± 4.9	47	42.7 ± 4.5	48
86	43.6 ± 4.1	47	43.2 ± 4.9	48	43.3 ± 5.0	47	42.6 ± 4.4	48
87	43.5 ± 4.0	47	43.2 ± 5.1	48	43.4 ± 5.2	47	42.8 ± 4.5	48
88	43.4 ± 3.9	47	42.9 ± 5.4	48	43.2 ± 5.1	47	42.6 ± 4.5	48
89	43.5 ± 3.9	47	42.9 ± 5.5	48	43.3 ± 5.2	47	42.5 ± 4.7	48
90	43.5 ± 4.1	47	42.8 ± 5.8	48	43.4 ± 5.5	47	42.7 ± 4.7	47
91	43.5 ± 4.1	47	42.8 ± 5.9	48	43.3 ± 5.6	47	42.7 ± 4.5	47
92	43.6 ± 4.1	47	43.3 ± 5.5	47	43.4 ± 5.6	47	42.7 ± 4.5	47
93	43.6 ± 4.2	47	43.1 ± 5.7	47	43.0 ± 5.7	47	42.5 ± 4.7	47
94	43.5 ± 4.2	47	43.4 ± 5.0	46	43.0 ± 5.7	46	42.4 ± 5.3	47
95	43.5 ± 4.3	47	43.5 ± 4.7	46	42.8 ± 5.9	46	42.8 ± 4.4	45
96	43.4 ± 4.2	47	43.4 ± 4.7	45	42.9 ± 6.1	45	42.8 ± 4.6	45
97	43.3 ± 4.4	47	43.3 ± 4.8	44	42.9 ± 6.4	44	42.7 ± 4.6	45
98	43.0 ± 4.4	47	43.4 ± 4.6	43	42.4 ± 6.4	43	42.6 ± 4.7	45
99	43.1 ± 4.2	46	43.2 ± 4.6	43	42.3 ± 6.5	43	42.4 ± 4.6	45
100	43.0 ± 4.3	46	43.3 ± 4.8	43	42.3 ± 6.6	43	42.3 ± 4.7	45
101	42.9 ± 4.4	45	42.9 ± 4.6	43	41.9 ± 6.6	43	41.7 ± 5.1	45
102	42.7 ± 4.4	44	42.8 ± 4.8	43	41.8 ± 6.9	43	41.9 ± 5.2	44
103	42.4 ± 4.4	44	42.4 ± 4.6	42	41.6 ± 6.8	43	41.7 ± 5.1	44
104	42.1 ± 4.6	44	42.2 ± 5.1	39	41.7 ± 6.5	43	41.4 ± 5.2	44
105	42.0 ± 4.6	44	42.1 ± 5.2	38	41.7 ± 6.5	40	41.2 ± 5.3	44
106	42.1 ± 4.7	44	42.1 ± 5.4	37	42.1 ± 5.9	39	41.2 ± 5.0	44
107	42.1 ± 4.8	43	41.8 ± 5.6	37	41.7 ± 6.5	38	41.1 ± 5.2	44
108	42.4 ± 4.9	43	41.6 ± 5.7	37	41.9 ± 6.5	36	41.1 ± 5.3	44
109	42.3 ± 4.9	42	41.5 ± 5.7	37	42.1 ± 6.7	36	41.1 ± 5.4	44
110	42.0 ± 4.8	42	41.0 ± 5.8	37	41.5 ± 6.6	36	40.8 ± 5.4	44

[a] Body weights are given in grams as mean ± standard deviation.
[b] Interim evaluation occurred when mice were 71 weeks old.

TABLE D6
Body Weights of Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

Weeks of Age	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg	
	Body Weight	n	Body Weight	n	Body Weight	n	Body Weight	n
4	14.4 ± 2.2	60	15.0 ± 2.4	60	14.6 ± 2.3	60	14.3 ± 2.3	60
5	18.9 ± 1.6	60	19.1 ± 2.9	60	19.6 ± 3.6	60	18.9 ± 1.6	60
6	20.3 ± 1.0	60	20.2 ± 1.3	60	20.1 ± 1.1	60	20.2 ± 0.7	60
7	20.4 ± 0.7	60	20.6 ± 0.7	60	20.2 ± 0.9	60	20.2 ± 1.1	60
8	20.0 ± 0.8	60	20.0 ± 0.8	60	19.8 ± 0.8	60	19.7 ± 0.9	60
9	21.6 ± 0.7	60	21.6 ± 0.8	60	21.3 ± 0.9	60	21.2 ± 1.0	60
10	22.9 ± 0.7	60	22.8 ± 0.9	60	22.7 ± 0.8	60	22.5 ± 0.9	60
11	23.4 ± 0.8	60	23.6 ± 0.8	60	23.2 ± 0.9	60	23.1 ± 0.6	60
12	23.7 ± 0.8	60	23.6 ± 0.8	60	23.4 ± 0.9	60	23.3 ± 0.6	60
13	24.0 ± 0.8	60	24.0 ± 0.9	60	23.6 ± 0.8	60	23.7 ± 0.8	60
14	24.2 ± 0.8	60	24.4 ± 0.9	60	24.0 ± 0.8	60	24.0 ± 0.8	60
15	25.3 ± 0.9	60	25.4 ± 1.0	60	25.1 ± 0.7	60	25.1 ± 0.8	60
16	25.9 ± 0.8	60	25.8 ± 1.0	60	25.5 ± 0.8	60	25.3 ± 0.8	60
17	26.1 ± 0.8	60	26.2 ± 0.9	60	25.9 ± 0.9	60	25.7 ± 0.8	60
18	26.4 ± 0.9	60	26.5 ± 0.9	60	26.1 ± 0.9	60	26.1 ± 0.9	60
19	27.1 ± 0.8	60	27.2 ± 0.9	60	26.7 ± 0.9	60	26.8 ± 1.0	60
20	27.3 ± 1.3	60	27.2 ± 1.2	60	26.7 ± 1.7	60	26.7 ± 1.2	60
21	27.4 ± 1.2	60	27.6 ± 1.5	60	27.2 ± 1.5	60	27.0 ± 1.4	60
22	27.9 ± 1.4	60	28.0 ± 1.3	60	27.6 ± 1.5	60	27.5 ± 1.7	60
23	28.7 ± 1.1	60	28.8 ± 1.3	60	28.5 ± 1.2	60	28.3 ± 1.3	60
24	28.9 ± 1.2	60	29.0 ± 1.4	60	28.7 ± 1.3	60	28.5 ± 1.3	60
25	29.1 ± 1.2	60	29.1 ± 1.3	60	28.8 ± 1.3	60	28.6 ± 1.4	60
26	29.7 ± 1.2	60	29.8 ± 1.5	60	29.4 ± 1.6	60	29.1 ± 1.5	60
27	29.8 ± 1.3	60	30.3 ± 1.5	59	29.8 ± 1.7	60	29.3 ± 1.5	60
28	30.2 ± 1.4	60	30.4 ± 1.7	59	30.0 ± 1.5	60	29.6 ± 1.6	60
29	30.6 ± 1.5	60	30.8 ± 1.7	59	30.4 ± 1.5	60	30.0 ± 1.7	60
30	30.9 ± 1.7	60	31.1 ± 1.7	59	30.7 ± 1.7	60	30.2 ± 1.8	60
31	31.2 ± 1.8	60	31.5 ± 1.8	59	31.0 ± 1.9	60	30.5 ± 1.9	60
32	31.3 ± 1.8	60	31.7 ± 1.8	59	31.2 ± 2.0	60	30.7 ± 2.0	60
33	31.7 ± 1.8	60	31.9 ± 1.9	59	31.4 ± 2.0	60	31.1 ± 2.1	60
34	31.9 ± 1.9	60	32.2 ± 1.9	59	31.7 ± 2.0	60	31.2 ± 2.1	60
35	32.0 ± 2.2	60	32.4 ± 1.9	59	31.8 ± 2.2	60	31.5 ± 2.3	60
36	32.3 ± 1.9	59	32.8 ± 2.1	59	32.0 ± 2.1	60	31.6 ± 2.2	60
37	32.6 ± 1.9	59	33.1 ± 2.2	59	32.3 ± 2.2	60	31.7 ± 2.2	60
38	32.7 ± 2.1	59	33.4 ± 2.0	59	32.5 ± 2.3	60	32.0 ± 2.1	60
39	32.8 ± 2.0	59	33.6 ± 2.1	59	32.8 ± 2.2	60	32.1 ± 2.3	60
40	32.7 ± 2.1	59	33.5 ± 2.2	59	32.8 ± 2.2	60	32.3 ± 2.3	60
41	32.9 ± 2.0	59	33.7 ± 2.1	59	32.8 ± 2.2	60	32.3 ± 2.3	60
42	33.0 ± 2.0	59	33.9 ± 1.9	59	33.0 ± 2.1	60	32.3 ± 2.1	60
43	33.4 ± 1.9	59	34.3 ± 2.2	59	33.2 ± 2.1	60	32.6 ± 2.1	60
44	33.8 ± 2.0	59	34.4 ± 2.0	59	33.6 ± 2.2	60	32.9 ± 2.1	60
45	33.9 ± 2.2	59	34.7 ± 2.0	59	33.6 ± 2.1	60	33.2 ± 2.2	59
46	34.2 ± 2.1	59	34.9 ± 1.9	59	34.0 ± 2.2	60	33.4 ± 2.3	59
47	34.4 ± 2.2	59	35.2 ± 2.1	59	34.0 ± 2.3	60	33.6 ± 2.4	59
48	34.7 ± 2.1	59	35.7 ± 2.1	59	34.7 ± 2.4	60	34.1 ± 2.5	59
49	35.1 ± 2.2	59	35.9 ± 1.9	59	34.8 ± 2.5	60	34.3 ± 2.4	59
50	35.2 ± 2.2	59	36.2 ± 2.2	59	34.8 ± 2.4	60	34.5 ± 2.4	59
51	35.2 ± 2.3	59	36.1 ± 2.1	59	34.8 ± 2.3	60	34.6 ± 2.4	59
52	35.5 ± 2.2	59	36.1 ± 2.2	59	35.0 ± 2.3	60	34.6 ± 2.6	59
53	35.4 ± 2.3	59	36.3 ± 2.2	59	35.3 ± 2.5	60	34.8 ± 2.6	59
54	35.6 ± 2.5	59	36.5 ± 2.4	59	35.7 ± 2.6	60	35.0 ± 2.5	59
55	35.8 ± 2.5	59	36.7 ± 2.4	59	35.7 ± 2.6	60	35.2 ± 2.7	59
56	35.9 ± 2.4	59	36.8 ± 2.3	59	35.8 ± 2.7	60	35.4 ± 2.8	59
57	36.0 ± 2.4	59	36.9 ± 2.5	59	35.8 ± 2.7	60	35.5 ± 2.7	59
58	36.1 ± 2.4	59	36.9 ± 2.3	59	36.0 ± 2.8	59	35.4 ± 2.7	59

TABLE D6
Body Weights of Dietary-Controlled Male Mice in the 2-Year Gavage Study of Chloral Hydrate

Weeks of Age	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg	
	Body Weight	n	Body Weight	n	Body Weight	n	Body Weight	n
59	36.1 ± 2.5	59	36.9 ± 2.3	59	36.0 ± 2.6	59	35.4 ± 2.8	59
60	36.1 ± 2.3	59	36.6 ± 2.1	59	35.8 ± 2.5	59	35.4 ± 2.7	59
61	35.9 ± 2.3	59	36.5 ± 2.0	59	35.6 ± 2.4	59	35.3 ± 2.6	59
62	35.9 ± 2.2	59	36.4 ± 2.1	59	35.6 ± 2.5	59	35.2 ± 2.7	59
63	35.9 ± 2.2	59	36.7 ± 2.1	59	35.5 ± 2.5	59	35.3 ± 2.6	59
64	36.0 ± 2.1	59	36.5 ± 2.0	59	35.7 ± 2.5	59	35.3 ± 2.5	59
65	36.2 ± 2.3	59	36.7 ± 2.0	59	35.8 ± 2.5	59	35.5 ± 2.6	59
66	36.1 ± 2.3	59	36.7 ± 2.1	59	35.7 ± 2.4	59	35.3 ± 2.5	59
67	36.2 ± 2.3	59	36.9 ± 2.0	59	35.8 ± 2.4	59	35.5 ± 2.5	59
68	36.1 ± 2.3	59	36.9 ± 2.0	59	35.8 ± 2.5	59	35.4 ± 2.7	59
69	36.4 ± 2.5	59	36.8 ± 2.1	59	35.9 ± 2.4	59	35.6 ± 2.7	59
70	36.5 ± 2.6	59	36.8 ± 2.1	59	36.1 ± 2.4	59	35.7 ± 2.7	58
71[b]	36.1 ± 2.6	59	36.9 ± 2.2	59	35.9 ± 2.2	59	35.8 ± 2.7	58
72	35.8 ± 2.4	47	36.4 ± 2.1	47	35.7 ± 2.0	47	35.5 ± 2.4	46
73	35.6 ± 2.3	47	36.3 ± 1.9	47	35.5 ± 1.9	47	35.1 ± 2.2	46
74	35.8 ± 2.3	47	36.3 ± 1.8	47	35.5 ± 2.0	47	35.4 ± 2.3	46
75	35.5 ± 2.3	47	36.5 ± 1.9	47	35.4 ± 2.1	47	35.3 ± 2.1	46
76	35.9 ± 2.4	47	36.5 ± 2.0	47	35.7 ± 2.2	47	35.4 ± 2.4	46
77	36.1 ± 2.5	47	36.7 ± 2.1	47	36.0 ± 2.3	47	35.7 ± 2.4	46
78	36.1 ± 2.4	47	36.9 ± 2.1	47	36.0 ± 2.2	47	35.7 ± 2.4	46
79	36.3 ± 2.7	47	37.0 ± 2.3	47	36.1 ± 2.2	47	36.0 ± 2.7	46
80	36.5 ± 2.5	47	37.1 ± 2.2	47	36.3 ± 2.2	47	36.1 ± 2.6	46
81	36.2 ± 2.9	47	36.8 ± 2.5	47	36.0 ± 2.5	47	35.9 ± 2.9	46
82	36.9 ± 2.9	47	37.6 ± 2.7	47	36.6 ± 2.6	47	36.5 ± 2.9	46
83	36.8 ± 3.1	47	37.3 ± 2.5	47	36.3 ± 2.5	47	36.2 ± 3.0	46
84	37.5 ± 2.9	47	38.2 ± 2.3	47	37.2 ± 2.4	47	36.9 ± 2.5	46
85	37.5 ± 2.7	47	38.1 ± 2.3	47	37.1 ± 2.4	47	37.1 ± 2.6	46
86	37.6 ± 3.0	47	38.3 ± 2.5	47	37.2 ± 2.4	47	37.3 ± 2.8	46
87	37.8 ± 3.0	47	38.3 ± 2.7	47	37.5 ± 2.6	47	37.6 ± 2.8	46
88	37.6 ± 2.9	47	38.3 ± 2.5	46	37.5 ± 2.6	47	37.5 ± 2.8	46
89	37.8 ± 3.0	47	38.7 ± 2.6	46	37.8 ± 2.5	47	37.4 ± 3.0	46
90	37.7 ± 2.9	47	38.5 ± 2.4	46	37.8 ± 2.5	47	37.6 ± 2.5	44
91	38.0 ± 2.9	47	38.2 ± 2.5	46	37.7 ± 2.5	47	37.6 ± 2.6	44
92	37.7 ± 2.8	47	38.2 ± 2.6	46	37.9 ± 2.4	47	37.8 ± 2.5	44
93	37.8 ± 2.9	47	38.5 ± 2.5	45	37.9 ± 2.5	47	37.7 ± 2.6	44
94	38.0 ± 3.1	47	38.4 ± 2.7	45	38.1 ± 2.5	47	37.9 ± 2.6	44
95	37.9 ± 3.0	47	38.5 ± 2.6	45	38.1 ± 2.6	47	37.7 ± 2.7	44
96	37.5 ± 2.8	47	38.3 ± 2.7	45	37.8 ± 2.3	47	37.6 ± 2.8	44
97	37.4 ± 2.7	47	38.0 ± 2.6	45	37.8 ± 2.2	47	37.2 ± 2.6	44
98	37.4 ± 2.6	47	37.8 ± 2.7	45	37.7 ± 2.1	47	37.2 ± 2.5	44
99	37.4 ± 2.4	47	37.8 ± 2.7	45	37.5 ± 2.1	47	37.1 ± 2.5	44
100	37.3 ± 2.5	47	37.6 ± 2.8	45	37.6 ± 2.2	47	37.0 ± 3.0	44
101	37.4 ± 2.6	47	37.6 ± 3.0	45	37.5 ± 2.4	47	37.1 ± 2.7	44
102	37.3 ± 2.6	47	37.4 ± 2.9	45	37.5 ± 2.5	47	37.1 ± 2.8	44
103	37.2 ± 2.5	47	37.3 ± 3.0	45	37.4 ± 2.3	47	37.1 ± 2.8	44
104	37.0 ± 2.8	47	37.2 ± 3.2	45	37.3 ± 2.3	47	36.9 ± 3.0	44
105	36.9 ± 2.8	47	37.2 ± 3.1	44	37.3 ± 2.5	47	36.8 ± 3.0	44
106	37.0 ± 3.0	47	37.4 ± 3.2	44	37.6 ± 2.7	47	37.1 ± 3.2	43
107	37.3 ± 2.9	47	37.6 ± 3.3	44	37.7 ± 2.6	47	37.0 ± 3.1	43
108	37.0 ± 2.6	46	37.2 ± 3.2	44	37.1 ± 2.3	47	36.6 ± 2.6	42
109	37.1 ± 2.4	46	37.2 ± 3.2	44	37.1 ± 2.1	47	36.4 ± 3.1	42
110	36.3 ± 2.5	46	36.7 ± 3.0	44	36.8 ± 2.4	47	35.8 ± 3.4	42

[a] Body weights are given in grams as mean ± standard deviation.
[b] Interim evaluation occurred when mice were 71 weeks old.

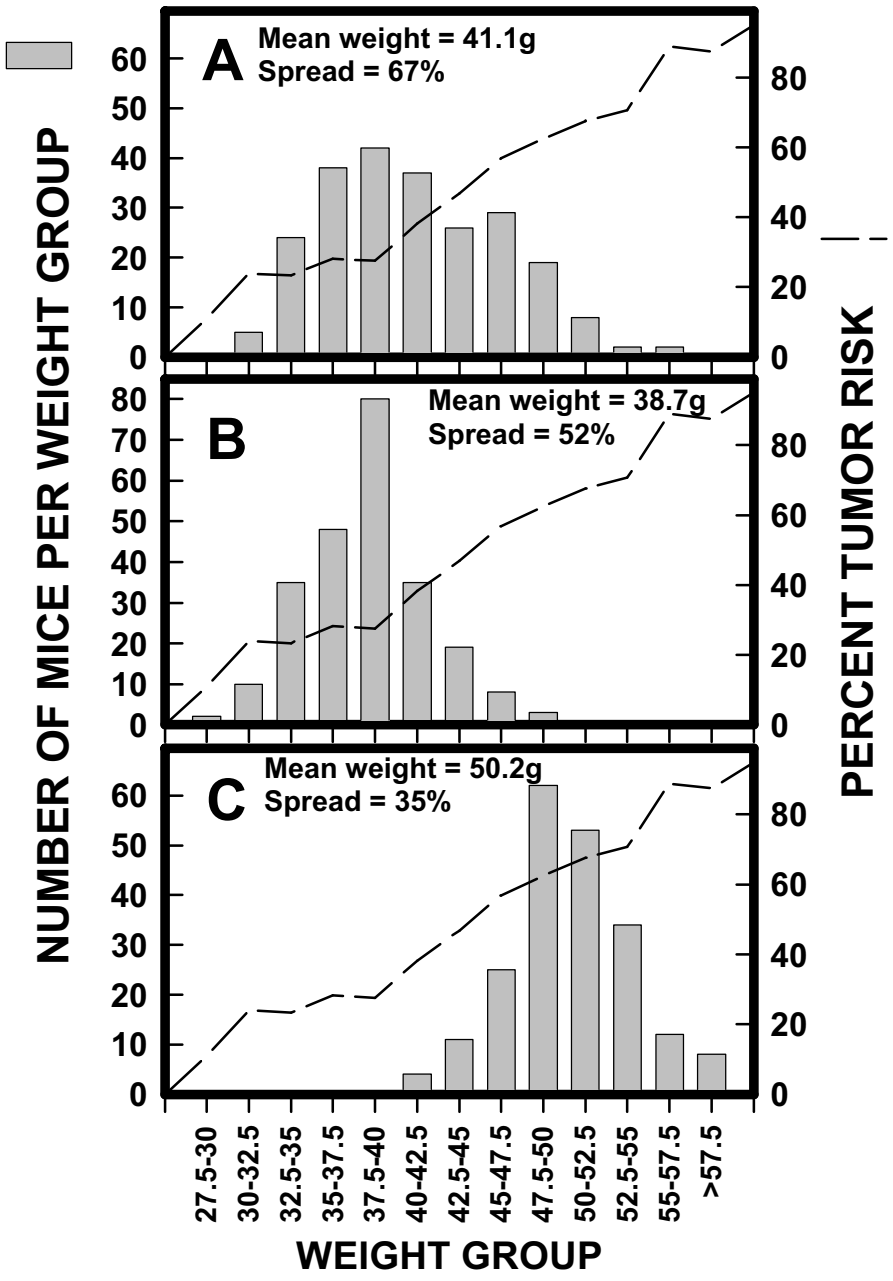


FIGURE D9
Comparison of *Ad Libitum*-Fed Mice with Previous NTP Studies

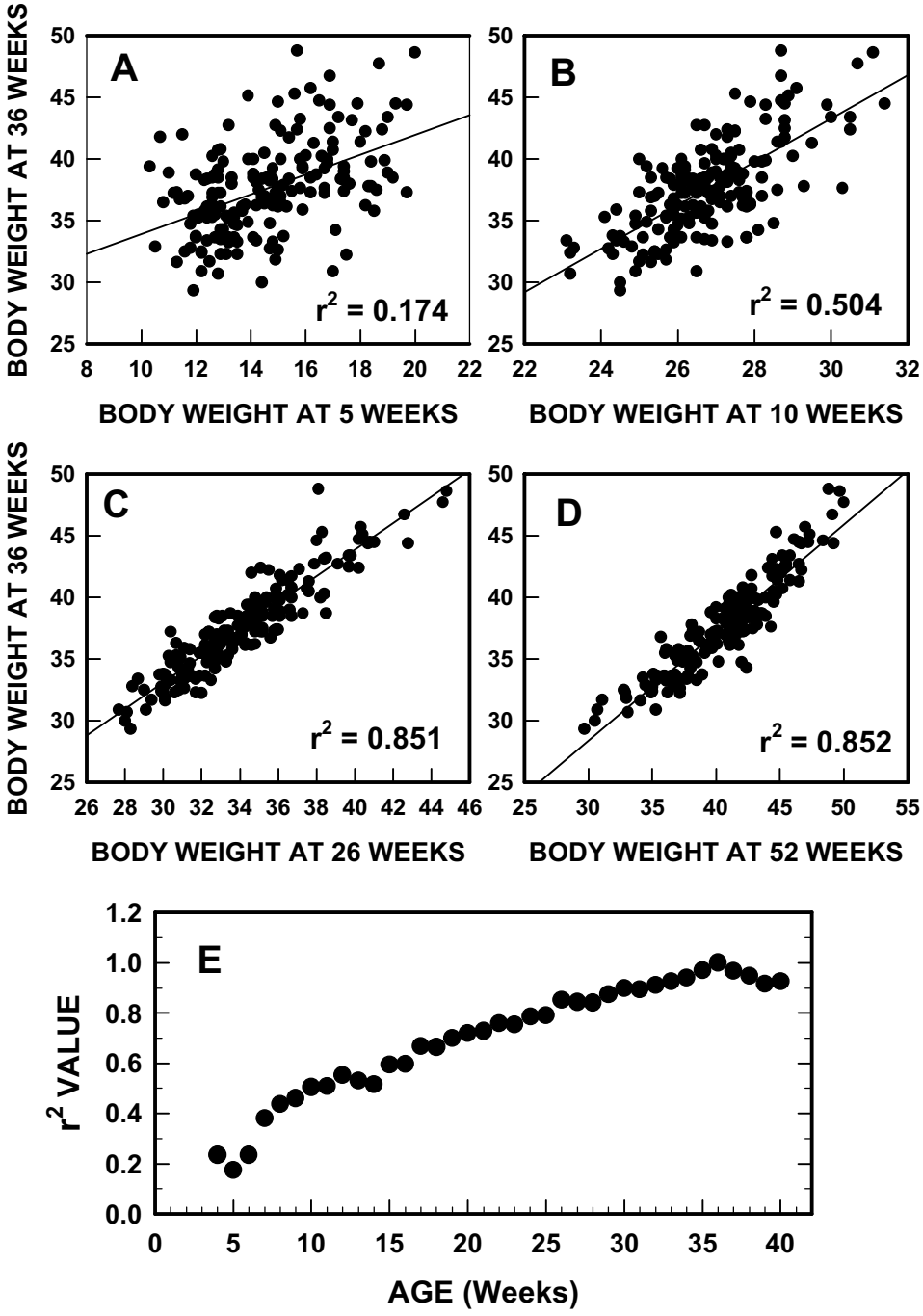


FIGURE D10
Correlation Between 36-Week Body Weights of *Ad Libitum*-Fed Mice and Body Weights at Other Ages

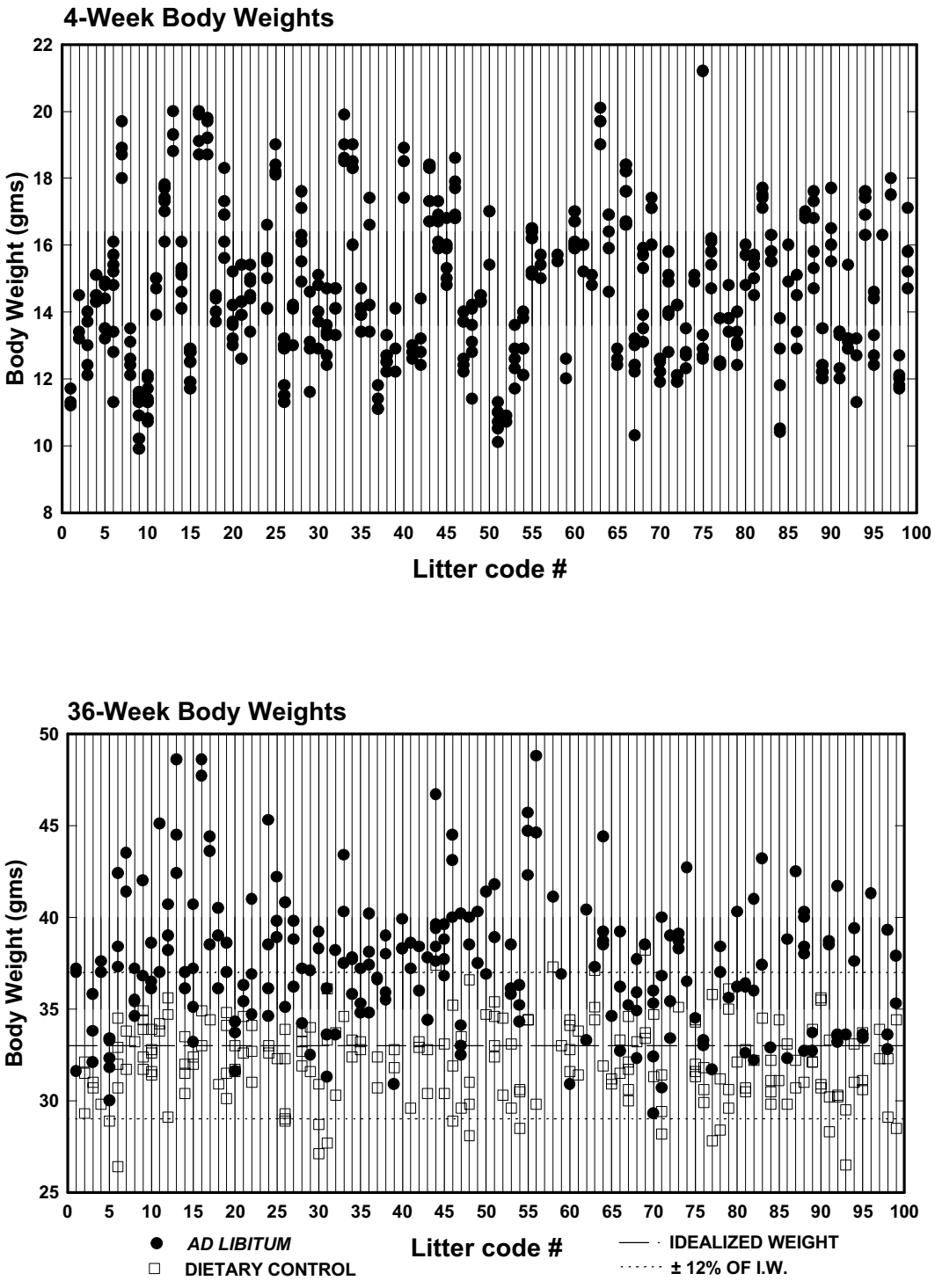


FIGURE D11
Influence of Birth Litter on Body Weight Variation at 4 and 36 Weeks of Age

NOTES FOR FIGURES D9, D10, AND D11

Figure D9 compares the body weight spread of the *ad libitum*-fed mice from the 2-year chloral hydrate study at 44 weeks with the body weight spread of mice from previous NTP bioassays. Panel A shows a group of NTP bioassays conducted in the early 1980s and Panel C shows a group of bioassays from the late 1980s when heavier mice were being used. Details of the actual studies are given on page 120. Panel B shows the body weight spread for the 2-year chloral hydrate study (i.e., the four *ad libitum*-fed dose groups combined). The figure demonstrates that the drift towards heavier mice that occurred during the 1980s and early 1990s was associated with a narrowing of the weight-spread (i.e., the range of body weights of the population as a percentage of its mean body weight) as well as an increase in mean body weight. The NCTR mice used in the 2-year chloral hydrate study resemble NTP mice from the early 1980s rather than those from the late 1980s. This could be because there has been no commercial pressure for the NCTR breeding services to produce highly fertile mice which grow fast.

Figure D10 investigates the relationship between body weight at weaning and subsequent body weight during the period when liver neoplasm risk is most influenced by body weight. The data used come from the *ad libitum*-fed 0, 25, and 50 mg/kg dose groups. The 100 mg/kg group was eliminated because there was a slight dose effect on body weight between 10 and 20 weeks of age. Although there is a strong correlation between body weights at 26 and 52 weeks with those at 36 weeks (panels C and D), there is little or no correlation between body weights at 4 or 10 weeks and those at 36 weeks (panels A and B). As shown in panel E, when all ages are compared with 36 weeks, the regression coefficient increases at an approximately constant rate between 8 and 36 weeks reaching 0.66 by 20 weeks. These data suggest that mice allocation body weights at the initiation of a chronic study cannot be used to select mice for a specified subsequent body weight and liver neoplasm incidence.

Figure D11 investigates the relationship between the litters in which the mice were born and subsequent body weight. Mice from all the dose and diet groups but not sentinel animals were used here. The mice were derived from a total of 122 litters. Only 100 litters are shown here for clarity. The remaining 22 litters were each represented by only one or two mice and were therefore less useful in investigating relative body weights of siblings. No statistical analysis was attempted but it can be seen from the plots that there is a closer relationship between sibling body weight at 4 weeks than at 36 weeks. The close relationship at 4 weeks could be due to either genetic or environmental effects (e.g., litter size). This figure also shows that there are a significant number of *ad libitum*-fed mice which weigh less than the idealized weight. It follows that approximately the same proportion of the dietary-controlled mice would not require dietary restriction to maintain their idealized weight.

Control of Variability

The dietary control paradigm successfully maintained all four of the chloral hydrate dose groups at or near their idealized target mean body weights. Chloral hydrate appeared to have only a minimal effect on food consumption and body weight, which made the control of body weight in the high dose groups relatively easy. Dietary control successfully reduced body weight variation as well as reducing mean body weights of the dose groups. This is illustrated in Figures D12 through D18. It can be seen in all of the seven ages illustrated in these figures that the weight-spread is larger for the *ad libitum*-fed groups than for the dietary control groups. For example, at 28 weeks of age, the individual body weights of the control *ad libitum*-fed group ranged between 25 and 45 g with more than 90% of the mice between 27.5 and 42.5 g, whereas the individual body weights for the corresponding dietary-controlled group ranged between 25 and 35 g with more than 90% of the mice ranging between 27.5 and 32.5 g.

Prediction of Tumor Risk

Risk curves, such as those used to create the idealized body weight curve (Figures D5, D6, and D7), can also be used to predict liver neoplasm risk of experimental groups of mice in bioassays. This is also illustrated in Figures D12 through D18. This approach potentially provides a more accurate baseline for evaluating bioassay data than the simple use of historical control data, which does not compensate for drift in body weight of the test animals. When this approach was used, it predicted the liver neoplasm outcome of the *ad libitum* control group with reasonable accuracy, but it underestimated the tumor outcome of the dietary-controlled group (Table D7). One significant problem associated with the experimental design of this dietary-control study was the weight profile of the NCTR male B6C3F$_1$ mouse colony. These mice are relatively small and are more similar to the commercially available mice used by the NTP in the early 1980s than those used in the early 1990s. This is illustrated in Figure D9. The idealized body weight curve was constructed using dietary restricted mice from a recent NTP studies with relatively large reductions in mean body weight and NCTR mice which were calorically restricted by 40%. These situations produced significant body weight reductions for all the mice. However, within the large range of body weights of NCTR *ad libitum* mouse population, some mice weigh less than the idealized weight without the need for dietary restriction. Because of this, these mice have a different liver neoplasm risk than mice which are small due to dietary restriction or other forced body weight loss. This is illustrated in Figure D19. There is overlap between the two weight groups (crosshatch), which suggests that a portion of the dietary controlled mice in the 27.5 to 30 g and 30 to 32.5 g weight groups will have a neoplasm risk corresponding to the *ad libitum* risk curve rather than the dietary restriction curve. When this effect is taken into consideration the predicted tumor incidence for the dietary control groups becomes closer to the observed values. This is illustrated in Table D8. Assuming all the dietary-controlled mice are dietary restricted underestimated the observed tumor rate by 4.2% and 2.8% for the 0 and 25 mg/kg dose groups respectively. Conversely, assuming all the dietary-controlled mice are *ad libitum*-fed overestimates the same tumor rates by 2.5% and 2.4% respectively. The assumption that mice that weighed less than the −5% confidence limit of the idealized weight curve were effectively *ad libitum* was based on the fact that these mice received an additional 1.0 or 1.5 g of food daily for each week they were below the −5% confidence limit. This resulted in a total food allocation that was greater than the observed mean *ad libitum* food consumption for the corresponding age. The use of this assumption resulted in neoplasm risk predictions that were remarkably close to the observed values.

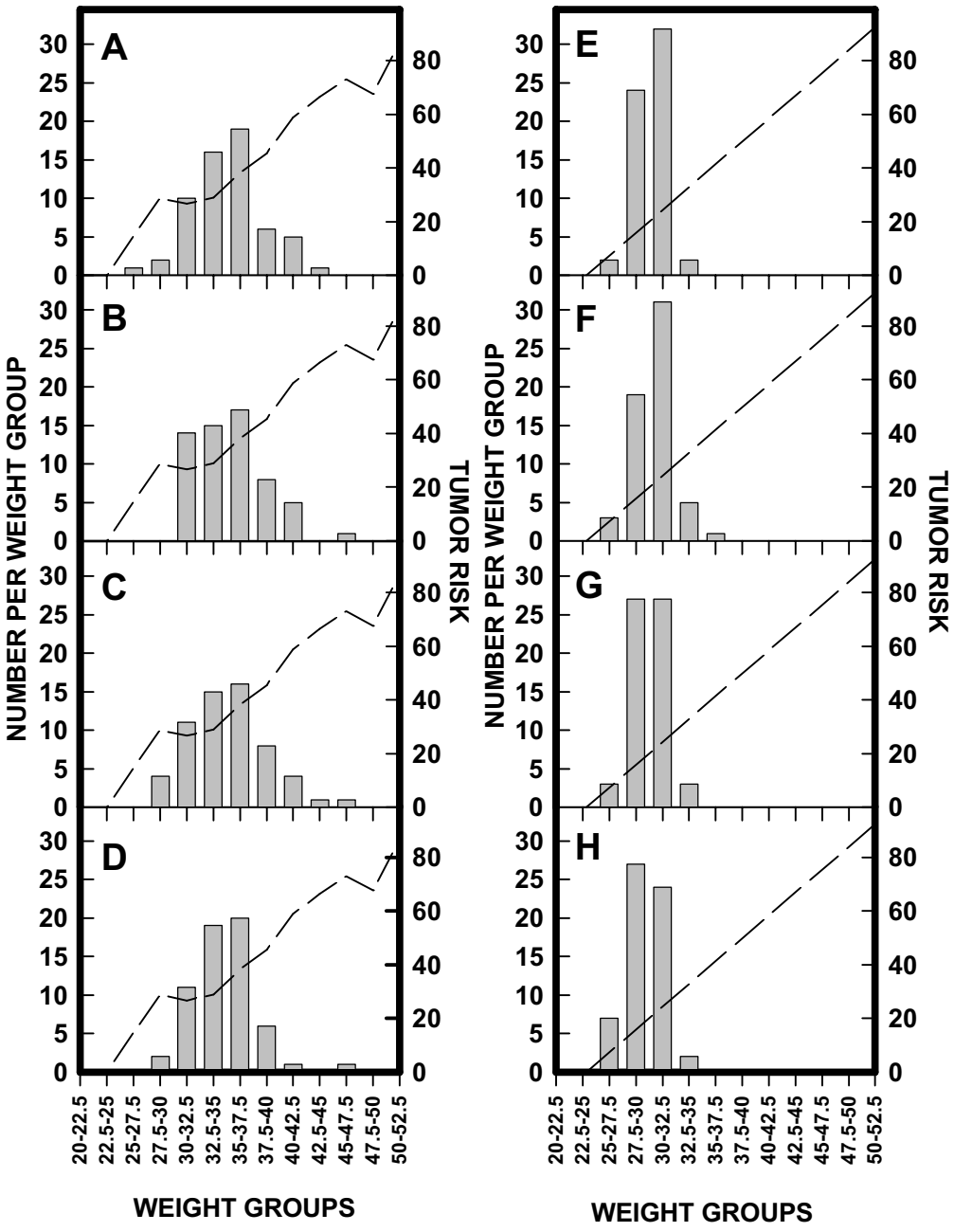


FIGURE D12
Body Weight Variation at 28 Weeks of Age in All Dose Groups of Male Mice Administered Chloral Hydrate

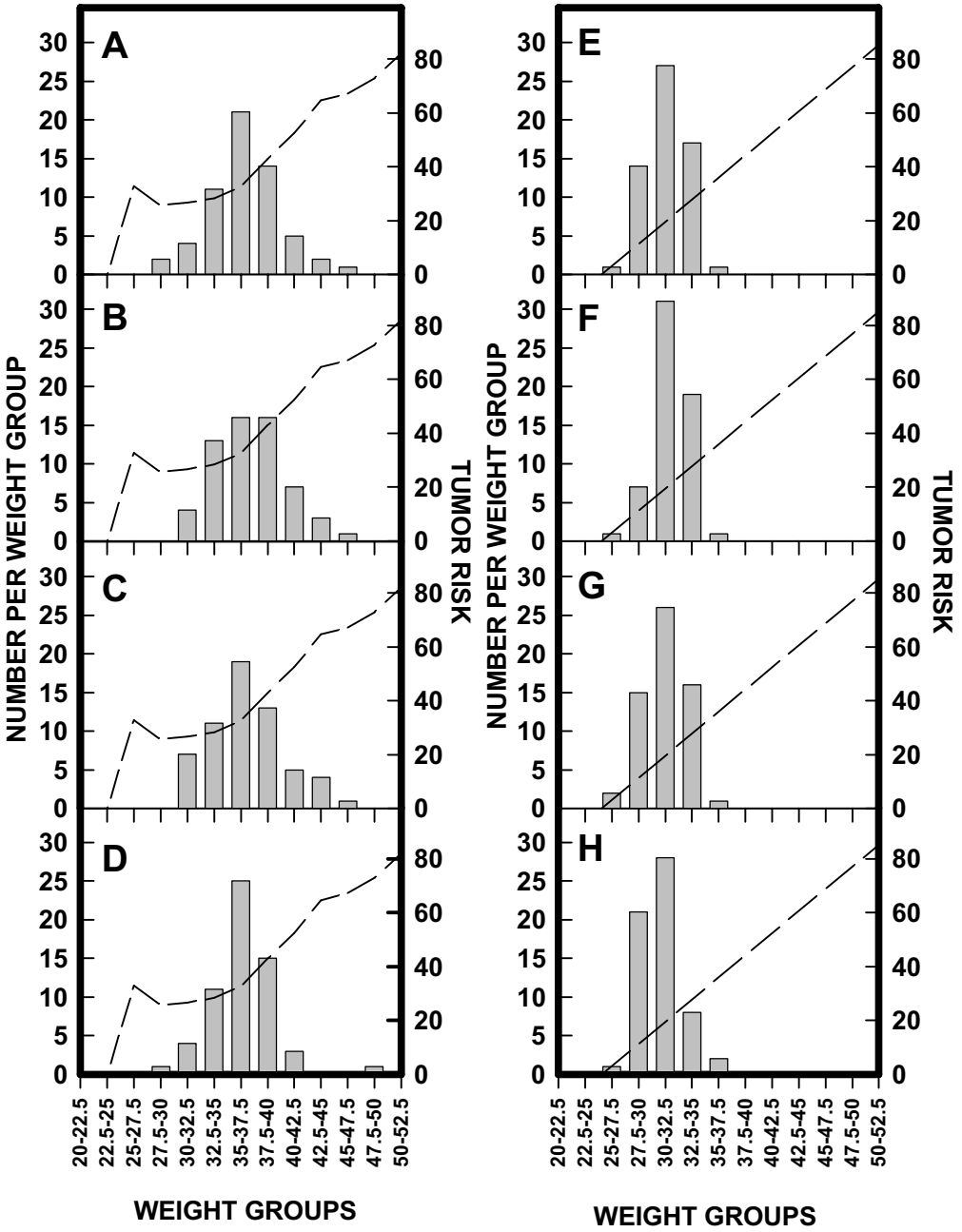


FIGURE D13
Body Weight Variation at 32 Weeks of Age in All Dose Groups of Male Mice Administered Chloral Hydrate

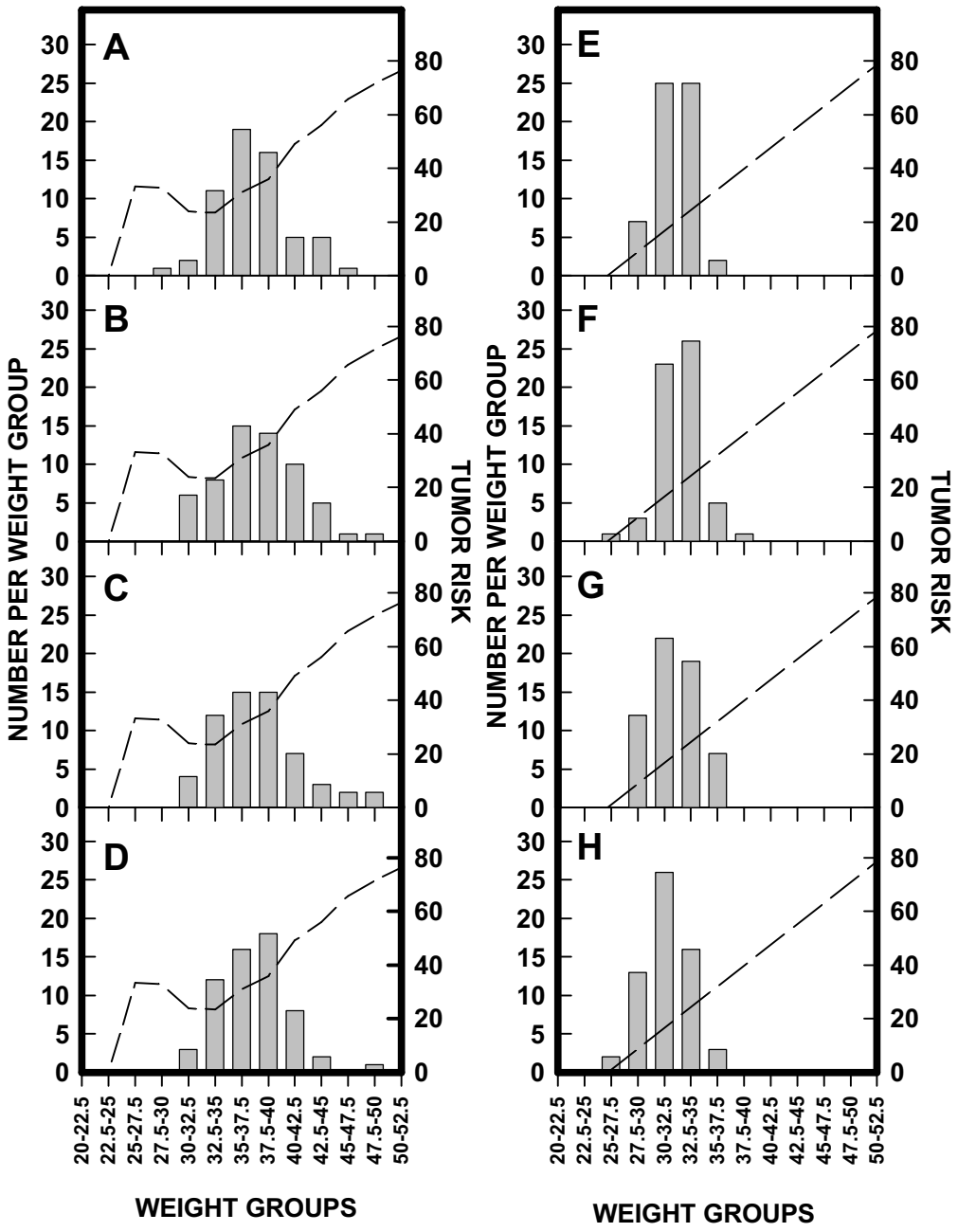


FIGURE D14
Body Weight Variation at 36 Weeks of Age in All Dose Groups of Male Mice Administered Chloral Hydrate

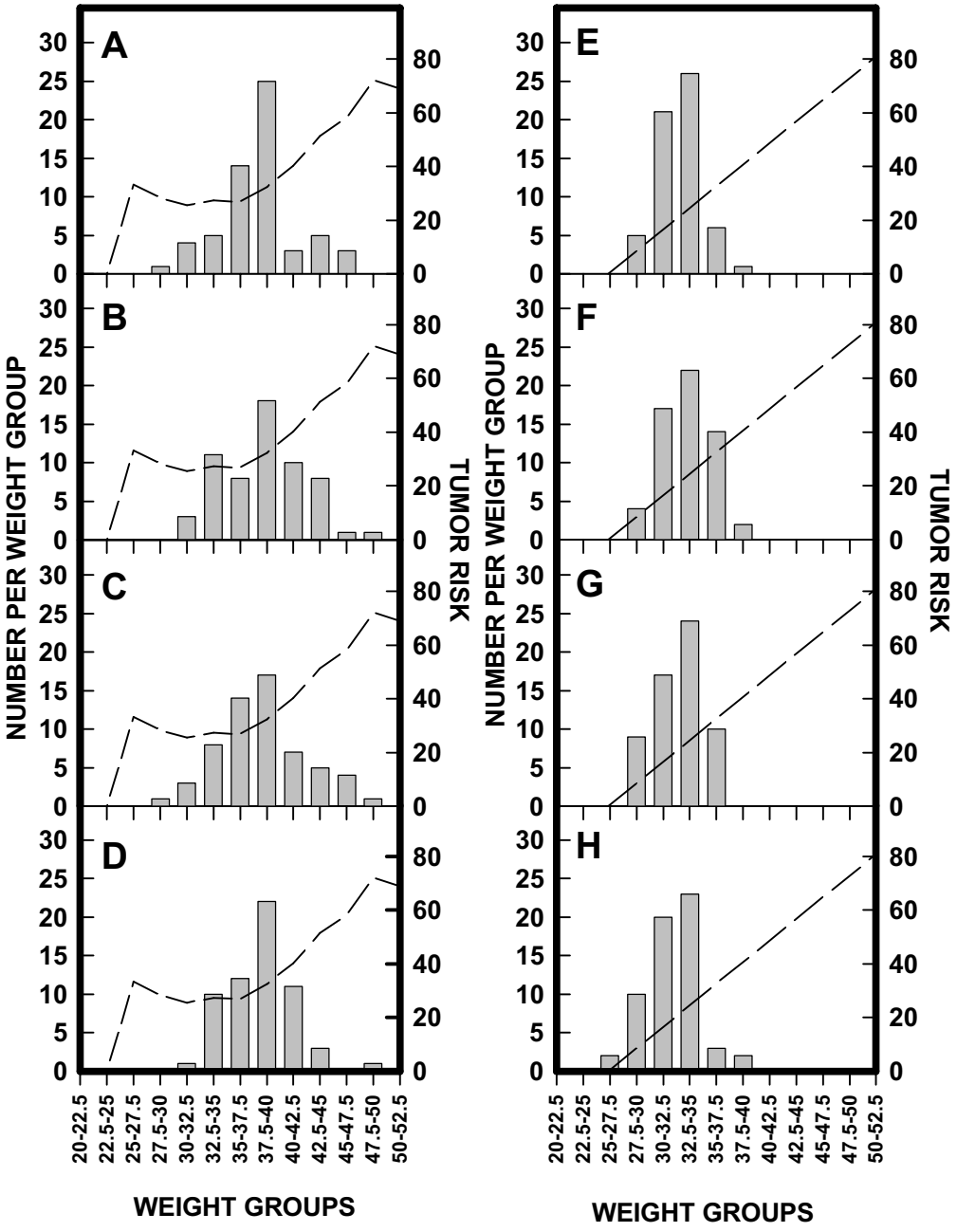


FIGURE D15
Body Weight Variation at 40 Weeks of Age in All Dose Groups of Male Mice Administered Chloral Hydrate

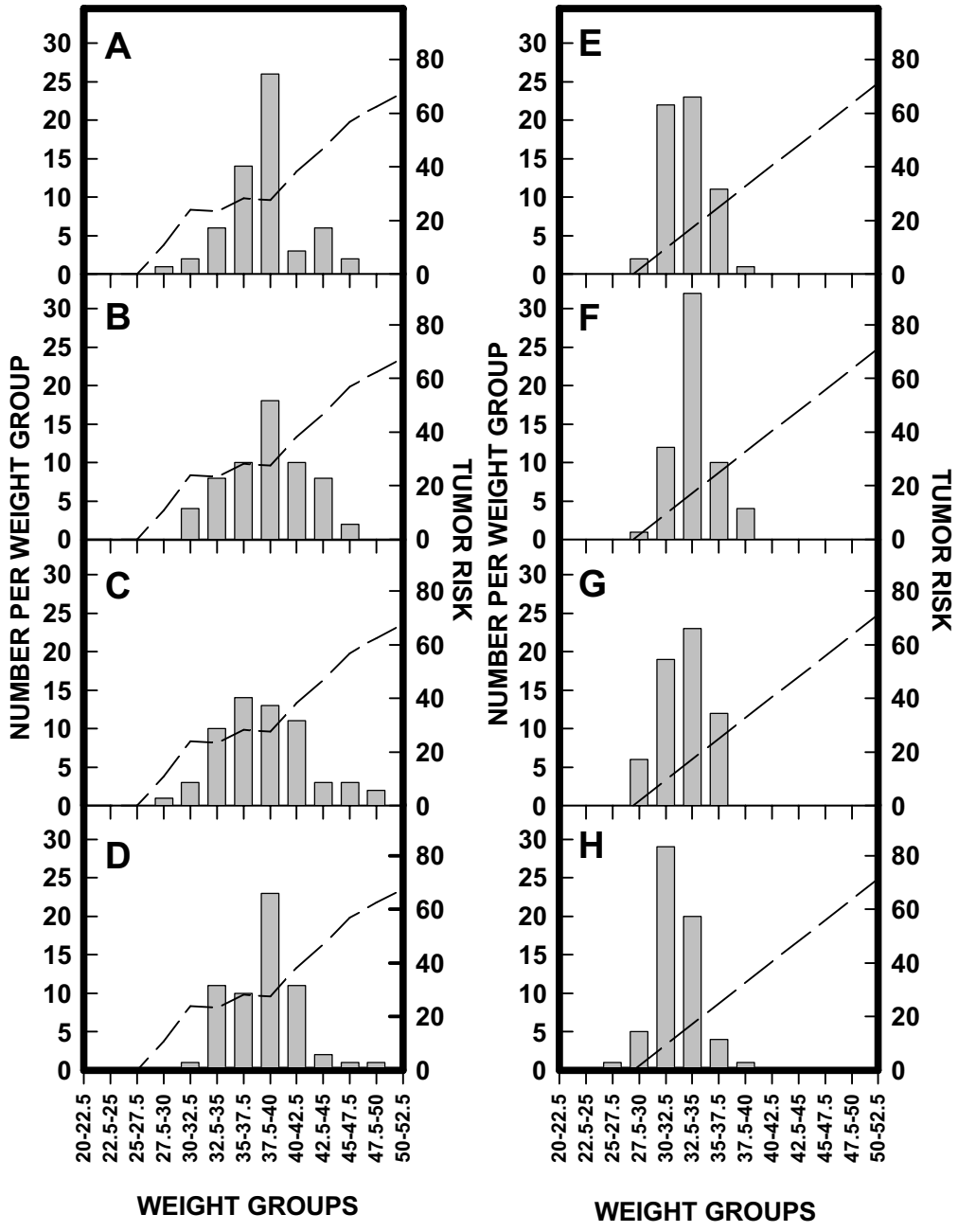


FIGURE D16
Body Weight Variation at 44 Weeks of Age in All Dose Groups of Male Mice Administered Chloral Hydrate

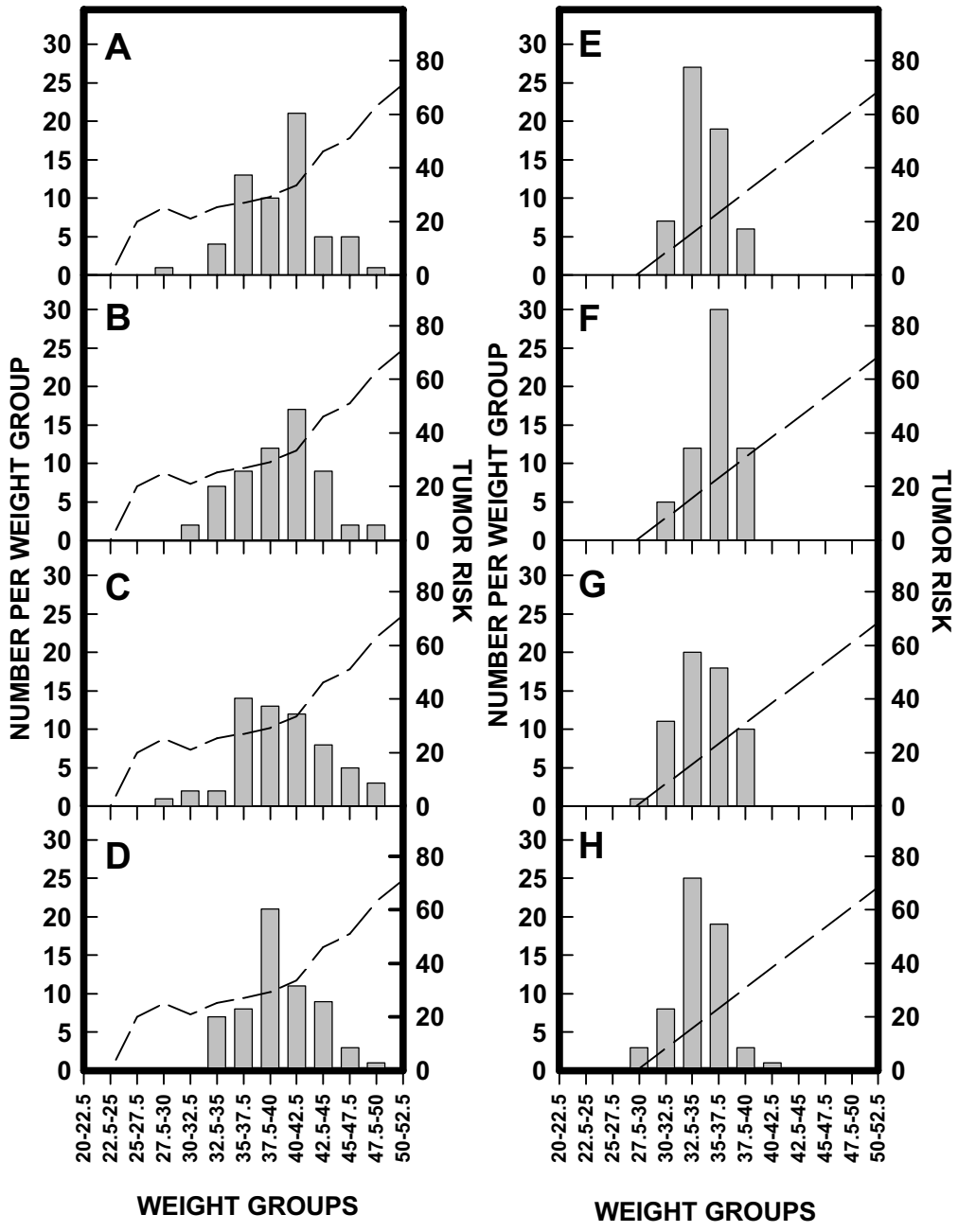


FIGURE D17
Body Weight Variation at 48 Weeks of Age in All Dose Groups of Male Mice Administered Chloral Hydrate

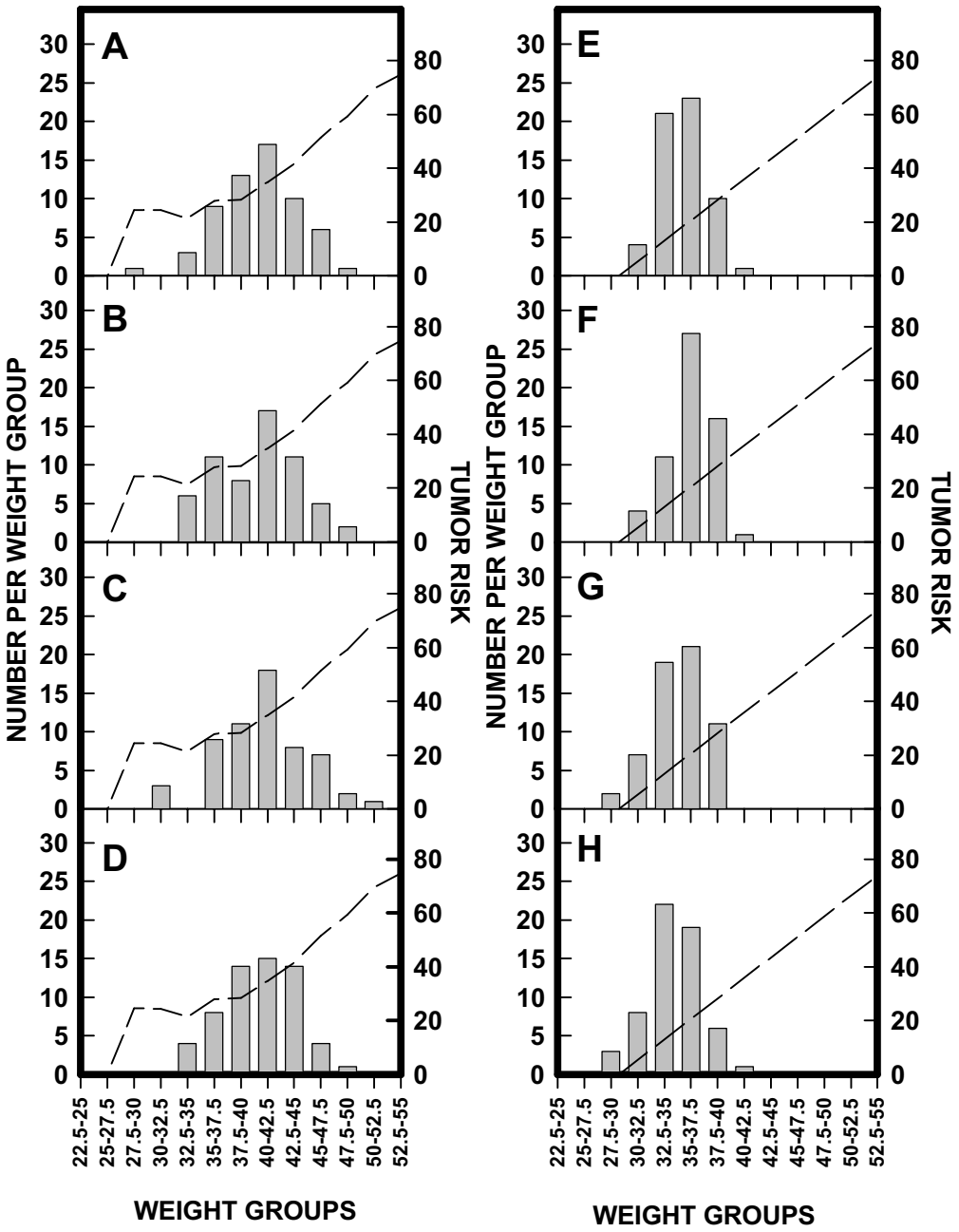


FIGURE D18
Body Weight Variation at 52 Weeks of Age in All Dose Groups of Male Mice Administered Chloral Hydrate

NOTES FOR FIGURES D12 THROUGH D18

Figures D12 through D18 show weight-group assignment plots of each of the eight experimental groups from the 2-year study at ages 28, 32, 36, 40, 44, 48, and 52 weeks respectively. Plots A through D show the *ad libitum*-fed groups and plots E through H show the dietary-controlled groups. Plots A and E, B and F, C and G, and D and H show the 0, 25, 50 and 100 mg/kg dose groups respectively. The dashed lines show relative neoplasm risk against weight group for either *ad libitum*-fed or dietary-controlled mice. The weight spread is less for the dietary-controlled mice than for the *ad libitum*-fed mice. For example, at 36 weeks and 0 mg/kg dosing, the *ad libitum*-fed and dietary-controlled diet groups ranged from 27.5 to 47.5 g and 27.5 to 37.5 g respectively. For the *ad libitum*-fed group, the estimated neoplasm risk for this weight range varied from 23% to 66%. For the dietary controlled group it varied from 9% to 32% with over 80% of the mice correlating to a neoplasm risk of 15% to 25%.

For each of the seven figures, the overall neoplasm risk of each dose group was calculated. This was achieved by multiplying the number of mice in each weight group by the neoplasm risk assigned to each weight group, then adding the products for all the weight groups together and dividing by the total number of mice in the dose group. The results are shown in Table D7, and the means for the seven ages calculated as the predicted neoplasm risk for each dose group. Although the predicted tumor risk for the *ad libitum*-fed control group is reasonably close to the observed value, the corresponding predicted value for the dietary control group underestimates the observed tumor rate by 4%. This is probably due to a small number of the dietary-controlled mice being too small to require dietary restriction to achieve their idealized weight and therefore having the higher tumor risk that is associated with the *ad libitum*-fed mice.

The difference between *ad libitum*-fed and weight-reduced male B6C3F$_1$ mice is shown in Figure D19. There is a large difference for mice weighing between 25 and 35 g due to the J-curve effect in the *ad libitum*-fed mice. The majority of the mice from the chloral hydrate study were in this weight range.

TABLE D7
Predicted and Observed Liver Neoplasm Incidence for Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

Weeks of Age	*Ad Libitum*-Fed Groups				Dietary-Controlled Groups			
	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Predicted								
28	36.00	36.45	36.52	34.47	20.66	21.75	20.09	18.82
32	36.90	38.52	37.73	35.56	19.95	21.21	19.40	18.03
36	34.91	36.80	36.13	34.55	19.45	21.02	19.27	17.21
40	33.41	35.31	34.88	33.38	21.46	23.64	21.25	19.37
44	30.31	32.17	32.08	30.48	15.46	17.69	14.72	12.63
48	33.71	33.77	34.85	33.46	18.86	22.05	18.94	17.59
52	35.20	34.75	36.31	34.55	18.40	20.46	17.11	15.67
Mean	34.35	35.40	35.50	33.78	19.18	21.12	18.68	17.04
SD	2.16	2.09	1.80	1.63	1.94	1.82	2.15	2.28
SEM	0.82	0.79	0.68	0.62	0.73	0.69	0.81	0.86
Observed								
	33.4	52.6	50.6	46.2	23.4	23.9	29.7	38.6

[a] Data are given as percentages.
SD Standard deviation
SEM Standard error of the mean

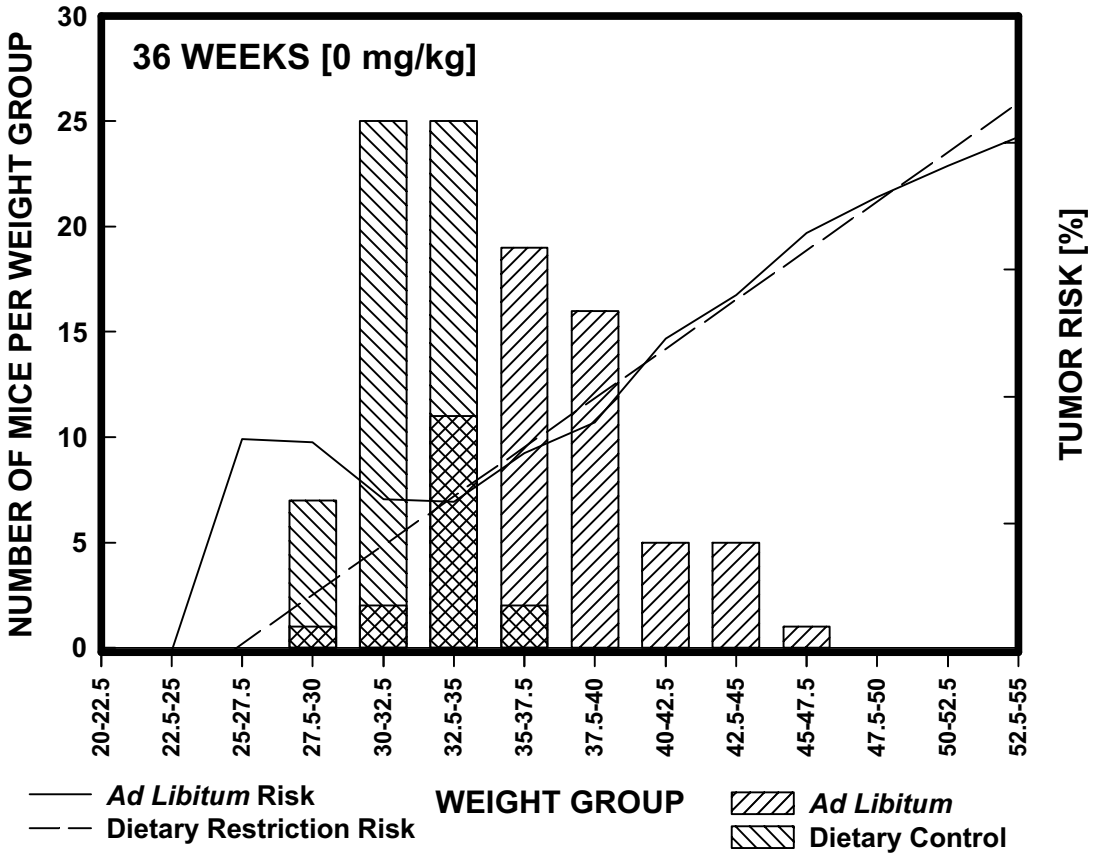


FIGURE D19
Weight Spread and Neoplasm Risk of Male B6C3F$_1$ Mice

TABLE D8
Predicted and Observed Liver Neoplasm Incidence Allowing for Small Body Weights of the NCTR Mouse Population

Weeks of Age	*Ad Libitum*-Fed Groups				Dietary-Controlled Groups			
	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg	Vehicle Control	25 mg/kg	50 mg/kg	100 mg/kg
Observed	33.4	52.6	50.6	46.2	23.4	23.9	29.7	38.6
Predicted								
All DC[a]	—	—	—	—	19.18	21.12	18.68	17.04
SD					1.94	1.82	2.15	2.28
-5% AL[b]	—	—	—	—	23.31	23.06	23.17	23.48
SD					1.49	1.21	2.72	1.60
#AL					18/48	6/48	18/48	20/48
All AL[c]	34.35	35.40	35.50	33.78	25.92	26.31	26.14	25.91
SD	2.16	2.09	1.80	1.63	1.18	1.14	1.41	1.56

[a] Predicted neoplasm risk for dietary-controlled groups calculated with the dietary restriction neoplasm risk database as for Table D7.

[b] DC=Dietary-controlled; AL=*ad libitum*
Predicted neoplasm risk calculated by separating out mice which weighed at or below the -5% confidence limit of the idealized weight curve for more than 50% of the weeks between age 20 weeks and age 60 weeks. The liver neoplasm risk of these mice was calculated with the *ad libitum* neoplasm risk database while the dietary restriction neoplasm risk database was used for the remaining mice. The neoplasm risk of both groups was then combined. #AL denotes the number of mice that were considered *ad libitum* for each dietary-controlled group.

[c] Predicted neoplasm risk calculated with the *ad libitum* neoplasm risk database for all the dietary controlled mice

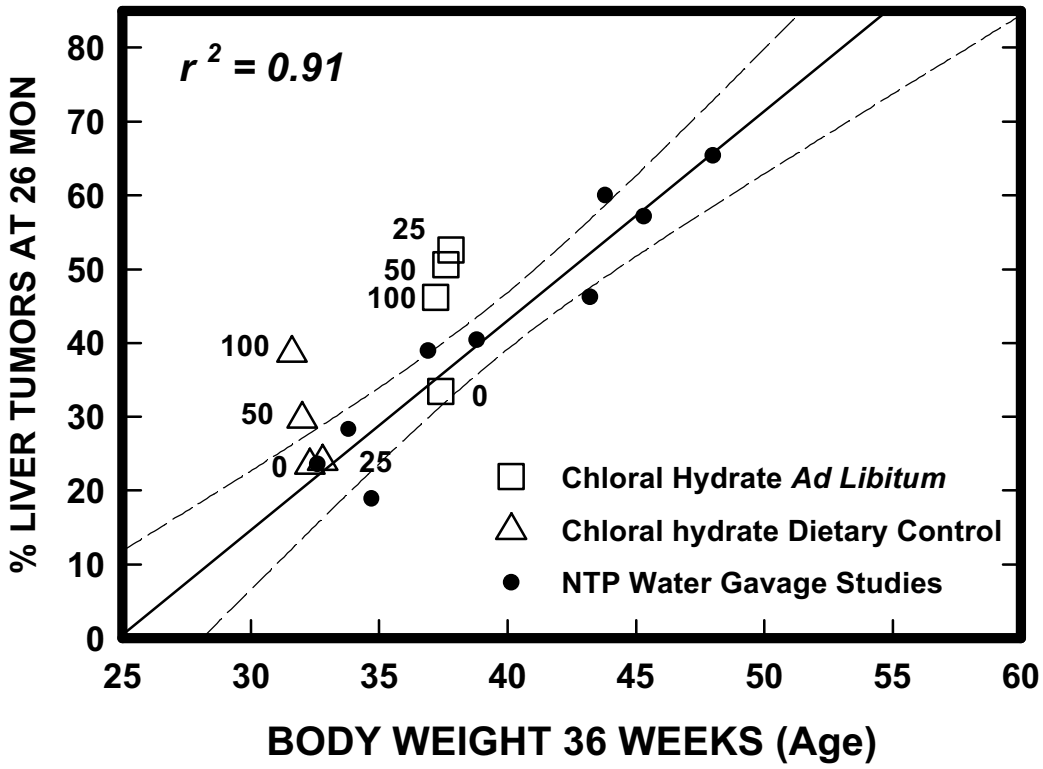


FIGURE D20
Comparison of the 2-Year Gavage Study of Chloral Hydrate with Other Recent NTP Studies That Used Water-Based Gavage Administered to Male B6C3F$_1$ Mice

The mean body weight values at approximately 36 weeks of age (29 to 30 weeks of dosing) of control groups from the nine water-based gavage studies listed in Table D3 were plotted against terminally adjusted liver neoplasm incidence (adenoma, carcinoma, or hepatoblastoma). The dashed lines represent 95% confidence limits for the linear regression (solid line). This type of plot produces good correlations when studies are segregated according to the method of dosing. When the control groups from the current chloral hydrate study were included, both the *ad libitum*-fed and dietary-controlled groups were found to closely fit the regression line of the previous studies. As expected, the *ad libitum*-fed 25 mg/kg group and the dietary-controlled 100 mg/kg group were both significantly above the regression line. The values for all three *ad libitum*-fed dosed groups and the dietary-controlled 50 and 100 mg/kg groups were also outside the confidence intervals.

CONCLUSIONS

Although moderate dietary restriction can enhance survival and decrease the incidence of background neoplasms, it can also inhibit chemically-induced carcinogenesis and therefore may also compromise the outcome of cancer bioassays. For example, one NTP study that tested the effect of 10% dietary restriction in $B6C3F_1$ mice on the hepatocarcinogenicity of salicylazosulfapyridine, suggested that dietary restriction would mask chemically induced carcinogenic effects (NTP, 1997a). However, the chemical itself caused significant body weight decreases, which made the study difficult to interpret.

The current study used an alternative procedure of dietary control to further investigate this problem. Rather than restricting dietary or caloric intake by a set percentage of *ad libitum* feed consumption, dietary control uses variable dietary restriction to manipulate the growth patterns of all the animals on the study to fit a predetermined (idealized) weight curve. The potential advantage of this approach is that it may decrease intra-assay and inter-assay variability, and control problems associated with body weight changes induced by the test chemical, as well as controlling survival and background neoplasm incidence. A major objective of this study was to test the feasibility of using dietary control to normalize body weight in a cancer bioassay, and the ability of the idealized weight curve to accurately predict liver neoplasm incidence.

The study demonstrated that dietary control decreased individual variation in the dose groups. This allowed the demonstration of effects on liver-weight-to-body-weight ratios and marker enzymes for peroxisome proliferation in the interim evaluation animals which could not be demonstrated in the *ad libitum*-fed mice. Although incidence of liver neoplasms was reduced by dietary control, a significant dose response to the hepatocarcinogenic effects of chloral hydrate was observed by trend analysis in the dietary controlled mice, but not in the *ad libitum*-fed mice.

The dietary control procedures used in this study maintained the mean body weights of all four dose groups at or near their target weights with a relatively small amount of additional animal husbandry hours. Because of the relatively small size of the NCTR $B6C3F_1$ mice used in this study, the amount of dietary restriction required to control body weight was small for most of the mice and some were essentially fed *ad libitum* through major periods of the study. While this minimized the possibility of large changes in metabolism or toxicokinetics occurring, it created problems of its own.

The relationship between liver neoplasms and body weight in male $B6C3F_1$ mice is complex. In large mice of both genders, the relationship appears linear. However, small male mice develop liver neoplasms at a rate of around 25% to 30% at 26 months apparently independently of body weight. There is therefore a sex difference in liver neoplasm incidence in small $B6C3F_1$ mice, which appears to be regulated by gonadal steroids (Kemp and Drinkwater, 1990; Poole and Drinkwater, 1995). There is little or no sex difference in the incidence of liver neoplasms in large $B6C3F_1$ mice which have been used in recent NTP studies. Caloric restriction decreases liver tumor incidence in small (Witt *et al.*, 1991), as well as in large (NTP, 1997a), male $B6C3F_1$ mice. Therefore, as illustrated in Figure D19, there is a large difference in the estimated neoplasm of small male mice depending on whether they are *ad libitum*-fed naturally small mice, or whether they are small because of stress or dietary restriction. The observed neoplasm incidence, food consumption and body weight data from the control groups of the current dietary control study tend to support this. Only a portion of the mice appeared to be dietary-restricted and the observed neoplasm incidence was intermediate between that predicted for *ad libitum*-fed and for dietary-restricted mice.

This difference in neoplasm risk of small male mice will create problems in interpreting bioassay data when the test chemical reduces body weight. Chemically induced body weight reductions can arise for a number of reasons, including decreased food consumption due to palatability problems in feed studies, anorexia due to toxic stress, disrupted intestinal absorption, or toxic wasting syndromes due to disruption of metabolism or endocrine systems. In most cases, plasma corticosteroid levels rise as part of the stress response, as also occurs

with dietary restriction (Leakey *et al.*, 1998). Chemically induced body weight changes have, in many cases, resulted in a decrease in mouse liver neoplasm incidence that is similar to decreases resulting from dietary restriction (NTP, 1995c, 1996a, 1997a,c), but there is not always a corresponding decrease in food consumption. If a chemically treated group exhibits a moderately reduced body weight during the middle period of a 2-year bioassay, it is difficult to determine whether the mice are smaller due to effects of the chemical or because of random assignment of small mice to the experimental group. Early body weight at ages when mice are assigned to studies on a randomized weight basis does not correlate well with later body weight which is associated with liver neoplasm risk (see Figure D10).

The use of larger B6C3F_1 mice, such as the mice that were commercially available in the early 1990s, would solve this problem if they were used in conjunction with dietary control. Body weight changes, whether due to animal selection, chemically induced stress, or dietary restriction, would occur in the weight range where the relationship between body weight and liver neoplasm incidence is more predictable. Such a paradigm would improve both the sensitivity and reproducibility of cancer bioassays in mice. However, mouse liver neoplasms are frequently induced epigenetically by chemicals which appear to not be carcinogenic for humans, and the appearance of a more sensitive mouse model might not be welcomed wholeheartedly within the regulatory community.

In answer to this, it must be stated that most other neoplastic lesions are also reduced by caloric restriction and related stress responses, but their low background levels mask any potential relationship to body weight. Uncontrolled chemically induced weight loss may complicate detection of neoplasia in tissues other than mouse liver. Moreover, as the current study suggests, the use of less variable, more standardized bioassay procedures will allow better correlation between cancer bioassays and accompanying mechanistic studies. This, in turn, may provide the regulatory scientists with improved information to determine whether the human carcinogenic risk of the chemical being tested is real or merely perceived.

The dietary control procedures were relatively easy to maintain, and most of the mice weighed relatively close to their target weights throughout the study. However, the observed background incidence of liver neoplasms was slightly higher than expected. Nevertheless, dietary control appeared to enhance rather than decrease the sensitivity of the bioassay. This effect was most likely due to dietary control causing a reduction in the individual variation of the test animals.

REFERENCES

Fu, P.P., Dooley, K.L., Von Tungeln, L.S., Bucci, T., Hart, R.W., and Kadlubar, F.F. (1994). Caloric restriction profoundly inhibits liver tumor formation after initiation by 6-nitrochrysene in male mice. *Carcinogenesis* **15**, 159-161.

Hart, R.W., Keenan, K., Turturro, A., Abdo, K.M., Leakey, J., and Lyn-Cook, B. (1995). Caloric restriction and toxicity. *Fundam. Appl. Toxicol.* **25**, 184-195.

Haseman, J.K. (1985). Issues in carcinogenicity testing: Dose selection. *Fundam. Appl. Toxicol.* **5**, 66-78.

Haseman, J.K. (1998). National Toxicology Program experience with dietary restriction: Does the manner in which reduced body weight is achieved affect tumor incidence? *Int. J. Toxicol.* **17** (Suppl. 2), 119-134.

Jeong, K.-H., Jacobson, L., Widmaier, E.P., and Majzoub, J.A. (1999). Normal suppression of the reproductive axis following stress in corticotropin-releasing hormone-deficient mice. *Endocrinology* **140**, 1702-1708.

Keenan, K.P., Soper, K.A., Hertzog, P.R., Gumprecht, L.A., Smith, P.F., Mattson, B.A., Ballam, G.C., and Clark, R.L. (1995). Diet, overfeeding, and moderate dietary restriction in control Sprague-Dawley rats: II. Effects on age-related proliferative and degenerative lesions. *Toxicol. Pathol.* **23**, 287-302.

Keenan, K.P., Ballam, G.C., Dixit, R., Soper, K.A., Laroque, P., Mattson, B.A., Adams, S.P., and Coleman, J.B. (1997). The effects of diet, overfeeding and moderate dietary restriction on Sprague-Dawley rat survival, disease and toxicology. *J. Nutr.* **127**, 851S-856S.

Kemp, C.J., and Drinkwater, N.R. (1990). The androgen receptor and liver tumor development in mice. In *Mouse Liver Carcinogenesis: Mechanisms and Species Comparisons*, pp. 203-214 (D.E. Stevenson, J.A. Popp, J.M. Ward, R.M. McClain, T.J. Slaga, and H.C. Pitot, Eds.), Alan R. Liss, New York.

Leakey, J.E.A., Seng, J.E., Barnas, C.R., Baker, V.M., and Hart, R.W. (1998). A mechanistic basis for the beneficial effects of dietary restriction on longevity and disease. Consequences for the interpretation of rodent toxicity studies. *Int. J. Toxicol.* **17** (Suppl. 2), 5-57.

National Toxicology Program (NTP) (1989a). Toxicology and Carcinogenesis Studies of *N*-Methylolacrylamide (CAS No. 924-42-5) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 352. NIH Publication No. 89-2807. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1989b). Toxicology and Carcinogenesis Studies of Dimethoxane (CAS No. 828-00-2) (Commercial Grade) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 354. NIH Publication No. 89-2809. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1989c). Toxicology and Carcinogenesis Studies of Pentaerythritol Tetranitrate (CAS No. 78-11-5) with 80% *d*-Lactose Monohydrate (PETN, NF) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 365. NIH Publication No. 89-2820. National Institute of Environmental Health Sciences, Research Triangle Park, NC.

National Toxicology Program (NTP) (1989d). Toxicology and Carcinogenesis Studies of Hydroquinone (CAS No. 123-31-9) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 366. NIH Publication No. 90-2821. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1989e). Toxicology and Carcinogenesis Studies of 4-Vinyl-1-cyclohexene Diepoxide (CAS No. 106-87-6) in F344/N Rats and B6C3F$_1$ Mice (Dermal Studies). Technical Report Series No. 362. NIH Publication No. 90-2817. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1990a). Toxicology and Carcinogenesis Studies of Benzaldehyde (CAS No. 100-52-7) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 378. NIH Publication No. 90-2833. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1990b). Toxicology and Carcinogenesis Studies of *d*-Carvone (CAS No. 2244-16-8) in B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 381. NIH Publication No. 90-2836. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1990c). Toxicology and Carcinogenesis Studies of Furfural (CAS No. 98-01-1) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 382. NIH Publication No. 90-2837. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1990d). Toxicology and Carcinogenesis Studies of Toluene (CAS No. 108-88-3) in F344/N Rats and B6C3F$_1$ Mice (Inhalation Studies). Technical Report Series No. 371. NIH Publication No. 90-2826. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1991a). Toxicology and Carcinogenesis Studies of *dl*-Amphetamine Sulfate (CAS No. 60-13-9) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 387. NIH Publication No. 91-2842. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1991b). Toxicology and Carcinogenesis Studies of Tris(2-Chloroethyl) Phosphate (CAS No. 115-96-8) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 391. NIH Publication No. 91-2846. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1991c). Toxicology and Carcinogenesis Studies of Probenecid (CAS No. 57-66-9) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 395. NIH Publication No. 91-2850. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992a). Toxicology and Carcinogenesis Studies of Monochloroacetic Acid (CAS No. 79-11-8) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 396. NIH Publication No. 92-2851. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992b). Toxicology and Carcinogenesis Studies of 2,4-Diaminophenol Dihydrochloride (CAS No. 137-09-7) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 401. NIH Publication No. 92-2856. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992c). Toxicology and Carcinogenesis Studies of Resorcinol (CAS No. 108-46-3) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 403. NIH Publication No. 92-2858. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992d). Toxicology and Carcinogenesis Studies of γ-Butyrolactone (CAS No. 96-48-0) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 406. NIH Publication No. 92-3137. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992e). Toxicology and Carcinogenesis Studies of C.I. Pigment Red 3 (CAS No. 2425-85-6) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 407. NIH Publication No. 92-3138. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992f). Toxicology and Carcinogenesis Studies of C.I. Pigment Red 23 (CAS No. 6471-49-4) in F344 Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 411. NIH Publication No. 93-3142. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992g). Toxicology and Carcinogenesis Studies of 4,4′-Diamino-2,2′-stilbenedisulfonic Acid, Disodium Salt (CAS No. 7336-20-1) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 412. NIH Publication No. 92-3143. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992h). Toxicology and Carcinogenesis Studies of Ethylene Glycol (CAS No. 107-21-1) in B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 413. NIH Publication No. 93-3144. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992i). Toxicology and Carcinogenesis Studies of Polysorbate 80 (CAS No. 9005-65-6) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 415. NIH Publication No. 92-3146. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1992j). Toxicology and Carcinogenesis Studies of Methyl Bromide (CAS No. 74-83-9) in B6C3F$_1$ Mice (Inhalation Studies). Technical Report Series No. 385. NIH Publication No. 92-2840. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993a). Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 394. NIH Publication No. 93-2849. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993b). Toxicology and Carcinogenesis Studies of Furan (CAS No. 110-00-9) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 402. NIH Publication No. 93-2857. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993c). Toxicology and Carcinogenesis Studies of Manganese (II) Sulfate Monohydrate (CAS No. 10034-96-5) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 428. NIH Publication No. 94-3159. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993d). Toxicology and Carcinogenesis Studies of Pentachloroanisole (CAS No. 1825-21-4) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 414. NIH Publication No. 93-3145. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993e). Toxicology and Carcinogenesis Studies of *o*-Nitroanisole (CAS No. 91-23-6) in F344 Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 416. NIH Publication No. 93-3147. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993f). Toxicology and Carcinogenesis Studies of *p*-Nitroaniline (CAS No. 100-01-6) in B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 418. NIH Publication No. 93-3149. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993g). Toxicology and Carcinogenesis Studies of Triamterene (CAS No. 396-01-0) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 420. NIH Publication No. 94-3151. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993h). Toxicology and Carcinogenesis Studies of Talc (CAS No. 14807-96-6) in F344/N Rats and B6C3F$_1$ Mice (Inhalation Studies). Technical Report Series No. 421. NIH Publication No. 93-3152. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993i). Toxicology and Carcinogenesis Studies of Coumarin (CAS No. 91-64-5) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 422. NIH Publication No. 93-3153. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993j). Toxicology and Carcinogenesis Studies of 3,4-Dihydrocoumarin (CAS No. 119-84-6) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 423. NIH Publication No. 93-3154. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993k). Toxicology and Carcinogenesis Studies of Promethazine Hydrochloride (CAS No. 58-33-3) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 425. NIH Publication No. 94-3156. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993l). Toxicology and Carcinogenesis Studies of Turmeric Oleoresin (CAS No. 8024-37-1) (Major Component 79%-85% Curcumin, CAS No. 458-37-7) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 427. NIH Publication No. 93-3158. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993m). Toxicology and Carcinogenesis Studies of Benzyl Acetate (CAS No. 140-11-4) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 431. NIH Publication No. 93-3162. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993n). Toxicology and Carcinogenesis Studies of 1,3-Butadiene (CAS No. 106-99-0) in B6C3F$_1$ Mice (Inhalation Studies). Technical Report Series No. 434. NIH Publication No. 93-3165. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1993o). Toxicology and Carcinogenesis Studies of Mercuric Chloride (CAS No. 7487-94-7) in F344 Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 408. NIH Publication No. 93-3139. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1994a). Toxicology and Carcinogenesis Studies of *o*-Benzyl-*p*-Chlorophenol (CAS No. 120-32-1) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 424. NIH Publication No. 94-3155. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1994b). Toxicology and Carcinogenesis Studies of Barium Chloride Dihydrate (CAS No. 10326-27-9) in F344/N Rats and B6C3F$_1$ Mice (Drinking Water Studies). Technical Report Series No. 432. NIH Publication No. 94-3163. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1994c). Toxicology and Carcinogenesis Studies of Tricresyl Phosphate (CAS No. 1330-78-5) in F344/N Rats and B6C3F$_1$ Mice (Gavage and Feed Studies). Technical Report Series No. 433. NIH Publication No. 94-3164. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1994d). Toxicology and Carcinogenesis Studies of 4,4′-Thiobis(6-*t*-butyl-*m*-cresol) (CAS No. 96-69-5) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 435. NIH Publication No. 95-3166. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1994e). Toxicology and Carcinogenesis Studies of Hexachlorocyclopentadiene (CAS No. 77-47-4) in F344/N Rats and B6C3F$_1$ Mice (Inhalation Studies). Technical Report Series No. 437. NIH Publication No. 94-3168. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1994f). Toxicology and Carcinogenesis Studies of *p*-Nitrobenzoic Acid (CAS No. 62-23-7) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 442. NIH Publication No. 95-3358. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1995a). Toxicology and Carcinogenesis Studies of *t*-Butyl Alcohol (CAS No. 75-65-0) in F344/N Rats and B6C3F$_1$ Mice (Drinking Water Studies). Technical Report Series No. 436. NIH Publication No. 95-3167. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1995b). Toxicology and Carcinogenesis Studies of Methylphenidate Hydrochloride (CAS No. 2981-59-9) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 439. NIH Publication No. 95-3355. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1996a). Toxicology and Carcinogenesis Studies of 1-trans-delta^9-Tetrahydrocannabinol (CAS No. 1972-08-3) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 446. NIH Publication No. 97-3362. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1996b). Toxicology and Carcinogenesis Studies of 1-Amino-2,4-dibromoanthraquinone (CAS No. 81-49-2) in F344/N Rats and B6C3F$_1$ Mice (Feed Studies). Technical Report Series No. 383. NIH Publication No. 96-2838. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1997a). Toxicology and Carcinogenesis Studies of Salicylsulfapyridine (CAS No. 599-79-1) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 457. NIH Publication No. 97-3373. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1997b). Toxicology and Carcinogenesis Studies of Scopolamine Hydrobromide Trihydrate (CAS No. 6533-68-2) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 445. NIH Publication No. 97-3361. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (1997c). Effect of Dietary Restriction on Toxicology and Carcinogenesis Studies in F344/N Rats and B6C3F$_1$ Mice. Technical Report Series No. 460. NIH Publication No. 97-3376. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (2000a). Toxicology and Carcinogenesis Studies of 2-Butoxyethanol (CAS No. 111-76-2) in F344/N Rats and B6C3F$_1$ Mice (Inhalation Studies). Technical Report Series No. 484. NIH Publication No. 00-3974. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

National Toxicology Program (NTP) (2000b). Toxicology and Carcinogenesis Studies of Methyleugenol (CAS No. 93-15-12) in F344/N Rats and B6C3F$_1$ Mice (Gavage Studies). Technical Report Series No. 491. NIH Publication No. 00-3950. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC.

Nohynek, G.J., Longeart, L., Geffray, B., Provost, J.P., and Lodola, A. (1993). Fat, frail and dying young: Survival, body weight and pathology of the Charles River Sprague-Dawley-derived rat prior to and since the introduction of the VAF^R variant in 1988. *Human Exp. Toxicol.* **12**, 87-98.

Poole, T.M., and Drinkwater, N.R. (1995). Hormonal and genetic interactions in murine hepatocarcinogenesis. In *Growth Factors and Tumor Promotion: Implications of Risk Assesment*, 187-194. Wiley-Liss, Inc., New York.

Roe, F.J.C. (1981). Are nutritionists worried about the epidemic of tumours in laboratory animals? *Proc. Nutr. Soc.* **40**, 57-65.

Roe, F.J.C. (1993). What does carcinogenicity mean and how should we test for it? *Food Chem. Toxicol.* **31**, 225-229.

Seilkop, S.K. (1995). The effect of body weight on tumor incidence and carcinogenicity testing in $B6C3F_1$ mice and F344 rats. *Fundam. Appl. Toxicol.* **24**, 247-259.

Sheldon, W.G., Bucci, T.J., Hart, R.W., and Turturro, A. (1995). Age-related neoplasia in a lifetime study of *ad libitum*-fed and food-restricted B6C3F1 mice. *Toxicol. Pathol.* **23**, 458-476.

Turturro, A., Duffy, P.H., and Hart, R.W. (1993). Modulation of toxicity by diet and dietary macronutrient restriction. *Mutat. Res.* **295**, 151-164.

Turturro, A., Duffy, P., Hart, R.W., and Allaben, W.T. (1996). Rational use of dietary control in toxicity studies – B6C3F1 mouse. *Toxicol. Pathol.* **24**, 769-775.

Witt, W.M., Sheldon, W.G., and Thurman, J.D. (1991). Pathological endpoints in dietary restricted rodents – Fischer 344 rats and $B6C3F_1$ mice. In *Biological Effects of Dietary Restriction* (L. Fishbein, Ed.) pp. 73-86. Springer-Verlag, New York.

APPENDIX E
HEPATIC ENZYME ANALYSIS

HEPATIC ENYZME ANALYSIS

INTRODUCTION

In addition to the standard serum enzyme clinical markers of liver function, several enzyme assays were performed directly on samples of livers from the mice evaluated at 15 months and from the mice used in the supplemental study. The enzymes were evaluated for three objectives:

1) Total cytochrome P450 content and CYP2B-dependent 7-pentoxyresorufin *O*-dealkylase activity were assayed because a previous report suggested that chloral hydrate induced cytochrome P450 and monooxygenase activities that are in part catalyzed by CYP2B isoforms (Sanders *et al.,* 1982). Preferential CYP2B isoform induction has been associated with increased liver tumor formation in mice that were chronically exposed to phenobarbital (Wolff *et al.,* 1991).

2) Cytosolic aldehyde and alcohol dehydrogenase activities and UDP-glucuronosyltransferase activity toward trichloroethanol were assayed to determine whether chloral hydrate induced the major enzyme systems associated with its own metabolism.

3) Lauric acid hydroxylase, CYP4A immunoreactive protein, and palmitoyl CoA fatty acid hydroxylase activities were assayed as marker enzymes for peroxisome proliferation. Trichloroacetic acid is well established as a peroxisome proliferator in rodents (Elcombe *et al.,* 1987; Austin *et al.,* 1995).

Decapitation without prior anesthesia or suffocation was used as the method of euthanasia because anesthetics and the hypoxia that occurs during slow asphyxiation by carbon dioxide cause changes in drug metabolizing enzyme activities, in cellular redox state (and hence levels of glutathione, NADPH, and UDPGA), and in serum corticosterone and ACTH levels. Decapitation allows the rapid removal of tissues immediately after the head is severed, and thus greatly reduces the biochemical degradation of the tissues (Liu *et al.,* 1993).

For the mice evaluated at 15 months, only six mice were used for most of the enzyme assays because this number is usually adequate to demonstrate biologically significant levels of enzyme induction. The six heaviest *ad libitum*-fed mice and the six lightest dietary-controlled mice were selected to maximize the difference between the two groups.

MATERIALS AND METHODS

Reagents: 7-Pentoxyresorufin was obtained from Molecular Probes Inc. (Junction City, OR) and resorufin was obtained from from Matherson, Coleman and Bell (Norwood, OH). Glucose-6-phosphate dehydrogenase was obtained from Calbiochem (La Jolla, CA). Organic solvents [high-performance liquid chromatography (HPLC) grade] were obtained from Baker Chemical Co. (Phillipsburg, NJ). All other chemical and biochemical reagents were obtained from Sigma Chemical Co. (St Louis, MO).

Tissue Preparation: Following decapitation of the animals, livers were rapidly excised from the carcasses, weighed, wrapped in labeled aluminum foil, and then immersed in liquid nitrogen. The frozen livers were then stored at −80° C. Portions were cut off each frozen liver sample on dry ice with a razor blade. The tissue pieces (approximately 5 g) were quickly weighed and placed back on dry ice to prevent thawing. The liver tissue was subsequently added to a conical Teflon® glass homogenizer tube containing 4 mL of ice-cold homogenization buffer [250 mM sucrose containing 25 mM potassium chloride, 1 mM DL-dithiothreitol, 0.5 mM EDTA, 10 mM HEPES, 20% glycerol, and 10 mg/500 mL PMSF (pH 7.4)], and rapidly homogenized while thawing. Liver microsomal and cytosolic fractions were prepared by differential centrifugation as described by Leakey *et al.* (1989). After centrifugation at 100,000 × g, the supernatant (cytosolic fraction) was removed, and the washed microsomal pellet was resuspended in 500 µL of 50 mM Tris-buffer (pH 7.4)

containing 20% glycerol. Microsomal and cytosolic protein concentrations were determined by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard. The remaining cytosol and microsome samples were rapidly frozen in liquid nitrogen and stored at –80° C after protein assay.

Enzyme Assays: Total cytochrome P450 and cytochrome B-5 were assayed by an adaptation of the method of Omura and Sato (1964) using a Beckman DU7 spectrophotometer. Cytochrome P420 concentrations were less than 0.02 nmol/mg protein in all samples assayed.

Dealkylation of 7-pentoxyresorufin was assayed by an adaptation of the direct fluorometric methods of Lubet *et al.* (1985). The reactions were carried out at 37° C in 100 mM phosphate buffer, pH 7.4, containing an NADPH-regenerating system (1.25 mM DL-isocitric acid, 0.5 mM NADP, 0.3 U/mL isocitrate dehydrogenase, and 1.25 M $MgCl_2$) 1 µM 7-ethoxyresorufin or 1.5 µM 7-benzoxyresorufin and approximately 400 µg protein in a total reaction volume of 1 mL. Reactions were initiated by the addition of the NADPH-regeneration system and monitored at an excitation wavelength of 550 nm and an emission wavelength of 585 nm on a Shimadzu RF5000U spectrofluorometer (Shimadzu, Columbia, MD). Resorufin (25 pmol) was used as the standard.

Alcohol dehydrogenase was assayed with a PC-30001 dual beam ultraviolet spectrophotometer (Shimadzu) by following the reduction of NAD at 340 nm at 37° C. The incubation mixture contained 200 µL cytosol, 90 mM sodium pyrophosphate buffer (pH 8.8), 200 µM ethanol, and 2 mM NAD in a total volume of 3 mL, and was placed in a quartz cuvette. The reference cuvette contained an identical 3-mL incubation solution except for the addition of the alcohol dehydrogenase inhibitor 4-methyl pyrazole at a final concentration of 1 mM. The reaction was initiated by adding the NAD to both cuvettes and activity was calculated by measuring the rate of absorbance increase and using the extinction coefficient for NADH of 6.6 $mM^{-1} \bullet cm^{-1}$.

Cytosolic aldehyde dehydrogenase was assayed by a similar procedure using an incubation mixture containing 200 µL of cytosol, 90 mM sodium pyrophosphate buffer (pH 8.8), 500 µM acetaldehyde, 500 µM 4-methyl pyrazole, and 2 mM NAD in a total volume of 3 mL. The acetaldehyde was omitted from the reference cuvette.

UDP-Glucuronosyltransferase activity toward trichloroethanol was assayed by an adaptation of the method of Coughtrie *et al.* (1986), using a Waters Module 1 automated HPLC system (Waters Corp., Milford, MA) and an A500 radio-chromatography detector (Packard Instruments, Meriden, CT).

CYP4A immunoreactive protein was assayed in the microsomal samples by SDS-PAGE and Western blotting according to the method of Laemmli (1970) modified by Seng *et al.* (1996) using a BioRad MiniGel Apparatus (BioRad Laboratories Inc., Richmond, CA). The separated proteins were then transferred electrophoretically from the polyacrylamide gel to an *Immobilon* membrane (Millipore Co., Bedford, MA). The membrane was then incubated with a rabbit polyclonal antibody for CYP4A1 (Daiiachi Pure Chemicals Company Ltd., distributed through GeneTest Inc., Woburn, MA). Blocking procedures were modified to include 2-hour and 30-minute incubations in 5% casein (in phosphate-buffered saline) pre- and post-primary antibody incubation, respectively. Primary antibody was detected with an alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody (BioRad) and a BioRad colorimetric alkaline phosphatase detection kit. Daiiachi liver microsomes from clofibrate-treated rats were provided with the primary antibody preparation as a positive control. Immunoblots were scanned with a Bio-Rad computing imaging densitometer (Model GS-670) and relative A_{540nm} was determined using the Molecular Analyst data analysis system.

Lauric acid hydroxylase activity was determined by measuring the conversion of lauric acid to hydroxylated product(s) by modifications of previously described methods (Bains *et al.,* 1985). Briefly, the reaction mixture contained approximately 200 µg of microsomal protein, an NADPH regenerating system (2.4 mM NADP,

4 U/mL glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, and 10 mM $MgCl_2$) and 100 µM [4-^{14}C]-lauric acid (1.2 µCi/µmol) in a total volume of 0.25 mL. Samples were then incubated for 5 minutes at 37° C. The reaction was terminated by the addition of 375 µL of 3.0 M HCl and samples were extracted with 1.25 mL of methylene chloride. The organic phase was evaporated under argon and the residue was stored at −20° C until analysis.

For analysis, the residue was resuspended in water:methanol:glacial acetic acid (45:55:5) and analyzed by HPLC. Lauric acid and its metabolites were resolved and quantified with a Waters Module 1 automated HPLC system (Waters Corp.) and an A500 radio-chromatography detector (Packard Instruments). The system utilized an UltraCarb 5 µm, 250 x 4.6 mm C-18 column (Phenomenex, Torrance, CA) at a flow rate of 1 mL/min. A 45-minute convex gradient was run starting with water containing 0.01% glacial acetic acid and ending with methanol containing 10% tetrahydrofuran. The 11- and 12-hydroxy lauric acid metabolites were quantitated by their radioactivity with the flow detector.

Palmitoyl CoA fatty acid hydroxylase was assayed by an adaptation of the method of Bronfman *et al.* (1979) using a Beckman DU-7 stable beam spectrophotometer.

Although cyanide-insensitive palmitoylCoA fatty acid hydroxylase is primarily a peroxisomal enzyme, it is released into the cytosolic fraction when liver is homogenized. Hence, the measured activity was several hundred times greater in the cytosolic fraction than in the washed microsomal fraction. The 1,000 µL incubation mixture contained 50 µM palmitoyl-CoA, 100 µM CoA, 10 µM FAD, 60 µg/mL bovine serum albumin, 200 µM NAD, 100 µM dithiothreitol, 1 mM potassium cyanide, and mouse liver cytosol (100 to 250 µg protein) in 94 mM tris-hydrochloride buffer, pH 8.3. The mixture without the cytosol was added to a 1 mL quartz cuvette and preheated to 37° C in the spectrophotometer while a baseline was obtained at 340 nm. The cytosol was then added and the rate of increase in absorbance recorded. The specific activity was calculated from the molar extinction coefficient of NADH measured with the same spectrophotometer.

RESULTS AND DISCUSSION

Chloral hydrate did not induce either total or CYP2B isoforms of cytochrome P450. Chronic exposure to phenobarbital results in a three- to sixfold increase in 7-pentoxyresorufin *O*-dealkylase activity (Wolff *et al.,* 1991). There was no evidence of a similar induction in any of the chloral hydrate-treated samples. There was a slight increase in the control activities of 7-pentoxyresorufin *O*-dealkylase in the calorically restricted mice. This is consistent with previous studies that showed caloric restriction causes a circadian-dependent induction of this activity in rats.

Chloral hydrate did not induce cytosolic alcohol or aldehyde dehydrogenase activities, suggesting that it did not induce its own metabolism. However, it is possible that specific isoforms of these enzymes might have been induced and this might not have been detected by the generic assays used. UDP-glucuronosyltransferase activity towards trichloroethanol was also not induced by chloral hydrate treatment. This activity was relatively high in the mouse liver and approximately fivefold greater than that observed with human liver samples in our laboratory. It is possible, therefore, that the explanation for the relatively low concentrations of trichloroethanol in mouse plasma is due to the rapid conjugation of chloral hydrate.

Chloral hydrate did induce the marker enzymes of peroxisome proliferation, cytochrome P450 CYP4A and its dependent activity, lauric acid hydroxylation, and cyanide-insensitive palmitoyl CoA fatty acid hydroxylase. For both the mice evaluated at 15 months and the mice from the supplemental study, there was greater induction of these activities in the dietary-controlled and calorically restricted mice than in the *ad libitum*-fed mice. These differences could not be completely explained by differences in plasma trichloroacetate levels (see Appendix J). The dose response of the dietary-controlled group exhibited better statistical significance

than that of the other two diet groups in the supplemental study and in the 15-month evaluation, as statistically significant increases in both CYP4A protein and lauric acid hydroxylase activities were observed in only the dietary-controlled mice. This increase coincided with significant induction of hepatocellular carcinoma in the 2-year study.

REFERENCES

Austin, E.W., Okita, J.R., Okita, R.T., Larson, J.L., and Bull, R.J. (1995). Modification of lipoperoxidative effects of dichloroacetate and trichloroacetate is associated with peroxisome proliferation. *Toxicology* **97**, 59-69.

Bains, S.K., Gardiner, S.M., Mannweiler, K., Gillett, D., and Gibson, G.G. (1985). Immunochemical study on the contribution of hypolipidaemic-induced cytochrome P-452 to the metabolism of lauric acid and arachidonic acid. *Biochem. Pharmacol.* **34**, 3221-3229.

Bronfman, M., Inestrosa, N.C., and Leighton, F. (1979). Fatty acid oxidation by human liver peroxisomes. *Biochem. Biophys. Res. Commun.* **88**, 1030-1036.

Coughtrie, M.W.H., Burchell, B., and Bend, J.R. (1986). A general assay for UDPglucuronosyltransferase activity using polar amino-cyano stationary phase HPLC and UDP[U-^{14}C]glucuronic acid. *Anal. Biochem.* **159**, 198-205.

Elcombe, C.R., Pratt, I.S., and Green, T. (1987). Species differences in biotransformation and peroxisome proliferation due to trichloroethylene. *Arch. Toxicol. Suppl.* **10**, 147 (Abstr.).

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leakey, J.E.A., Cunny, H.C., Bazare, J., Jr., Webb, P.J., Feuers, R.J., Duffy, P.H., and Hart, R.W. (1989). Effects of aging and caloric restriction on hepatic drug metabolizing enzymes in the Fischer 344 rat. I. The cytochrome *P*-450 dependent monooxygenase system. *Mech. Ageing Devel.* **48**, 145-155.

Liu, P.T., Ioannides, C., Shavila, J., Symons, A.M., and Parke, D.V. (1993). Effects of ether anaesthesia and fasting on various cytochromes P450 of rat liver and kidney. *Biochem. Pharmacol.* **45**, 871-877.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin and phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Lubet, R.A., Mayer, R.T., Cameron, J.W., Nims, R.W., Burke, M.D., Wolff, T., and Guengerich, F.P. (1985). Dealkylation of pentoxyresorufin: A rapid and sensitive assay for measuring induction of cytochrome(s) *P*-450 by phenobarbital and other xenobiotics in the rat. *Arch. Biochem. Biophys.* **238**, 43-48.

Omura, T., and Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239**, 2370-2378.

Sanders, V.M., Kauffmann, B.M., White, K.L., Jr., Douglas, K.A., Barnes, D.W., Sain, L.E., Bradshaw, T.J., Borzelleca, J.F., and Munson, A.E. (1982). Toxicology of chloral hydrate in the mouse. *Environ. Health Perspect.* **44**, 137-146.

Seng, J.E., Gandy, J., Turturro, A., Lipman, R., Bronson, R.T., Parkinson, A., Johnson, W., Hart, R.W., and Leakey, J.E.A. (1996). Effects of caloric restriction on expression of testicular cytochrome P450 enzymes associated with the metabolic activation of carcinogens. *Arch. Biochem. Biophys.* **335**, 42-52.

Wolff, G.L., Leakey, J.E.A., Bazare, J.J., Harmon, J.R., Webb, P.J., and Law, M.G. (1991). Susceptibility to phenobarbital promotion of hepatotumorigenesis: Correlation with differential expression and induction of hepatic drug metabolizing enzymes in heavy or light male (C3H × VY) F1 hybrid mice. *Carcinogenesis* **12**, 911-915.

TABLE E1
Liver-Weight-to-Body-Weight Ratios in 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate[a]

Dose (mg/kg)	*Ad Libitum*-Fed	Dietary-Controlled	Calorically Restricted
0	71.41 ± 5.54	67.07 ± 8.87	67.28 ± 8.13
50	64.42 ± 5.72	65.23 ± 8.06	71.33 ± 7.56
100	60.89 ± 6.28	69.65 ± 7.76	72.71 ± 8.13
250	65.01 ± 4.71	69.56 ± 6.07	72.32 ± 5.22
500	59.95 ± 6.86[b]	66.27 ± 7.47[c]	81.44 ± 8.09[b]

[a] Ratio is given as mg liver weight per g body weight (mean ± standard error of the mean). Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose. There were no significant differences between dose groups (P<0.05); n=6 unless otherwise noted.
[b] n=4
[c] n=5

TABLE E2
Hepatic Microsomal Cytochrome P450 Concentration in 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate[a]

Dose (mg/kg)	*Ad Libitum*-Fed	Dietary-Controlled	Calorically Restricted
0	0.64 ± 0.06	0.51 ± 0.03	0.67 ± 0.11
50	0.50 ± 0.03	0.44 ± 0.02	0.71 ± 0.12
100	0.49 ± 0.03	0.53 ± 0.10	0.59 ± 0.11
250	0.54 ± 0.03	0.44 ± 0.04	0.50 ± 0.05
500	0.56 ± 0.09[b]	0.45 ± 0.03[c]	0.62 ± 0.16[b]

[a] Concentration is expressed as nmol cytochrome P450 per mg microsomal protein (mean ± standard error of the mean) as assessed from the CO-cytochrome P450 binding spectrum. Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose. There were no significant differences between dose groups (P<0.05); n=6 unless otherwise noted.
[b] n=4
[c] n=5

TABLE E3
Hepatic Microsomal 7-Pentoxyresorufin *O*-Dealkylase Activity in 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate[a]

Dose (mg/kg)	*Ad Libitum*-Fed	Dietary-Controlled	Calorically Restricted
0	17.8 ± 4.1[b]	21.4 ± 5.8	30.2 ± 7.4
50	15.3 ± 3.2[b]	15.8 ± 3.5	26.4 ± 5.0
100	12.2 ± 1.9[b]	35.7 ± 13.2	14.1 ± 2.9
250	17.9 ± 2.3[b]	24.7 ± 11.6	19.7 ± 6.9
500	10.2 ± 2.5[c]	16.4 ± 3.6[d]	21.1 ± 10.6[b]

[a] Activity is expressed as nmol resorufin formed per minute per mg microsomal protein (mean ± standard error of the mean). Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose. There were no significant differences between dose groups (P<0.05); n=6 unless otherwise noted.
[b] n=4
[c] n=2
[d] n=5

TABLE E4
Hepatic Microsomal Lauric Acid ω-Hydroxylase Activity in 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate[a]

Dose (mg/kg)	*Ad Libitum*-Fed			Dietary-Controlled			Calorically Restricted		
0	1.22 ± 0.25	A	A	1.26 ± 0.07	A	A	2.33 ± 0.23	A	B
50	0.95 ± 0.21	A	A	1.59 ± 0.12	AB	AB	2.50 ± 0.57	AB	B
100	1.42 ± 0.37	AB	A	2.30 ± 0.18	B	B	3.80 ± 0.42	BC	B
250	2.38 ± 0.57	ABC	A	3.36 ± 0.12[c]	C	B	5.49 ± 0.51	C	B
500	3.95 ± 0.22[b]	C	A	7.71 ± 0.51[c]	D	B	8.16 ± 0.99[b]	D	B

[a] Activity is expressed as nmol ω-12-hydroxylaurate formed per minute per mg microsomal protein (mean ± standard error of the mean). Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose (first column), and each dose group was compared by diet (second column). Means that do not share the same letter are significantly different (P<0.05); n=6 unless otherwise noted.
[b] n=4
[c] n=5

TABLE E5
Hepatic Peroxisomal Palmitoyl CoA Fatty Acid Hydroxylase Activity in 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate[a]

Dose (mg/kg)	*Ad Libitum*-Fed			Dietary-Controlled			Calorically Restricted		
0	20.2 ± 1.6	A	A	23.6 ± 1.9	A	A	29.2 ± 2.4	A	A
50	25.7 ± 1.9	A	A	26.3 ± 4.2	A	A	24.3 ± 1.7	A	A
100	28.6 ± 6.0	A	A	30.5 ± 2.6	A	A	31.5 ± 3.3	A	A
250	56.8 ± 18.4	A	A	66.8 ± 13.3[c]	B	A	66.8 ± 6.1[b]	B	A
500	46.5 ± 7.0[b]	A	A	82.9 ± 14.0[c]	B	B	101.5 ± 18.0[b]	C	B

[a] Activity is expressed as nmol NAD reduced per minute per mg cytosolic protein (mean ± standard error of the mean). Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose (first column), and each dose group was compared by diet (second column). Means that do not share the same letter are significantly different (P<0.05); n=6 unless otherwise noted.
[b] n=4
[c] n=5

TABLE E6
Hepatic Cytosolic Alcohol Dehydrogenase Activity in 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate[a]

Dose (mg/kg)	*Ad Libitum*-Fed	Dietary-Controlled	Calorically Restricted
0	2.32 ± 0.53	3.15 ± 0.41	2.76 ± 0.48
50	2.07 ± 0.31	2.71 ± 0.46	3.25 ± 0.48
100	1.38 ± 0.28*	2.84 ± 0.38	3.33 ± 0.36
250	2.43 ± 0.45[b]	3.16 ± 0.36[b]	2.23 ± 0.42[b]
500	2.02 ± 0.43[c]	1.91 ± 0.33[b]	3.04 ± 0.48[b]

* Significantly different (P<0.05) from the corresponding dietary-controlled and calorically restricted groups
[a] Activity is expressed as nmol NAD reduced per minute per mg cytosolic protein (mean ± standard error of the mean). Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose. There were no significant differences between dose groups (P<0.05); n=6 unless otherwise noted.
[b] n=5
[c] n=4

TABLE E7
Hepatic Cytosolic Aldehyde Dehydrogenase Activity in 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate[a]

Dose (mg/kg)	*Ad Libitum*-Fed	Dietary-Controlled	Calorically Restricted
0	1.69 ± 0.17	2.20 ± 0.23	2.37 ± 0.41
50	1.54 ± 0.30	2.32 ± 0.33	2.15 ± 0.25[b]
100	1.79 ± 0.23	2.03 ± 0.09	2.24 ± 0.29[b]
250	1.79 ± 0.24	2.43 ± 0.50[b]	1.51 ± 0.14
500	1.53 ± 0.17[c]	1.79 ± 0.20[b]	1.58 ± 0.52[c]

[a] Activity is expressed as nmol NADH oxidized per minute per mg of cytosolic protein (mean ± standard error of the mean). Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose. There were no significant differences between dose or diet groups ($P<0.05$); n=6 unless otherwise noted.
[b] n=5
[c] n=4

TABLE E8
Liver-Weight-to-Body-Weight Ratios in Male Mice Evaluated at 15 Months in the 2-Year Gavage Study of Chloral Hydrate[a]

	Ad Libitum-Fed				Dietary-Controlled			
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	12	12	12	12	12	12	12	12
Mean	47.08	46.96	40.87	51.11	35.63	37.46	38.31	39.55
SD[b]	17.59	17.40	4.13	19.58	1.02	1.37	2.09	2.29
SEM[c]	5.08	5.02	1.19	5.65	0.30	0.39	0.60	0.66
Tukey's test[d]	A	A	A	A	A	AB	BC	C
Dunnett's test[e]					0.0001	0.0394	0.0017	0.0000

[a] Ratio is given as mg liver weight per g body weight.
[b] Standard deviation
[c] Standard error of the mean
[d] Each diet group was analyzed by a separate ANOVA; dose group means not sharing the same letter are significantly different from each other ($P<0.05$)
[e] Beneath the vehicle control group mean is the P value associated with the trend analysis. Beneath the dosed group means are the P values corresponding to pairwise comparisons between the vehicle controls and that dosed group.

TABLE E9
Hepatic Microsomal Cytochrome P450 Concentration in Male Mice Evaluated at 15 Months in the 2-Year Gavage Study of Chloral Hydrate[a]

	Ad Libitum-Fed				Dietary-Controlled			
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	6	6	6	6	6	6	6	6
Mean	0.71	0.74	0.74	0.67	0.78	0.74	0.77	0.77
SEM[b]	0.07	0.06	0.09	0.05	0.06	0.07	0.06	0.06
Tukey's test[c]	A	A	A	A	A	A	A	A

[a] Concentration is expressed as nmol cytochrome P450 per mg microsomal protein as assessed from the CO-cytochrome P450 binding spectrum.
[b] Standard error of the mean
[c] Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose; means not sharing the same letter are significantly different (P<0.05).

TABLE E10
Hepatic Microsomal 7-Pentoxyresorufin *O*-Dealkylase Activity in Male Mice Evaluated at 15 Months in the 2-Year Gavage Study of Chloral Hydrate[a]

	Ad Libitum-Fed				Dietary-Controlled			
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	6	6	6	6	6	6	6	6
Mean	19.1	22.6	19.9	16.4	21.0	20.9	18.9	19.2
SEM[b]	2.3	3.3	4.3	1.8	4.1	1.4	2.9	3.2
Tukey's test[c]	A	A	A	A	A	A	A	A

[a] Activity is expressed as nmol resorufin formed per minute per mg microsomal protein.
[b] Standard error of the mean
[c] Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose; means not sharing the same letter are significantly different (P<0.05).

TABLE E11
Hepatic Microsomal Lauric Acid ω-Hydroxylase Activity in Male Mice Evaluated at 15 Months in the 2-Year Gavage Study of Chloral Hydrate[a]

	Ad Libitum-Fed				Dietary-Controlled			
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	11	8	10	9	10	10	11	12
Mean	1.30	1.38	1.40	1.77	1.46	1.38	1.40	2.25
SEM[b]	0.20	0.13	0.16	0.19	0.09	0.12	0.16	0.19
Tukey's test[c]	A	A	A	A	A	A	A	B

[a] Activity is expressed as nmol ω-12-hydroxylaurate formed per minute per mg microsomal protein.
[b] Standard error of the mean
[c] Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose; means not showing the same letter are significantly different (P<0.05).

TABLE E12
Hepatic Microsomal CYP4A Concentration in Male Mice Evaluated at 15 Months in the 2-Year Gavage Study of Chloral Hydrate[a]

	Ad Libitum-Fed				Dietary-Controlled			
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	6	6	6	6	6	6	6	6
Mean	72.1	82.0	79.0	91.4	67.6	66.0	71.6	100.4
SEM[b]	3.2	8.1	10.5	9.3	5.5	8.5	4.7	6.8
Tukey's test[c]	A	A	A	A	A	A	A	B

[a] Concentration is expressed as the percentage of the integrated area (i.e., area × intensity) of a standard microsomal sample from clofibrate-treated rat liver adjusted per mg microsomal protein.
[b] Standard error of the mean
[c] Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose; means not sharing the same letter are significantly different (P<0.05).

TABLE E13
Hepatic Microsomal UDP-Glucuronosyltransferase Activity Toward Trichloroethanol in Male Mice Evaluated at 15 Months in the 2-Year Gavage Study of Chloral Hydrate[a]

	Ad Libitum-Fed				Dietary-Controlled			
	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
n	6	6	6	6	6	6	6	6
Mean	1.14	0.88	0.59	0.89	1.08	0.79	0.90	0.91
SEM[b]	0.31	0.27	0.16	0.29	0.27	0.07	0.25	0.30
Tukey's test[c]	A	A	A	A	A	A	A	A

[a] Activity is expressed as nmol 1,1,1-trichloroethonyl-β-D-glucuronide formed per minute per mg microsomal protein.
[b] Standard error of the mean
[c] Statistical analysis was performed by a SAS GLM-Tukey's test. Each diet group was compared by dose; means not sharing the same letter are significantly different (P<0.05).

TABLE E14
Serum Hepatic Enzyme Data for Male Mice in the 2-Year Gavage Study of Chloral Hydrate[a]

	0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
***Ad Libitum*-Fed**				
n	41	35	35	44
Alanine aminotransferase (IU/L)	59.52 ± 19.12	72.44 ± 16.07	70.23 ± 13.92[b]	39.92 ± 5.29
Amylase (IU/L)	2,203.55 ± 97.75	2,207.25 ± 90.17[c]	2,200.63 ± 95.09[c]	2,093.15 ± 82.74[d]
Aspartate aminotransferase (IU/L)	69.89 ± 8.88	126.24 ± 46.70*	68.40 ± 7.92	52.33 ± 5.49
Lactate dehydrogenase (IU/L)	359.87 ± 62.49	643.16 ± 262.47	409.54 ± 79.49	322.89 ± 50.54[e]
Dietary-Controlled				
n	45	44	47	41
Alanine aminotransferase (IU/L)	30.37 ± 3.25[f]	64.75 ± 18.19	39.29 ± 7.78	36.72 ± 5.06
Amylase (IU/L)	1,934.16 ± 38.81[f]	1,997.17 ± 50.01	2,014.43 ± 48.27[g]	1,895.88 ± 36.73[d]
Aspartate aminotransferase (IU/L)	59.26 ± 7.84	64.33 ± 7.73	66.65 ± 8.93	57.12 ± 7.04
Lactate dehydrogenase (IU/L)	271.05 ± 23.29	363.53 ± 72.01	275.02 ± 29.29[g]	257.83 ± 33.90

* Significantly different (P<0.05) from the vehicle control group by Dunnett's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n = 36
[c] n = 33
[d] n = 39
[e] n = 41
[f] n = 44
[g] n = 46

APPENDIX F
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF CHLORAL HYDRATE

Chloral hydrate was obtained from Amend Drug and Chemical Company, Inc. (Irvington, NJ), in one lot (Z57601P28), which was used in the 2-year study. Identity, purity and stability analyses were conducted by the study laboratory.

Upon receipt, the chloral hydrate was pulverized with a mortar and pestle. Lot Z57601P28, a white, crystalline powder, was identified as chloral hydrate with gas chromatography/mass spectrometry by system A (Table F1) and ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy (NMR). The NMR spectra were consistent with the structure of chloral hydrate; the mass spectrum was consistent with a literature spectrum (NIST Mass Spectral Reference Library) of chloral hydrate. Some relatively minor resonance peaks indicated the presence of toluene at a concentration of approximately 0.02% (200 ppm). The spectra are presented in Figures F1, F2, and F3.

The purity of lot Z57601P28 was determined with gas chromatography with flame ionization detection by system B, with a reference standard of chloral hydrate in diethyl ether, and with high-performance liquid chromatography (HPLC). HPLC was performed with a Waters™ Symmetry C_8 105 mm × 3.9 mm column (Waters-Millipore, Milford, MA) using photo diode array detection at 254 nm. The flow rate was 1.0 mL/minute.

One minor peak that represented approximately 0.18% of the total peak area was detected by gas chromatography. A standard of toluene in diethyl ether was prepared and injected into the gas chromatograph under identical conditions. The retention time matched that of the impurity. Peak height comparison indicated that approximately 170 µg/g toluene was present in the chloral hydrate. A comparison of peak areas by HPLC indicated that 171 µg/g toluene was present in the chloral hydrate. The overall purity was determined to be greater than 99%.

Portions of the bulk chemical were stored in three amber glass bottles. The bottles were placed in a dessicator containing dry indicating silica gel and stored at room temperature. The remaining bulk chemical was stored in an amber glass bottle in a plastic bag containing a drying agent, Drierite™ (Aldrich Chemical Co. Inc., Milwaukee, WI). The bag was placed in a plastic drum and stored at room temperature.

Stability of the bulk chemical was analyzed at the end of the 2-year study using gas chromatography by system B, gas chromatography/mass spectrometry by system C, and ^{1}H-NMR. These analyses showed the compound had remained stable throughout the study. Gas chromatography indicated a reduced level of toluene in comparison to the original evaluation, and ^{1}H-NMR confirmed the toluene had dissipated to approximately 70 ppm. Gas chromatography also indicated a few additional minor peaks with a combined area of approximately 0.4% relative to the major peak area; these impurities were tentatively identified as metachloral, a trimer of chloral, and similar polymers and were present at a concentration of approximately 4,000 ppm. The chloral hydrate was estimated to be approximately 99.6% pure at the end of the 2-year study.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

Dose formulations were prepared by dissolving chloral hydrate crystals in distilled water (Table F2). The solution was purged of toluene by sparging with a stream of nitrogen for at least 30 minutes and filtered through 0.45-µm nylon filters. Aliquots of this solution were diluted with water to form the appropriate concentrations. Dose formulations were prepared every 4 weeks. The dose formulations were stored in amber glass bottles, at room temperature, for up to 4 weeks.

Stability studies of 0.85, 1.4, 2.5, and 5 mg/mL formulations were performed by the study laboratory using gas chromatography by system C. Stability was confirmed for 24 days for the 0.85 mg/mL formulation, 78 days for the 2.5 mg/mL formulation, 6 months for the 5 mg/mL formulation and 7 months for the 1.4 mg/mL formulation when stored in amber glass vials at room temperature.

Periodic analyses of the dose formulations were conducted by the study laboratory using gas chromatography by system D. At the beginning of the 2-year study, analyses were performed on all dose formulations. The first four were found to be within 10% of the target concentration; thereafter, analyses were performed every 3 months. Animal room samples were analyzed every 6 months. Of the dose formulations analyzed, 98% (41/42) were within 10% of the target concentrations, with no value greater than 103% of the target concentration.

TABLE F1
Gas Chromatography Systems Used in the 2-Year Gavage Study of Chloral Hydrate[a]

Detection System	Column	Carrier Gas	Oven Temperature Program
System A			
Quadrupole mass spectrometry with electron impact ionization (70 eV)	DB5ms, 0.25 mm × 30 m, 0.25 µm film (J&W Scientific, Folsom, CA)	Helium at 12 psi head pressure	40° C for 5 minutes, then 20° C/minute to 250° C, held for 5 minutes
System B			
Flame ionization	J&W DB 1701, 30 m × 0.25 mm, 0.25 µm film (J&W Scientific)	Helium at 12 psi head pressure; flow of approximately 0.8 mL/minute	40° C for 5 minutes, then 20° C/minute to 200° C, held for 5 minutes
System C			
Mass spectrometer with electron impact ionization (70 eV)	DB5ms, 0.25 mm × 30 m, 0.25 µm film (J&W Scientific)	Helium at 10 psi head pressure	50° C for 2 minutes, then 20° C/minute to 280° C, held for 10 minutes
System D			
Flame ionization	Carbopak B/5% Carbowax 20M, 6 ft × 2 mm (Supelco, Bellefonte, PA)	Nitrogen or helium at approximately 20 mL/minute	110° C (isothermal)

[a] The mass spectrometers used in systems A (Finnigan 4500) and C (Finnigan TSQ 700) were manufactured by Finnigan MAT Corp. (San Jose, CA); the gas chromatographs were manufactured by Varian, Inc. (Palo Alto, CA) (systems A and C) and Hewlett-Packard (Palo Alto, CA) (systems B and D).

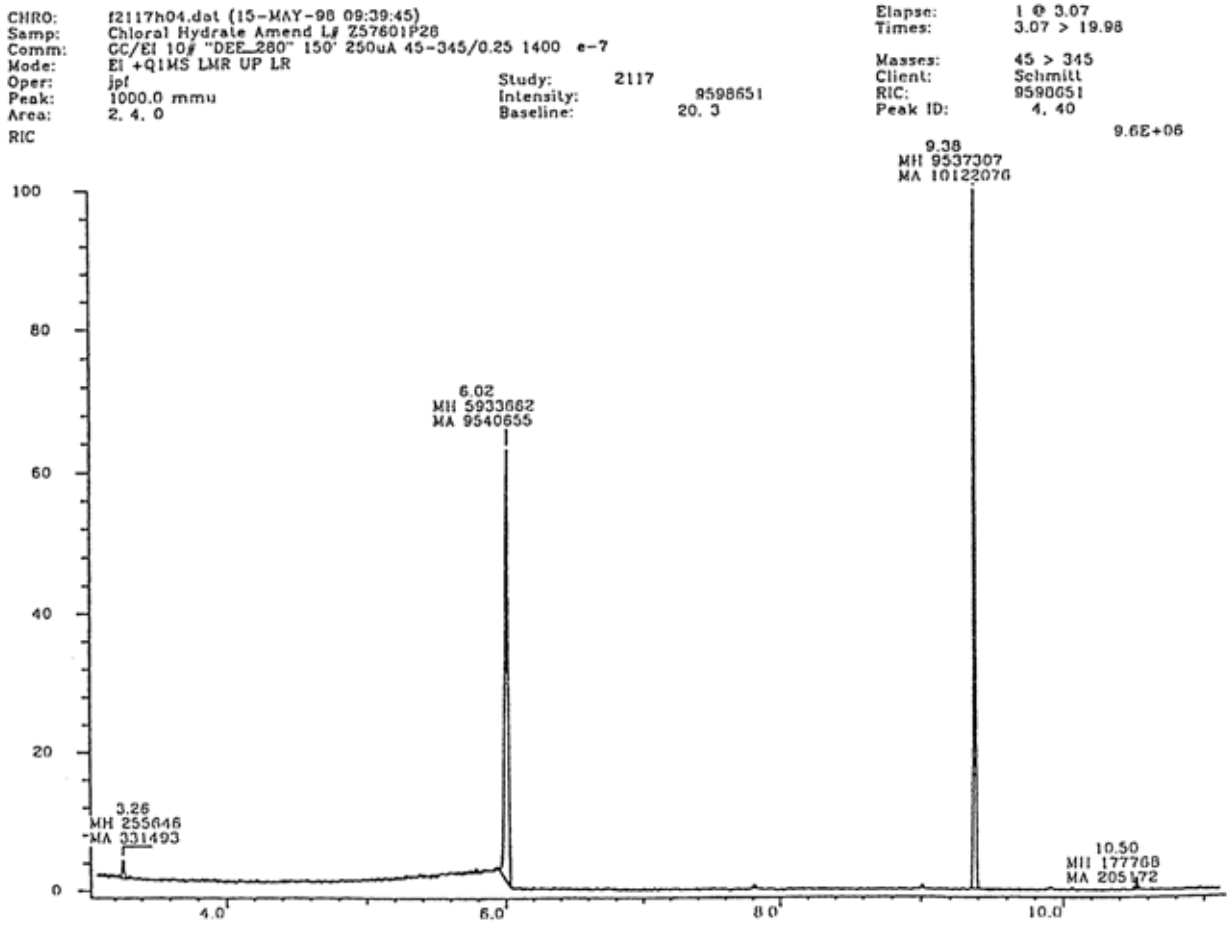


FIGURE F1
Gas Chromatography-Mass Spectrum of Chloral Hydrate
Peak 1 eluting at 3.26 minutes was a minor chlorinated contaminant(s) which was not identified; peak 2 at 6.02 minutes, was choral hydrate; peak 3 at 9.38 minutes was consistent with butylated hydroxytoluene; and peak 4 and 10.50 minutes was identified as toluene.

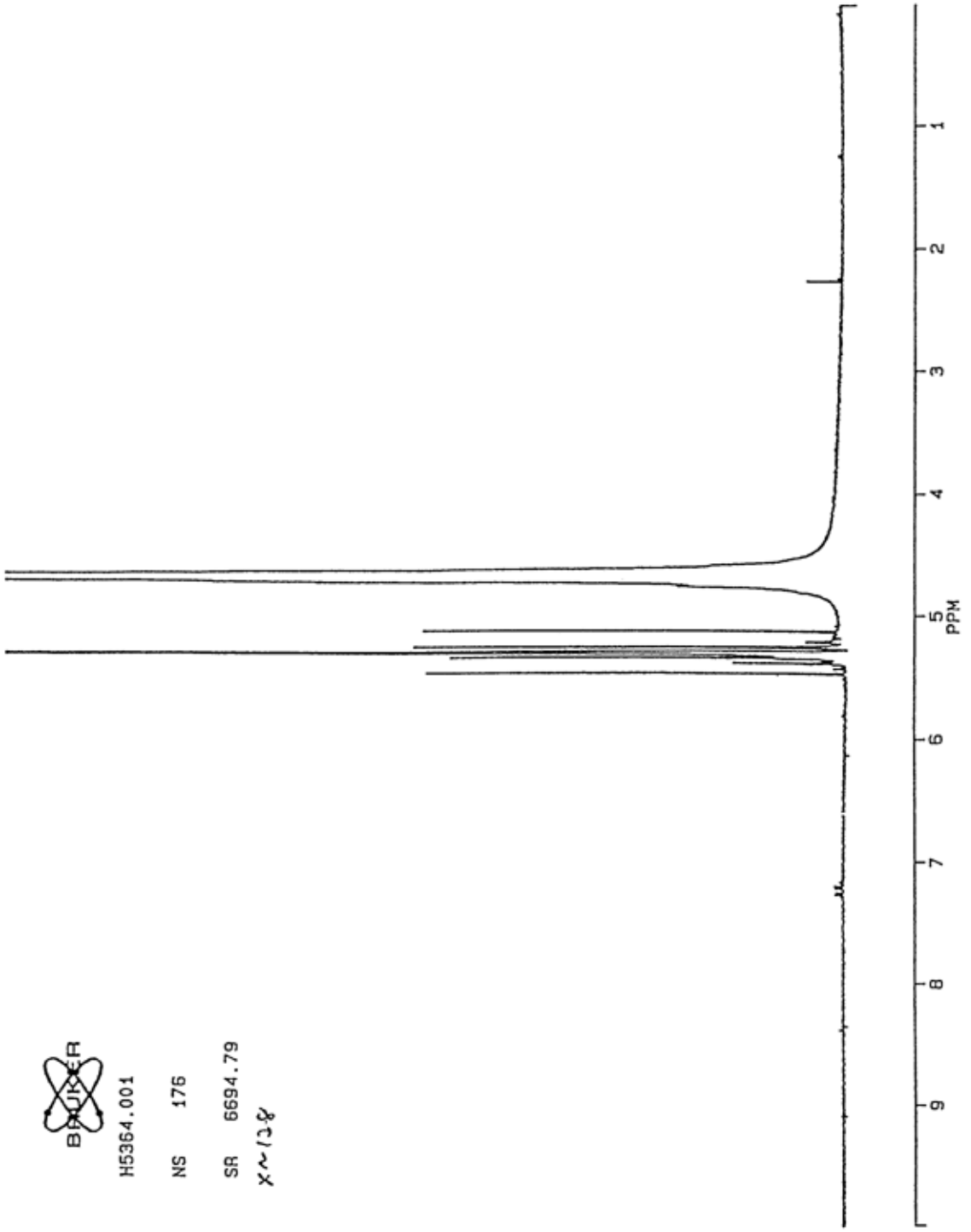


FIGURE F2
^{1}H-Nuclear Magnetic Resonance Spectrum of Chloral Hydrate

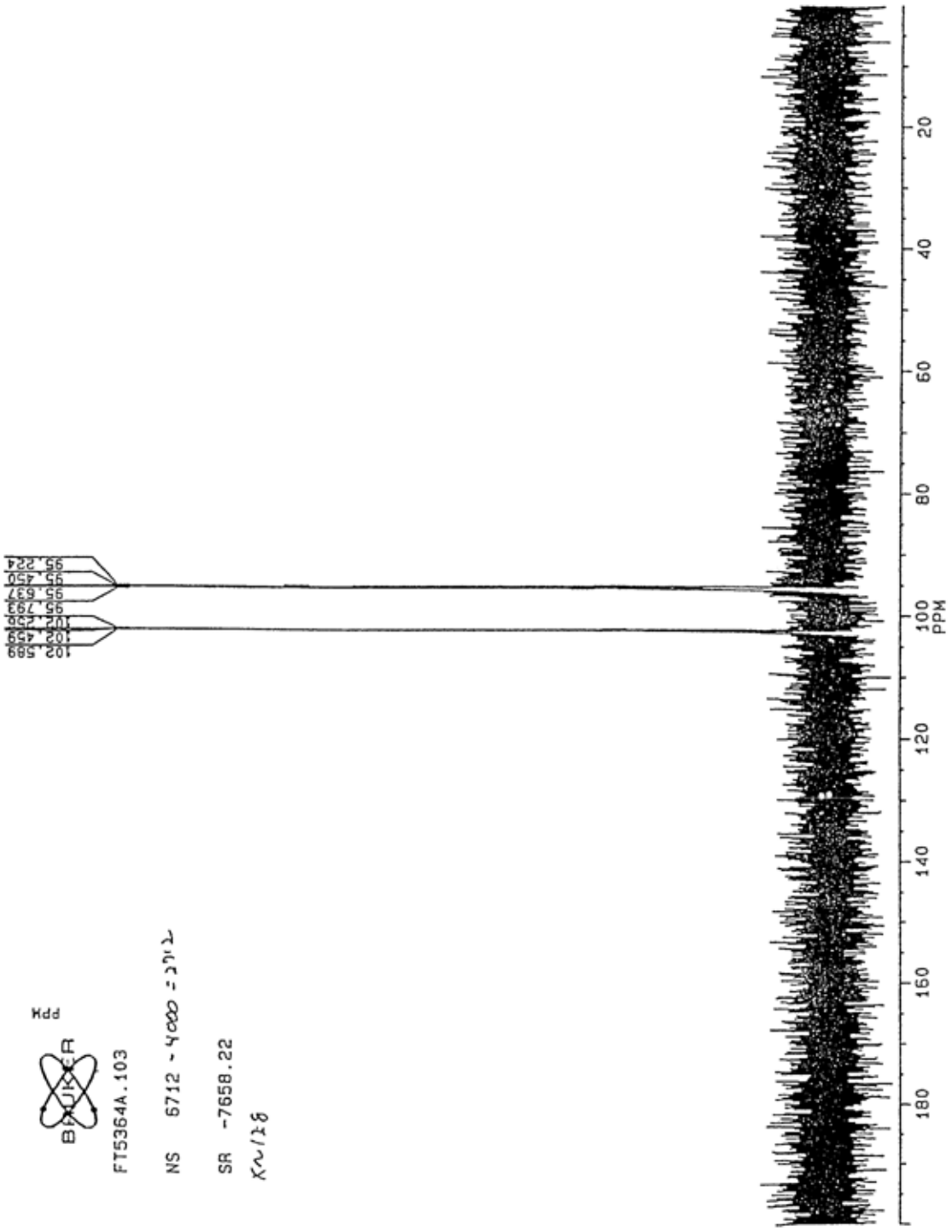


FIGURE F3
^{13}C-Nuclear Magnetic Resonance Spectrum of Chloral Hydrate

TABLE F2
Preparation and Storage of Dose Formulations in the 2-Year Gavage Study of Chloral Hydrate

Preparation
A stock solution was prepared by dissolving chloral hydrate crystals in distilled water. The stock solution was sparged with a stream of nitrogen for at least 30 minutes to purge toluene and filtered (0.45µm). Aliquots of this solution were diluted with water to form the desired concentrations. Dose formulations were prepared every 4 weeks.

Chemical Lot Number
Z57601P28

Maximum Storage Time
4 weeks

Storage Conditions
Amber glass bottles at room temperature

Study Laboratory
National Center for Toxicological Research (Jefferson, AR)

TABLE F3
Results of Analyses of Dose Formulations Administered to Mice in the 2-Year Gavage Study of Chloral Hydrate

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
January 22, 1996	January 24, 1996	5	5.02	0
		10	10.06	+1
		20	20.08	0
February 9, 1996	February 14, 1996	5	5.09	+2
		10	10.23	+2
		20	19.92	0
February 23, 1996	February 26, 1996	5	5.07	+1
		10	10.08	+1
		20	20.36	+2
March 1, 1996	March 12, 1996	5	5.06	+1
		10	10.15	+2
		20	19.93	0
March 12, 1997	March 24, 1997	5	5.04	+1
		10	9.99	0
		20	20.20	+1
March 18, 1996	July 9, 1996	5	5.06	+1
April 22, 1996	July 9, 1996	5	5.01	0
		10	10.19	+2
		20	20.39	+2
May 22, 1996	July 9, 1996	5	5.12	+2
		10	10.14	+1
June 6, 1996	July 9, 1996	5	4.83	−3
		10	9.87	−1
		20	20.20	+1
August 21, 1996	October 3, 1996	5	5.08	+2
		10	9.80	−2
		20	19.73	−1
November 19, 1996	November 26, 1996[b]	5	4.96	−1
		5	4.91	−2
		5	4.88	−2
		5	4.87	−3
		5	4.86	−3
		5	4.97	−1
		20	19.66	−2
		20	19.71	−1
		20	19.41	−3
		20	19.84	−1
		20	19.60	−2
		20	19.55	−2

TABLE F3
Results of Analyses of Dose Formulations Administered to Mice in the 2-Year Gavage Study of Chloral Hydrate

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
December 19, 1996	January 10, 1997	5	5.15	+3
		10	9.90	−1
		20	19.85	−1
December 31, 1996	January 10, 1997	5	4.97	−1
		10	9.94	−1
		20	19.69	−2
May 6, 1997	May 23, 1997[b]	5	5.14	+3
		5	5.14	+3
		5	4.91	−2
		5	5.06	+1
		5	4.85	−3
		5	4.82	−4
		20	20.15	+1
		20	19.77	−1
		20	19.55	−2
		20	19.24	−4
		20	19.47	−3
		20	19.60	−2
June 4, 1997	June 9, 1997[b]	5	4.94	−1
		5	5.01	0
		5	4.93	−1
June 4, 1997	June 18, 1997	5	4.98	0
		10	9.86	−1
		20	19.95	0
August 25, 1997	January 7, 1998	5	4.37	−13
		10	9.95	0
		20	19.74	−1
December 18, 1997	January 7, 1998	5	5.07	+1
		10	10.12	+1
		20	19.64	−2
January 14, 1998	January 23, 1998[b]	5	5.03	+1
		5	5.04	+1
		5	4.99	0
		5	5.01	0
		5	5.10	+2
		5	4.89	−2
		20	20.02	0
		20	19.69	−2
		20	19.87	−1
		20	19.98	0
		20	19.96	0
		20	19.75	−1

TABLE F3
Results of Analyses of Dose Formulations Administered to Mice in the 2-Year Gavage Study of Chloral Hydrate

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration (mg/mL)	Difference from Target (%)
February 10, 1998	March 4, 1998[b]	5	5.11	+2
		5	5.05	+1
		5	4.98	0
		20	20.41	+2
		20	20.38	+2
		20	20.07	+0

[a] Results of duplicate (dose formulations) or single (animal room samples) analyses
[b] Animal room samples

APPENDIX G
FEED CONSUMPTION
IN THE 2-YEAR GAVAGE STUDY
OF CHLORAL HYDRATE

TABLE G1
Feed Consumption by *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

Week	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg	
	Feed (g/day)[a]	Body Weight (g)	Feed (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)
1	4.4	23.5	4.4	23.4	4.4	23.3	4.2	23.1
2	4.5	24.8	4.4	24.7	4.6	24.5	4.3	24.4
3	4.6	26.0	4.4	25.8	4.6	25.7	4.4	25.4
4	4.7	26.9	4.7	26.7	4.7	26.5	4.6	26.2
5	4.8	27.7	4.7	27.5	4.8	27.4	4.6	27.0
6	4.9	28.3	4.8	28.1	4.8	27.7	4.7	27.4
7	5.0	28.7	4.9	28.5	4.8	28.2	4.8	27.9
8	5.0	29.1	4.8	29.0	4.9	28.7	4.8	28.2
9	5.0	29.4	4.8	29.3	4.9	29.0	4.8	28.6
10	5.0	29.8	4.8	29.7	4.8	29.4	4.8	28.9
11	5.0	30.3	4.8	30.2	4.8	29.8	4.8	29.4
12	5.0	30.8	4.8	30.7	4.9	30.3	4.8	29.9
13	5.1	31.1	4.7	31.1	4.8	30.7	4.8	30.4
14	5.0	31.4	4.8	31.7	4.8	31.2	4.7	30.8
15	5.0	31.9	4.8	32.0	4.9	31.5	4.7	31.2
16	4.9	32.3	4.8	32.5	4.9	32.2	4.8	31.8
17	5.0	32.9	4.8	33.0	4.8	32.6	4.8	32.2
18	5.0	33.3	4.8	33.5	4.9	32.9	4.7	32.7
19	4.9	33.5	4.7	33.8	4.9	33.5	4.7	33.1
20	4.9	34.0	4.8	34.3	4.8	33.9	4.6	33.5
21	5.0	34.5	4.8	34.9	4.8	34.5	4.8	34.0
22	4.9	34.9	4.8	35.3	4.9	35.0	4.8	34.8
23	4.9	35.4	4.9	35.8	4.8	35.6	4.6	35.2
24	4.9	35.7	4.8	36.3	4.8	35.8	4.7	35.7
25	5.0	36.1	4.9	36.8	4.9	36.4	4.7	36.1
26	5.1	36.5	4.9	37.0	4.9	36.6	4.7	36.4
27	5.0	36.8	4.9	37.3	4.8	36.9	4.7	36.5
28	5.0	36.9	4.9	37.5	4.8	37.2	4.8	36.8
29	5.0	37.2	4.9	37.8	4.8	37.4	4.9	37.2
30	5.0	37.2	4.9	37.9	4.9	37.5	4.9	37.2
31	5.0	37.4	4.9	38.1	5.0	37.7	4.9	37.6
32	5.1	37.8	4.9	38.4	4.9	38.0	4.8	37.9
33	5.0	37.8	5.0	38.6	4.9	38.2	4.8	38.2
34	5.0	37.7	4.9	38.6	4.8	38.3	4.8	38.2
35	5.0	37.8	4.9	38.5	4.9	38.3	4.8	38.2
36	5.0	37.9	4.9	38.7	4.9	38.2	4.8	38.2
37	5.0	38.1	4.8	38.9	4.8	38.3	4.7	38.3
38	5.0	38.6	4.8	39.0	4.8	38.6	4.7	38.4
39	4.9	38.5	4.7	39.0	4.8	38.7	4.7	38.5
40	4.9	38.6	4.8	39.1	4.8	39.0	4.6	38.8
41	4.9	39.0	4.8	39.3	4.8	39.2	4.7	39.1
42	4.9	39.5	4.8	39.7	4.7	39.7	4.7	39.5
43	4.9	39.8	4.7	40.1	4.7	40.0	4.7	39.6
44	4.9	39.9	4.7	40.2	4.8	40.2	4.7	39.8
45	5.0	40.1	4.7	40.2	4.9	40.4	4.7	40.1
46	4.9	40.2	4.8	40.6	4.9	40.8	4.7	40.4
47	5.0	40.3	4.9	40.8	4.8	41.1	4.7	40.8

TABLE G1
Feed Consumption by *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

Week	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg	
	Feed (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)
48	5.1	40.8	4.8	41.0	4.7	41.3	4.7	40.8
49	4.9	41.1	4.7	41.2	4.7	41.3	4.7	40.9
50	4.8	41.3	4.7	41.4	4.8	41.6	4.7	41.1
51	5.0	41.4	4.7	41.4	4.7	41.6	4.7	41.2
52	4.8	41.2	4.7	41.6	4.7	41.6	4.6	41.3
53	4.8	41.2	4.7	41.3	4.8	41.5	4.6	41.2
54	4.8	41.0	4.8	41.4	4.8	41.5	4.6	41.0
55	4.9	41.0	4.7	41.3	4.8	41.4	4.7	41.0
56	4.9	41.1	4.7	41.3	4.8	41.4	4.7	41.1
57	4.9	41.2	4.8	41.4	4.8	41.6	4.7	41.1
58	4.9	41.2	4.8	41.5	4.8	41.6	4.7	41.4
59	4.9	41.4	4.8	41.7	4.8	41.9	4.7	41.5
60	4.8	41.5	4.8	41.9	4.8	42.0	4.5	41.6
61	4.8	41.6	4.7	42.0	4.8	41.9	4.5	41.6
62	4.7	41.6	4.8	42.2	4.9	42.1	4.6	41.5
63	4.8	41.5	4.8	42.1	4.9	42.0	4.6	41.5
64	4.9	41.8	4.7	42.3	4.9	42.2	4.6	41.7
65	4.9	41.7	4.8	42.1	4.9	42.3	4.7	41.7
66	5.0	41.9	4.8	42.5	4.8	42.4	4.7	42.0
67	4.9	42.0	4.8	42.4	4.8	42.4	4.8	41.7
68	5.0	42.2	4.8	42.7	4.8	42.4	4.7	41.9
69	5.1	42.4	4.8	42.8	4.9	42.6	4.8	42.0
70	5.1	42.3	4.9	42.7	4.9	42.6	4.9	42.0
71	4.9	42.5	4.9	42.9	4.9	42.8	4.9	42.1
72	5.0	42.6	5.0	43.0	4.9	42.9	4.9	42.2
73	5.1	42.8	4.9	43.2	5.0	43.1	4.9	42.4
74	5.0	43.0	4.9	43.1	4.9	43.3	4.8	42.6
75	5.0	42.9	4.9	43.1	4.9	43.2	4.8	42.4
76	5.1	43.3	4.9	43.2	4.9	43.2	4.9	42.6
77	5.0	43.2	4.9	43.1	5.0	43.2	4.9	42.5
78	5.0	43.4	4.9	43.3	4.9	43.1	4.9	42.7
79	5.0	43.4	4.8	43.3	4.9	43.1	4.9	42.7
80	5.1	43.6	4.9	43.2	5.0	43.2	4.9	42.6
81	5.1	43.5	4.9	43.2	5.1	43.4	4.9	42.8
82	5.1	43.4	4.9	42.9	5.1	43.1	4.8	42.6
83	5.1	43.5	4.9	42.9	5.0	43.2	4.9	42.5
84	5.1	43.4	4.9	42.8	5.0	43.3	4.9	42.7
85	5.1	43.5	4.9	42.8	5.1	43.3	4.9	42.8
86	5.1	43.6	4.8	42.9	5.1	43.4	4.9	42.8
87	5.1	43.5	4.9	42.7	5.0	43.1	4.8	42.6
88	5.1	43.4	5.0	43.4	5.0	43.1	4.7	42.8
89	5.1	43.4	5.0	43.4	4.9	42.9	4.8	43.0
90	5.0	43.3	5.0	43.4	4.8	43.0	4.9	42.9
91	5.0	43.2	5.0	43.2	4.8	43.1	4.8	42.9
92	5.0	42.9	5.0	43.3	4.9	42.7	4.9	42.7
93	5.0	42.9	5.1	43.2	5.0	42.6	4.9	42.6
94	5.0	42.8	5.0	43.2	4.8	42.6	4.8	42.4

TABLE G1
Feed Consumption by *Ad Libitum*-Fed Male Mice in the 2-Year Gavage Study of Chloral Hydrate

Week	Vehicle Control		25 mg/kg		50 mg/kg		100 mg/kg	
	Feed (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)
95	5.0	42.9	5.0	42.8	4.8	42.2	4.7	42.2
96	5.1	42.6	5.1	42.6	4.9	41.9	4.8	42.2
97	5.1	42.3	5.0	42.2	4.8	41.8	4.8	42.0
98	5.2	42.0	5.0	41.9	4.8	41.7	4.7	41.6
99	5.2	41.9	5.0	41.8	5.0	41.4	4.8	41.4
100	5.1	42.1	5.0	41.7	5.0	42.5	4.9	41.6
101	5.1	42.1	5.0	41.5	5.0	41.9	4.9	41.3
102	5.1	42.3	5.0	41.3	5.1	41.9	4.7	41.3
103	5.1	42.2	5.0	41.3	5.1	42.1	4.7	41.2
104	5.3	40.7	5.0	42.0	5.1	41.3	4.7	41.2
Mean for weeks								
1-13	4.9	28.2	4.7	28.1	4.8	27.8	4.6	27.4
14-52	5.0	37.3	4.8	37.7	4.5	37.5	4.7	37.2
52-104	5.0	42.4	4.9	42.4	4.9	42.4	4.8	42.0

[a] Grams of feed consumed per animal per day

APPENDIX H
INGREDIENTS, NUTRIENT COMPOSITION, AND CONTAMINANT LEVELS IN NIH-31 RAT AND MOUSE RATION

TABLE H1
Ingredients of NIH-31 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground #2 yellow shelled corn	21.0
Ground whole hard wheat	35.5
Ground whole oats	10.0
Soybean meal (49% protein)	5.0
Fish meal (60% protein)	9.0
Wheat middlings	10.0
Alfalfa meal (17% protein)	2.0
Corn gluten meal (60% protein)	2.0
Soy oil	1.5
Dried brewer's yeast	1.0
Dicalcium phosphate (food grade)	1.5
Ground limestone	0.5
Salt	0.5
Premixes (vitamin and mineral)	0.5

[a] Ingredients were ground to pass through a U.S. Standard Screen No. 16 before being mixed.

TABLE H2
Vitamins and Minerals in NIH-31 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
A	22,000,000 IU	Vitamin A palmitate or acetate
D_3	3,800,000 IU	D-activated animal sterol
K_3	20 g	Menadione activity
d-α-Tocopheryl acetate	15 g	
Choline	700 g	Choline chloride
Folic acid	1 g	
Niacin	20 g	
d-Pantothenic acid	25 g	*d*-Calcium pantothenate
Riboflavin	5 g	
Thiamine	65 g	Thiamine mononitrate
B_{12}	14 g	
Pyridoxine	2 g	Pyridoxine hydrochloride
Biotin	0.120 g	*d*-Biotin
Minerals		
Iron	60 g	Iron sulfate
Magnesium	400 g	Magnesium oxide
Manganese	100 g	Manganous oxide
Zinc	10 g	Zinc oxide
Copper	4 g	Copper sulfate
Iodine	1.5 g	Calcium iodate
Cobalt	0.4 g	Cobalt carbonate

[a] Per ton (2,000 lb) of finished product

TABLE H3
Nutrient Composition of NIH-31 Rat and Mouse Ration[a]

Nutrient	Mean ± Standard Deviation	Number of Lots
Crude protein (% by weight)	18.8 ± 1.0	16
Crude fat (% by weight)	5.48 ± 1.1	16
Moisture (%)	6.04	1
Vitamins		
A (µg/g)	11.9 ± 1.1	16
E (ppm)	62.3 ± 6.6	16
Thiamine (mg/g)	0.090 ± 0.019	16
Mineral		
Selenium (ppm)	0.36 ± 0.10	16

[a] Post autoclaving

TABLE H4
Contaminant Levels in NIH-31 Rat and Mouse Ration

	Mean ± Standard Deviation[a]	Number of Lots
Contaminants		
Arsenic (ppb)	113 ± 40	16
Cadmium (ppb)	70 ± 24	16
Lead (ppm)	0.41 ± 0.22	16
Aflatoxin B_1 (ppb)	<0.25	16
Aflatoxin B_2 (ppb)	<0.25	16
Aflatoxin G_1 (ppb)	<0.25	16
Aflatoxin G_2 (ppb)	<0.12	16
Fumonisin B_1 (ppb)	41.3 ± 25.8	16
Total fumonisin (ppb)	69.7 ± 38.2	9
Volatiles (%)	6.88 ± 1.0	15
Pesticides (ppb)		
Heptachlor	<10.0	6
Total DDT[b]	<5.0	6
Dieldrin	<5.0	6
PCB	32.8 ± 29	6
Malathion	79.8 ± 41	6
Lindane	<1.0	6
Methoxychlor	<10.0	1

[a] For values less than the limit of detection, the detection limit is given as the mean.
[b] DDE+DDT+DDD

APPENDIX I
SENTINEL ANIMAL PROGRAM

SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the Carcinogenesis Program of the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of chemical compounds.

Serum samples were collected from randomly selected mice during the 2-year study. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and sent to the Surveillance/Diagnostic Program, Division of Microbiology, at NCTR for determination of antibody titers. The serum samples were analyzed using a commercially prepared murine antibody test kit (Organon Teknika Corp., Durham, NC). Serum samples with positive titers were sent to Microbiological Associates, Inc. (Bethesda, MD), for verification. The laboratory serology methods and viral agents for which testing was performed are tabulated below; the times at which blood was collected during the studies are also listed.

Method and Test	Time of Analysis
MICE	
ELISA	
Ectromelia virus	6, 12, and 18 months, study termination
GDVII (mouse encephalomyelitis virus)	6, 12, and 18 months, study termination
LCM (lymphocytic choriomeningitis virus)	6, 12, and 18 months, study termination
MVM (minute virus of mice)	6, 12, and 18 months, study termination
MHV (mouse hepatitis virus)	6, 12, and 18 months, study termination
Mycoplasma arthritidis	6, 12, and 18 months, study termination
Mycoplasma pulmonis	6, 12, and 18 months, study termination
PVM (pneumonia virus of mice)	6, 12, and 18 months, study termination
Polyoma virus	6, 12, and 18 months, study termination
Reovirus 3	6, 12, and 18 months, study termination
Sendai	6, 12, and 18 months, study termination

Results of serology tests are presented in Table I1.

TABLE I1
Murine Virus Antibody Determinations for Male Mice in the 2-Year Gavage Study of Chloral Hydrate

Interval	Incidence of Antibody in Sentinel Animals	Positive Serologic Reaction for
6 Months	2/4[a]	Mouse hepatitis virus
12 Months	0/4	None
18 Months	0/4	None
Study termination	0/1	None

[a] The presence of antibodies to mouse hepatitis virus was confirmed at Microbiological Associates, Inc., using ELISA and immunofluorescent assays.

APPENDIX J
SUPPLEMENTAL STUDY

SUPPLEMENTAL STUDY

INTRODUCTION

Because dietary restriction is known to alter the pharmacokinetics and toxicity of many chemicals and is generally assumed to raise the minimally toxic dose of test chemicals (Hart *et al.*, 1995), a supplemental acute study was performed by the study laboratory (NCTR) while the chronic study was ongoing. This supplemental study investigated the effects of dietary restriction on chloral hydrate pharmacokinetics and toxicokinetics. Three feed regimens were used: *ad libitum* feeding, dietary control identical to the chronic study, and 40% caloric restriction with vitamin-fortified NIH-31 diet as was used with previous dietary restriction studies at the NCTR (Sheldon *et al.,* 1995). For the calorically restricted group, the diet was supplemented with vitamins so only the caloric component of the diet was reduced. The calorically restricted group was included to determine whether any observed effects with dietary control would be amplified with the more extreme 40% caloric restriction. The mice were dosed with a range of five doses of chloral hydrate and assayed for survival, blood metabolite concentrations, and liver enzyme activities.

MATERIALS AND METHODS

Male B6C3F$_1$/Nctr BR (C57BL/6N × C3H/HeN MTV$^-$) mice were obtained from the NCTR breeding colony. On receipt, the mice were 4 weeks old. Mice were distributed randomly into groups of approximately equal mean body weight.

Groups of 18 male mice were administered 0, 50, 100, 250, 500, or 1,000 mg chloral hydrate/kg body weight in distilled water by gavage for 14 days, excluding weekends, beginning when the mice were 10 weeks old. Each animal was given a total of 10 doses, including the 5 consecutive days before necropsy. Six mice from each dose group were assigned to each feed regimen. Prior to the beginning of dosing, the *ad libitum*-fed group had 10 extra mice and the dietary-controlled group had six extra mice. In those groups, mice with abnormal body weights were eliminated at 10 weeks of age, prior to dosing.

Ad libitum-fed mice had standard NIH-31 feed available *ad libitum.* Dietary-controlled mice received standard NIH-31 feed in measured daily amounts calculated to maintain body weight on the same idealized body weight curve used in the 2-year study (Appendix D). Calorically restricted mice had feed allocations calorically reduced by 40% compared with historical *ad libitum* feed consumption from the B6C3F$_1$/Nctr BR mouse colony. The latter group was fed vitamin-fortified NIH-31 pellets (Witt *et al.*, 1991) and was included to provide a graded response to dietary restriction. Feed allocation for the dietary-controlled and calorically restricted groups began when the animals were 5 weeks old. Mice were housed individually in divided cages. Water was available *ad libitum*.

The study began in three stages approximately 2 to 4 weeks apart, with two mice from each experimental group represented at each stage. This allowed blood collection and euthanasia to be performed according to the required time schedule.

Mice were decapitated 2 hours after the final chloral hydrate dose. This method was used to preserve hepatic enzymes and cofactors, which can be depleted following carbon dioxide asphyxiation. The liver was removed, weighed, and frozen in liquid nitrogen. Full details of the enzyme assays are given in Appendix E.

Toxicokinetic Studies

Blood samples were collected at 15 and 30 minutes and l, 3, 6, and 24 hours after the penultimate dose of chloral hydrate. The mice were bled consecutively at each time point by use of tail clipping as outlined below. All mice were bled at each time point. Trunk blood was also collected at sacrifice (i.e., 2 hours after the final dose) to provide a 2-hour time point.

To obtain venous tail blood, animals were restrained in customized restraining devices developed at the NCTR and made of opaque 0.75-inch PVC tubing attached by retort clamps to a metal stand. The mice were allowed to freely enter the tube, which had perforations to allow adequate ventilation. A plexiglass piece fit into the end of the tube. This piece had a small groove in its base to allow the mouse's tail to extend out of the tube. To prevent clotting and reduce pain, tails were dipped in a solution of 50 mM EDTA, 0.33 mM salicylic acid, and 0.133 mg/mL atropine sulfate. The EDTA inhibited clotting factors, while the salicylic acid inhibited platelet aggregation and the atropine prevented vasoconstriction. The three ingredients of this dipping solution did not interfere with the gas chromatography (GC) analyses of chloral hydrate or its metabolites when spiked into serum samples. The epidermis from the tip of the tail was removed with a scalpel blade that had been wetted with the dipping solution. Care was taken not to expose the tail cartilage. Once bleeding commenced the first drop was discarded. Approximately 30 to 40 µL of blood was collected in a 50-µL capillary tube (Drummond Scientific, Broomall, PA). Bleeding was encouraged by gentle massaging of the tail, and additional dipping solution was applied if the bleeding stopped prematurely. Using this procedure, bleeding could be continued for several minutes and relatively large amounts of blood collected, if necessary. Once a sufficient amount of blood had been collected, the capillary tubes were sealed with Hemato-Seal® clay (Fisher Scientific Co., Pittsburgh, PA) and centrifuged for 2 minutes in a microhematocrit centrifuge. Further bleeding was inhibited by placing a small piece of surgical adhesive tape on the tip of the tail. Once centrifuged, the capillary tubes were scored at the plasma phase and collected. Two aliquots of plasma (4 µL each) were pipetted into labeled 300 µL crimp vials (Chromacol, Inc., Trumbull, CT). The vials were sealed with a Teflon®-lined septum under argon, flash frozen with liquid nitrogen, and stored at –80° C until assayed. Trunk blood (50 µL) was collected at sacrifice and processed in the same way as the tail blood.

Plasma concentrations of chloral hydrate and its metabolites were determined by GC using electron-capture detection (GC-ECD) by a modification of the methods of Gorecki *et al.* (1990). Care was taken to prevent the artifactual conversion of trichloroacetic acid into dichloroacetic acid by the use of modifications recommended by Ketcha *et al.* (1996). This included ensuring that all samples were frozen and thawed prior to assay. Gorecki *et al.* (1990) used 50 µL of plasma extracted with 5.0 mL of diethyl ether and injected 1 µL of the ether extract onto the GC. Because of the small amounts of blood available, the assay was scaled down using approximately the same proportions used by Gorecki *et al.* (1990). A 4-µL plasma aliquot from each blood sample was assayed for trichloroacetic acid, dichloroacetic acid, and free trichloroethanol. The other 4-µL plasma aliquot was assayed for chloral hydrate and total trichloroethanol.

Assay standards were prepared by dissolving aqueous solutions of trichloroacetic acid, dichloroacetic acid, chloral hydrate, or trichloroethanol glucuronide or a methanolic solution of trichloroethanol in mouse serum (Pel-Freez Biologicals, Rogers, AR) to achieve concentrations of 400 or 800 µM. Serial dilutions were then prepared from the 400-µM solutions to produce additional stock solutions of 10, 20, 50, and 100 µM. Series of standards of all six concentrations were then prepared by pipetting 4-µL aliquots of the stock solutions into 300-µL crimp vials. The vials were then purged with argon and crimp-sealed. They were then stored at –80° C along with the plasma samples from the experiment. An internal standard solution was prepared by diluting a 100 mM solution of 1,1,2,2-tetrachloroethane in acetone 1,000-fold into 50% mouse serum in water. The resulting 100 µM internal standard solution was stored in 1.5 mL aliquots at –80° C. A fresh aliquot was used for each assay. Trichloroacetic acid, dichloroacetic acid, and trichloroethanol were obtained from Aldrich Chemical Company (Milwaukee, WI). Chloral hydrate was obtained from Amend Drug and Chemical Company (Irvington, NJ) and was from the same batch used for dosing the mice.

[^{14}C]-Trichloroethanol glucuronide was prepared by incubating trichloroethanol with ^{14}C-labeled UDP-glucuronic acid and guinea pig microsomes for 16 hours. Unconjugated trichloroethanol was removed by extraction into methyl-tertiary-butyl ether (MTBE; Aldrich Chemical Co.), and the trichloroethanol was purified by passing it through a C-18 Sep-Pac (Millipore, Inc., Milford, MA). Purity was confirmed by high-performance liquid chromatography using the method outlined in Appendix F, and the trichloroethanol

glucuronide concentration was determined by liquid scintillation counting. The glucuronide solution was diluted to 1 mM with deionized water and stored at –80° C. Diazomethane was prepared by the action of base on 1-methyl-3-nitro-1-nitrosoguanidine using a generator supplied by Aldrich Chemical Company. The diazomethane formed as a solution in MTBE inside the apparatus and was used within 2 hours of preparation. β-Glucuronidase (Type VIIA) was obtained from Sigma Chemical Company (St. Louis, MO) and was diluted to 500 IU/mL in 250 mM potassium phosphate buffer at pH 6.8. It was stored in aliquots at –80° C.

The samples were analyzed in sets of approximately 100. These sets consisted of all the available time points from one mouse from each of the dose groups for all three diet groups. A few samples were lost when the capillary tubes broke in the centrifuge. Each set contained duplicate ranges of the appropriate standards. For the analysis of plasma trichloroacetate, dichloroacetate and free trichloroethanol, 5 µL of internal standard was initially added to each sample or standard vial by means of a repeating syringe pipette (Hamilton Inc., Reno, NV) attached to a 26 gauge needle while the contents of the vials were still frozen. The samples were then acidified by the addition of 10 µL of 2 M sulfuric acid. This was followed by 150 µL of MTBE. Both additions were made through the septum with syringe pipettes. The vials were then shaken on a mechanical shaker for 10 minutes and centrifuged at 2,500 × g for 5 minutes. After centrifugation, the crimp seals were removed from the vials and 100 µL of the ether phase was transferred to 100-µL vial inserts (01-CVG, Chromacol, Inc.) containing 5 µL of diazomethane. The vial inserts were crimp-sealed and loaded onto the GC-Autoinjector.

For the analysis of chloral hydrate and total trichloroethanol, 10 µL of β-glucuronidase solution was added to each sample or standard vial with a repeating syringe pipette and the vials were incubated in a water bath at 37° C for 10 hours. Internal standard and MTBE were added, and the vials were then shaken and centrifuged as for the trichloroacetate analysis. After centrifugation, 100 µL of the MTBE phase was added to vial inserts without diazomethane.

The samples were analyzed on a Hewlett Packard Model 5890 Series II gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a ^{63}Ni ECD and a DB 1701 column as described in Appendix F. The retention times for chloral hydrate, dichloroacetate-methyl ester, trichloroacetate-methyl ester, and trichloroethanol were 2.2, 4.1, 4.8, and 5.7 minutes, respectively. The practical limit of detection was 1 µM in plasma under the conditions of the assay.

RESULTS

Survival and Body Weight Effects

This study was designed to test the effect of the mild dietary restriction used to control body weight for the dietary-controlled mice in the 2-year study on the toxicokinetic and minimally toxic dose of chloral hydrate. The mice were divided into three diet groups: *ad libitum*-fed, dietary control identical to the 2-year study, and 40% caloric restriction. The latter group provided a high level of dietary restriction to explore potential dose responses.

Both the 500 and 1,000 mg/kg doses were toxic and most of the mice from the 1,000 mg/kg group died during treatment (Figure J1). All mice in the other dose groups in all three diet groups survived. The LD_{50} of CD mice administered chloral hydrate by gavage had previously been reported to be 1,442 mg/kg (Sanders *et al.*, 1982). Therefore, the B6C3F$_1$ mice used in this study appeared to be more susceptible. The 1,000 mg/kg dietary-controlled mice survived for a few more days of treatment than the *ad libitum*-fed or calorically restricted mice.

Chloral hydrate had only a weak sedative action on the mice. This was examined in detail on the initial day of dosing for the first set of mice. There was no observed effect in any of the 50 or 100 mg/kg groups fed any of

the three diets. For the six mice receiving 250 mg/kg, motor coordination was affected 10 minutes after dosing, resulting in staggering and poor righting reflexes. All diet groups appeared to be affected similarly, and the mice returned to normal behavior by 20 minutes after dosing. Similar behavior lasting up to 30 minutes was observed in the mice receiving 500 mg/kg. In addition, one *ad libitum*-fed and one calorically restricted mouse appeared fully sedated and were prostrate for approximately 1 hour. All the mice had fully recovered by 90 minutes after dosing. The six mice receiving 1,000 mg/kg initially exhibited staggering behavior after dosing. Both the *ad libitum*-fed and calorically restricted mice became fully sedated and remained prostrate until approximately 90 minutes after dosing and then slowly recovered. The two dietary-controlled mice remained upright but had poor coordination and were sluggish during this period.

There was no evidence that either dietary control or caloric restriction significantly increased the minimally toxic dose for chloral hydrate. The body weight profiles of the *ad libitum*-fed and dietary-controlled groups (Table J1) were similar to those of the 2-year study (Figure J2 and Table J2) until the initiation of dosing at 10 weeks. At this point, the body weight gain decreased to a larger extent in the *ad libitum*-fed mice than in the other two diet groups.

Water or chloral hydrate gavage suppressed body weight increases over the 2-week dosing periods in all *ad libitum*-fed groups. The effect was greatest in the 500 and 1,000 mg/kg dose groups. This did not occur in the dietary-controlled and calorically restricted groups, where body weight decreased only in the dietary-controlled, 1,000 mg/kg group.

Hepatic Enzyme Analysis

Induction of hepatic microsomal CYP4A and lauric acid ω-hydroxylase by chloral hydrate was further investigated to determine the dose response of this cytochrome P450 isoform to acute exposure to chloral hydrate. Lauric acid ω-hydroxylase activity and CYP4A immunoreactive protein were increased by chloral hydrate treatment in all three diet groups, but there was a greater induction and a steeper dose response in the dietary-controlled and caloric restriction groups than in the *ad libitum*-fed groups (Figure J3). Further details are given in Appendix E.

The acitivity of cyanide-insensitive palmitoyl CoA fatty acid hydroxylase, another peroxisomal marker enzyme, was also assayed in the liver samples. It was also induced by chloral hydrate and had dose responses in the three diet groups similar to the responses to lauric acid ω-hydroxylase (Figure J4). These enzyme induction studies suggested that the threshold dose for induction of peroxisome proliferation by chloral hydrate in the dietary-controlled or calorically restricted mice was around 100 mg/kg and that chloral hydrate was a less efficient inducer of peroxisome proliferation in *ad libitum*-fed mice than in dietary-restricted mice.

Toxicokinetics

Plasma concentrations of chloral hydrate and its metabolites, trichloroacetic acid, trichloroethanol, and trichloroethanol glucuronide, were assayed in mice 15 and 30 minutes and 1, 2, 3, 6, and 24 hours after dosing. Mice from the three diet groups and from the 50, 100, 250, and 500 mg/kg groups were assayed. The metabolite concentrations for all three diet groups are given in Tables J3 through J6 and partly shown in Figures J5 through J10. There was a large variation in individual samples that may have resulted partly from the assay technique. The samples were assayed in six individual runs comprising all the plasma samples from one mouse from each diet and dose group. This protected the data from artifactual dose or diet effects due to interassay variation but increased the individual variation within each dose group. Plasma trichloroacetate, which was the major metabolite, exhibited a concentration profile with a double peak, suggesting that enterohepatic recirculation was taking place as was observed by Stenner *et al.* (1998). There were no significant differences due to diet at any of the four doses. Figure J11 compares the plasma trichloroacetate concentrations observed in 100 mg/kg dietary-controlled mice in this study, extrapolated to five daily doses, with observed plasma trichloroacetate concentrations in a human neonate and adult male who were

administered typical therapeutic oral doses of chloral hydrate (50 and 11 mg/kg, respectively). Although the peak concentrations of plasma trichloroacetate are at least fivefold greater in the mouse than in the human, the area-under-the-curve (AUC) values do not show as great a difference due to the slower clearance of trichloroacetate from human plasma.

The plasma trichloroacetate AUC values for all the diet and dose groups are shown in Table J7. There were no significant differences between the diet groups at any of the doses, but the AUC increased by a greater and more statistically significant amount with dose in the dietary-controlled and calorically restricted groups than in the *ad libitum*-fed group. When the plasma trichloroacetate AUC values were compared with the induction of hepatic lauric acid ω-hydroxylase activities (Figure J12), the data suggested that differences in plasma trichloroacetate could not account for all of the differences in hydroxylase induction between the three diet groups. The *ad libitum*-fed mice showed a lower induction response than the other two diet groups.

REFERENCES

Gorecki, D.K.J., Hindmarsh, K.W., Hall, C.A., Mayers, D.J., and Sankaran, K. (1990). Determination of chloral hydrate metabolism in adult and neonate biological fluids after single-dose administration. *J. Chromatogr.* **528**, 333-341.

Hart, R.W., Keenan, K., Turturro, A., Abdo, K.M., Leakey, J., and Lyn-Cook, B. (1995). Caloric restriction and toxicity. *Fundam. Appl. Toxicol.* **25**, 184-195.

Ketcha, M.M., Stevens, D.K., Warren, D.A., Bishop, C.T., and Brashear, W.T. (1996). Conversion of trichloroacetic acid to dichloroacetic acid in biological samples. *J. Anal. Toxicol.* **20**, 236-241.

Sanders, V.M., Kauffmann, B.M., White, K.L., Douglas, K.A., Barnes, D.W., Sain, L.E., Bradshaw, T.J., Borzelleca, J.F., and Munson, A.E. (1982). Toxicology of chloral hydrate in the mouse. *Environ. Health Perspect.* **44**, 137-146.

Sheldon, W.G., Bucci, T.J., Hart, R.W., and Turturro, A. (1995). Age-related neoplasia in a lifetime study of *ad libitum*-fed and food-restricted B6C3F1 mice. *Toxicol. Pathol.* **23**, 458-476.

Stenner, R.D., Merdink, J.L., Fisher, J.W., and Bull, R.J. (1998). Physiologically-based pharmacokinetic model for trichloroethylene considering enterohepatic recirculation of major metabolites. *Risk Anal.* **18**, 261-269.

Witt, W.M., Sheldon, W.G., and Thurman, J.D. (1991). Pathological endpoints in dietary restricted rodents – Fisher 344 rats and B6C3F$_1$ mice. In *Biological Effects of Dietary Restriction* (L. Fishbein, Ed.), pp. 73-86. Springer-Verlag, New York.

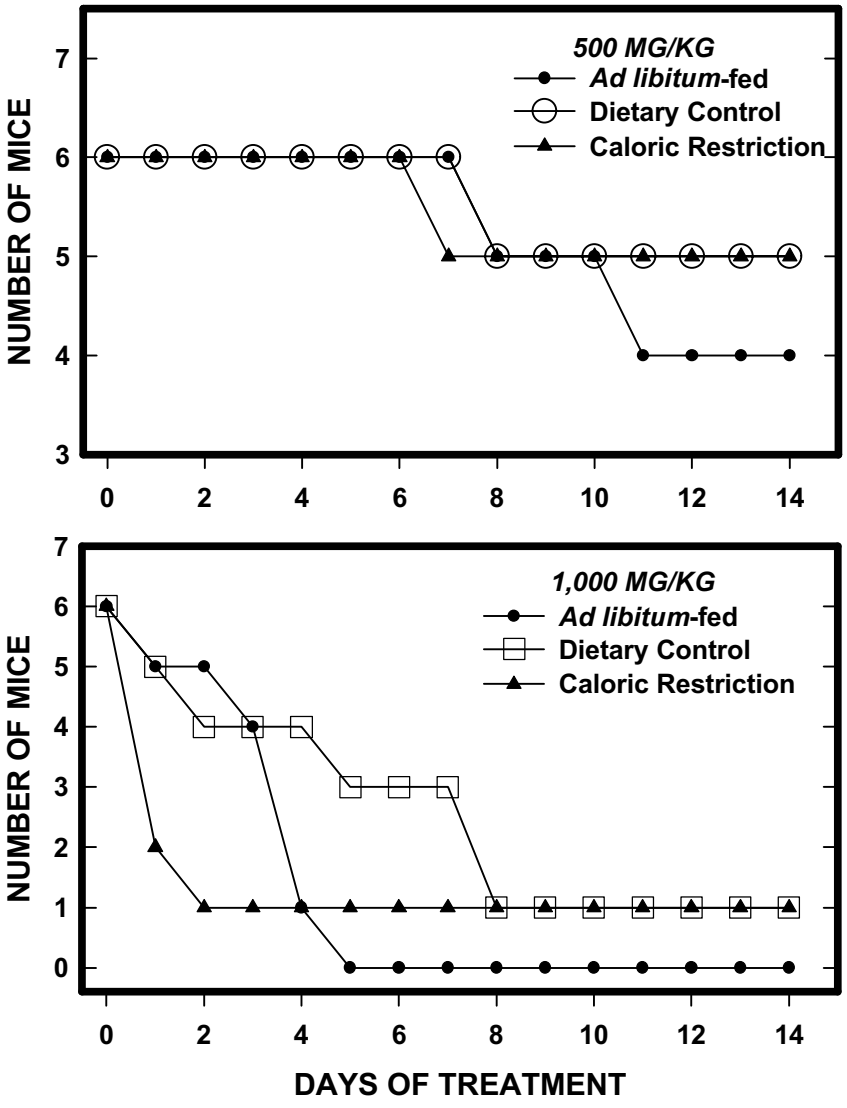


FIGURE J1
Survival of Male Mice Treated with 500 or 1,000 mg/kg Chloral Hydrate by Gavage

TABLE J1
Body Weights and Feed Consumption for Male Mice in the Supplemental Study of Chloral Hydrate[a]

Weeks of Age	Target Weights		Food Consumption/Allocation			Observed Mean Body Weight		
	Dietary-Controlled	Calorically Restricted	*Ad Libitum*	Dietary-Controlled	Calorically Restricted	*Ad Libitum*	Dietary-Controlled	Calorically Restricted
5	17.6	18.2	4.0	4.0	4.0	19.5	20.4	21.0
6	21.4	20.2	4.0	3.5	3.5	22.0	19.6	20.7
7	23.4	21.0	4.3	3.0	3.0	23.8	20.4	20.7
8	25.1	22.5	4.6	3.5	3.0	25.0	20.7	20.0
9	26.3	23.3	4.8	3.5	2.5	26.0	21.8	19.4
10	27.4	23.8	5.1	3.5	2.5	26.5	23.8	18.8
11	28.3	24.3	5.4	3.5	2.5	26.7	23.3	19.2
12	28.8	24.8	5.9	3.5	2.5	26.4	23.7	19.4

[a] Target and observed mean body weights are given in grams. Feed consumption/allocation is given in grams per day. Feed allocation values are rounded to the nearest 0.5 g.

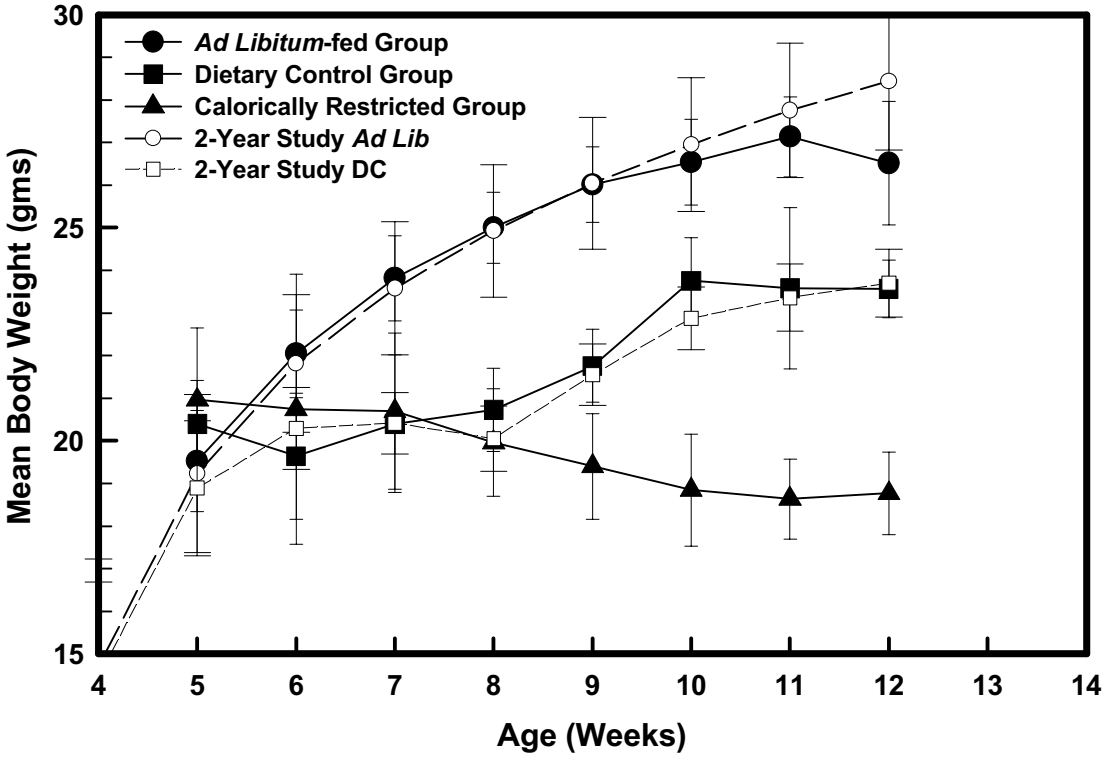


FIGURE J2
Comparison of Mean Body Weight Values Between the Supplemental Study and the 2-Year Gavage Study of Chloral Hydrate
The body weight values for the supplemental study were a reasonable match to those from the 2-year study. The standard deviation was less for the *ad libitum*-fed mice from the supplemental study than for those from the 2-year study because redundant mice were added to this group in the supplemental study so that mice showing abnormal weight gain could be eliminated from the study at 10 weeks, prior to dosing.

TABLE J2
Body Weights of Male Mice in the Supplemental Study of Chloral Hydrate[a]

Weeks	Dose (mg/kg)	*Ad Libitum*-Fed	Dietary-Controlled	Calorically Restricted
n		6	6	6
10	0	26.1 ± 0.43	24.1 ± 0.67	19.0 ± 0.41
11	0	27.1 ± 0.38	23.6 ± 0.77	18.6 ± 0.38
12	0	26.5 ± 0.59	23.6 ± 0.28	18.8 ± 0.40
10	50	26.2 ± 0.27	24.1 ± 0.40	19.0 ± 0.49
11	50	27.1 ± 0.36	23.6 ± 0.51	19.0 ± 0.31
12	50	26.4 ± 0.29	23.7 ± 0.16	18.9 ± 0.22
10	100	26.7 ± 0.46	23.8 ± 0.33	18.6 ± 0.38
11	100	27.7 ± 0.45	22.9 ± 0.58	19.1 ± 0.43
12	100	27.1 ± 0.46	22.9 ± 0.27	19.2 ± 0.43
10	250	26.4 ± 0.46	23.6 ± 0.26	19.4 ± 0.61
11	250	26.8 ± 0.44	23.7 ± 0.49	20.0 ± 0.28
12	250	26.6 ± 0.35	23.7 ± 0.28	20.1 ± 0.20
10	500	26.9 ± 0.48	23.8 ± 0.31	18.7 ± 0.60
11	500	25.6 ± 1.43	24.2 ± 0.73	19.7 ± 0.70
12	500	25.4 ± 1.59[b]	24.5 ± 0.65[b]	19.9 ± 0.91[b]
10	1,000	27.0 ± 0.35	23.3 ± 0.48	18.5 ± 0.77
11	1,000	24.4 ± 0.05[c]	20.6 ± 2.06[d]	18.1 ± 1.05[e]
12	1,000	—[f]	24.6[g]	19.7[g]

[a] Body weights are given in grams (mean ± standard error of the mean).
[b] n=5
[c] n=2
[d] n=3
[e] n=4
[f] n=0
[g] n=1

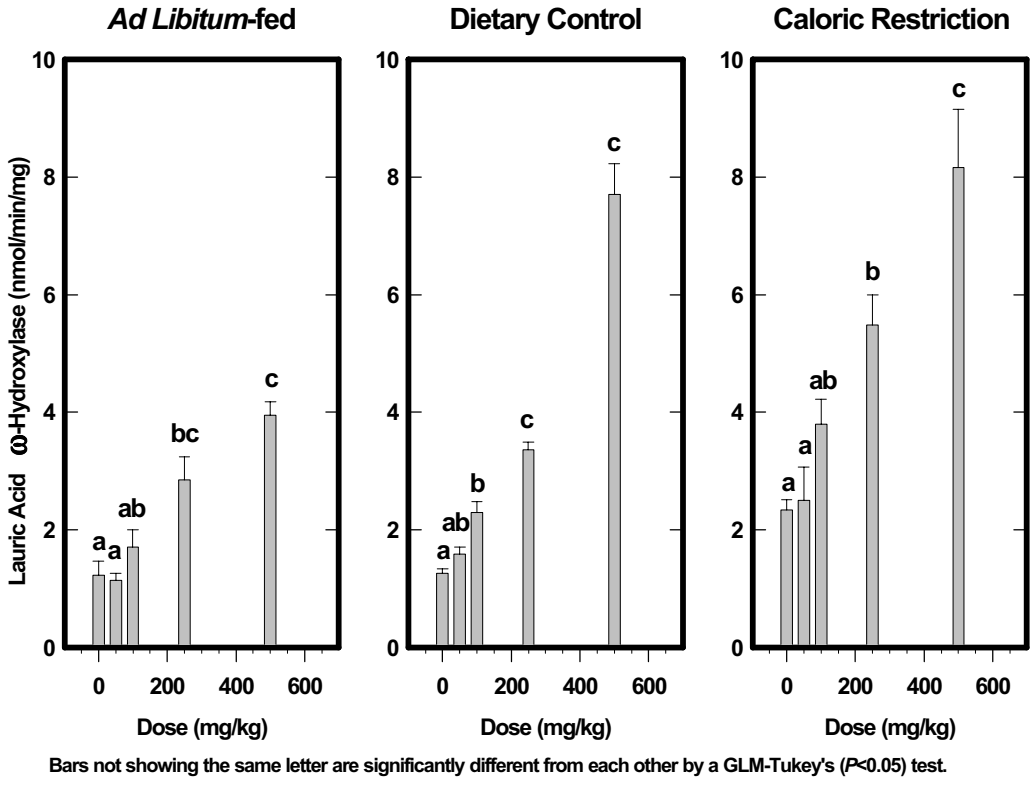


FIGURE J3
Induction of CYP4A-Dependent Lauric Acid ω-Hydroxylase Activity in Hepatic Microsomes from 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate

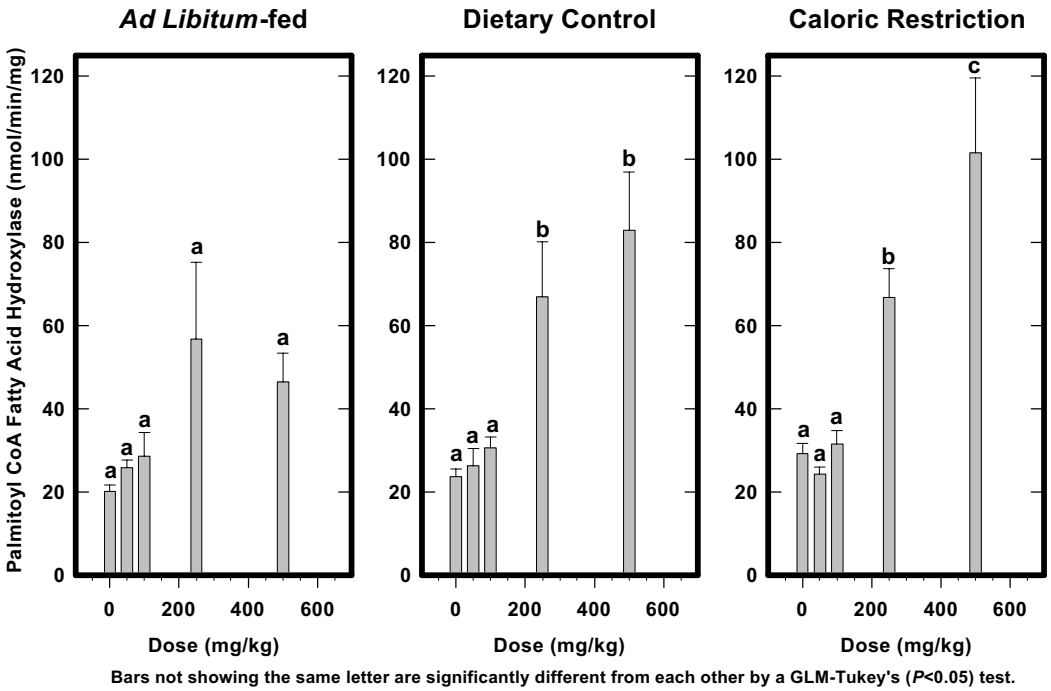


FIGURE J4
Induction of Cyanide-Insensitive Palmitoyl CoA Fatty Acid Hydroxylase Activity in Hepatic Cytosol from 12-Week-Old Male Mice in the Supplemental Study of Chloral Hydrate

TABLE J3
Plasma Concentrations of Chloral Hydrate and Its Metabolites in 50 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate[a]

	15 Min	30 Min	1 Hour	2 Hours	3 Hours	6 Hours	24 Hours
***Ad Libitum*-Fed**							
n	6	6	6	6	6	6	5
Chloral hydrate	21 ± 11	2.7 ± 1.8	2.9 ± 2.9	0.0	0.0	0.0	0.0
Trichloroacetic acid	238 ± 55	504 ± 139	423 ± 140	152 ± 44	319 ± 121	262 ± 156	34 ± 8
Free trichloroethanol	14.4 ± 2.3	6.3 ± 1.7	8.3 ± 2.7	3.6 ± 0.4	4.7 ± 1.1	23 ± 13	3.2 ± 0.6
Trichloroethanol-glucuronide	59 ± 9	16.0 ± 4.1	4.7 ± 2.1	4.2 ± 2.1	3.9 ± 2.2	4.2 ± 3.8	0.3 ± 0.3
Dietary-Controlled							
n	6	5	6	6	6	6	6
Chloral hydrate	11.2 ± 2.7	5.5 ± 2.3	0.0	0.9 ± 0.4	0.0	0.0	0.0
Trichloroacetic acid	274 ± 23	817 ± 422	527 ± 263	140 ± 29	425 ± 125	76 ± 39	67 ± 18
Free trichloroethanol	16.0 ± 1.5	16.0 ± 7.6	9.1 ± 3.3	3.6 ± 0.3	4.5 ± 0.9	3.5 ± 0.6	3.2 ± 0.7
Trichloroethanol-glucuronide	43.3 ± 8.4	25.8 ± 4.4	1.7 ± 0.7	2.3 ± 0.6	1.8 ± 0.8	4.3 ± 3.3	0.0
Calorically Restricted							
n	6	5	6	6	6	6	6
Chloral hydrate	24.4 ± 9.0	10.8 ± 3.6	0.0	0.7 ± 0.5	0.0	0.0	0.3 ± 0.3
Trichloroacetic acid	287 ± 36	927 ± 441	591 ± 327	299 ± 83	314 ± 38	86 ± 24	56 ± 6
Free trichloroethanol	18.0 ± 1.5	29.1 ± 17	6.8 ± 4.0	3.3 ± 0.5	4.0 ± 0.6	3.3 ± 0.7	3.5 ± 0.6
Trichloroethanol-glucuronide	45.4 ± 18.3	26.5 ± 11	8.6 ± 5.7	1.0 ± 0.7	2.4 ± 1.5	3.8 ± 2.6	0.0

[a] Values are expressed as μM concentrations in plasma (mean ± standard error of the mean). Dichloroacetic acid was not detected in any plasma sample.

TABLE J4
Plasma Concentrations of Chloral Hydrate and Its Metabolites in 100 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate[a]

	15 Min	30 Min	1 Hour	2 Hours	3 Hours	6 Hours	24 Hours
***Ad Libitum*-Fed**							
n	5	6	6	6	6	6	6
Chloral hydrate	34 ± 13	7.6 ± 2.5	1.9 ± 1.9	1.6 ± 0.6	0.0	0.0	0.0
Trichloroacetic acid	357 ± 71	895 ± 287	1,054 ± 795	199 ± 79	461 ± 116	94 ± 53	34 ± 12
Free trichloroethanol	27.0 ± 6	18 ± 4	4.5 ± 1.4	4.9 ± 0.8	4.3 ± 1.0	3.7 ± 0.6	3.6 ± 0.9
Trichloroethanol-glucuronide	73 ± 19	39 ± 12	4.8 ± 1.7	9.7 ± 3.6	2.4 ± 1.3	2.9 ± 2.2	0.0
Dietary-Controlled							
n	5	6	4	6	6	6	6
Chloral hydrate	29 ± 7	11.0 ± 2.1	2.7 ± 1.0	0.6 ± 0.4	0.2 ± 0.2	0.0	0.0
Trichloroacetic acid	275 ± 61	816 ± 171	340 ± 259	318 ± 107	531 ± 185	134 ± 45	59 ± 20
Free trichloroethanol	17 ± 3	28 ± 6	11.5 ± 4.1	6.3 ± 1.4	4.0 ± 1.0	3.5 ± 0.7	3.8 ± 1.0
Trichloroethanol-glucuronide	47 ± 19	64 ± 16	11.6 ± 4.9	3.5 ± 1.3	8.5 ± 4.0	1.8 ± 1.1	0.4 ± 0.2
Calorically Restricted							
n	4	6	6	6	6	6	6
Chloral hydrate	21 ± 10	12 ± 3.0	0.0	3.0 ± 1.6	0.1 ± 0.1	0.0	0.0
Trichloroacetic acid	505 ± 94	958 ± 293	1,240 ± 863	301 ± 74	661 ± 194	168 ± 67	96 ± 29
Free trichloroethanol	43 ± 19	52 ± 16	10.3 ± 4.0	4.4 ± 0.4	4.0 ± 0.8	3.6 ± 0.7	3.1 ± 0.6
Trichloroethanol-glucuronide	34 ± 20	66 ± 20	3.2 ± 1.6	5.3 ± 1.3	6.0 ± 5.4	3.9 ± 3.0	1.2 ± 1.0

[a] Values are expressed as μM concentrations in plasma (mean ± standard error of the mean). Dichloroacetic acid was not detected in any plasma sample.

TABLE J5
Plasma Concentrations of Chloral Hydrate and Its Metabolites in 250 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate[a]

	15 Min	30 Min	1 Hour	2 Hours	3 Hours	6 Hours	24 Hours
***Ad Libitum*-Fed**							
n	4	6	5	6	6	6	6
Chloral hydrate	106 ± 32	21.0 ± 5.8	4.2 ± 1.2	4.2 ± 2.0	2.1 ± 1.8	0.0	0.0
Trichloroacetic acid	537 ± 83	1,268 ± 217	841 ± 452	317 ± 110	747 ± 207	266 ± 108	65 ± 27
Free trichloroethanol	48.4 ± 6.1	93 ± 25	8.2 ± 1.6	10.0 ± 2.5	5.9 ± 1.4	4.2 ± 0.8	4.2 ± 1.7
Trichloroethanol-glucuronide	83 ± 33	242 ± 54	28 ± 9	27 ± 14	12.8 ± 4.7	2.9 ± 29	0.1 ± 0.1
Dietary-Controlled							
n	4	6	5	6	6	6	6
Chloral hydrate	92 ± 16	22 ± 8	5.7 ± 1.6	5.5 ± 1.0	1.7 ± 1.4	0.0	0.0
Trichloroacetic acid	1,200 ± 336	1,496 ± 425	1,445 ± 807	785 ± 251	1,156 ± 109	271 ± 103	176 ± 59
Free trichloroethanol	87 ± 27	161 ± 84	20.1 ± 5.2	17.1 ± 3.6	8.1 ± 3.9	4.7 ± 0.4	4.3 ± 1.5
Trichloroethanol-glucuronide	41 ± 18	121 ± 62	26 ± 10.2	32.2 ± 9.2	12.9 ± 3.9	1.7 ± 1.1	0.6 ± 0.4
Calorically Restricted							
n	5	6	6	6	5	6	6
Chloral hydrate	100 ± 37	25.7 ± 9.0	6.4 ± 2.6	6.9 ± 1.8	0.0	0.0	0.0
Trichloroacetic acid	1,348 ± 514	1,876 ± 566	1,605 ± 765	866 ± 301	1,174 ± 419	267 ± 93	191 ± 73
Free trichloroethanol	60 ± 15	116 ± 26	14.4 ± 4.2	23.8 ± 7.5	4.4 ± 1.0	3.4 ± 0.5	3.3 ± 0.8
Trichloroethanol-glucuronide	90 ± 41	132 ± 29	111 ± 68	41 ± 12	7.5 ± 4.2	3.6 ± 2.4	2.0 ± 1.3

[a] Values are expressed as μM concentrations in plasma (mean ± standard error of the mean). Dichloroacetic acid was not detected in any plasma sample.

TABLE J6
Plasma Concentrations of Chloral Hydrate and Its Metabolites in 500 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate[a]

	15 Min	30 Min	1 Hour	2 Hours	3 Hours	6 Hours	24 Hours
***Ad Libitum*-Fed**							
n	5	5	5	2	5	4	4
Chloral hydrate	395 ± 139	177 ± 136	79 ± 71	1.6 ± 1.6	30 ± 21	0.0	0.0
Trichloroacetic acid	944 ± 401	1,985 ± 570	1,230 ± 433	820 ± 353	1,401 ± 337	514 ± 232	219 ± 97
Free trichloroethanol	108 ± 25	345 ± 119	80 ± 60	7.9 ± 1.3	88 ± 72	3.6 ± 0.6	3.6 ± 0.8
Trichloroethanol-glucuronide	91 ± 38	112 ± 72	191 ± 135	29 ± 17	128 ± 49	12.8 ± 5.8	0.2 ± 0.2
Dietary-Controlled							
n	4	4	5	3	5	3	5
Chloral hydrate	251 ± 178	54 ± 15	13 ± 3	15.5 ± 0.4	6.6 ± 2.2	0.0	0.0
Trichloroacetic acid	1,572 ± 508	2,133 ± 597	2,696± 1,367	1,241 ± 290	1,682 ± 410	482 ± 131	163 ± 63
Free trichloroethanol	116 ± 58	256 ± 64	32 ± 5.0	42.9 ± 6.3	12.9 ± 2.8	3.0 ± 1.3	3.4 ± 0.7
Trichloroethanol-glucuronide	67.3 ± 25	962 ± 900	152 ± 46	30 ± 13	58 ± 16	5.6 ± 5.6	0.0
Calorically Restricted							
n	3	4	5	3	5	3	5
Chloral hydrate	113 ± 53	98 ± 15	65 ± 50	36 ± 32	2.5 ± 1.9	0.0	0.5 ± 0.5
Trichloroacetic acid	1,528 ± 806	3,640± 1,806	2,771 ± 1,029	1,513 ± 213	1,413 ± 214	670 ± 163	249 ± 108
Free trichloroethanol	154 ± 41	351 ± 179	38 ± 10	78 ± 28	11.8 ± 5.3	3.0 ± 0.3	3.7 ± 1.0
Trichloroethanol-glucuronide	59 ± 31	349 ± 40	304 ± 179	42 ± 42	25 ± 15	7.7 ± 4.9	2.9 ± 1.8

[a] Values are expressed as μM concentrations in plasma (mean ± standard error of the mean). Dichloroacetic acid was not detected in any plasma sample.

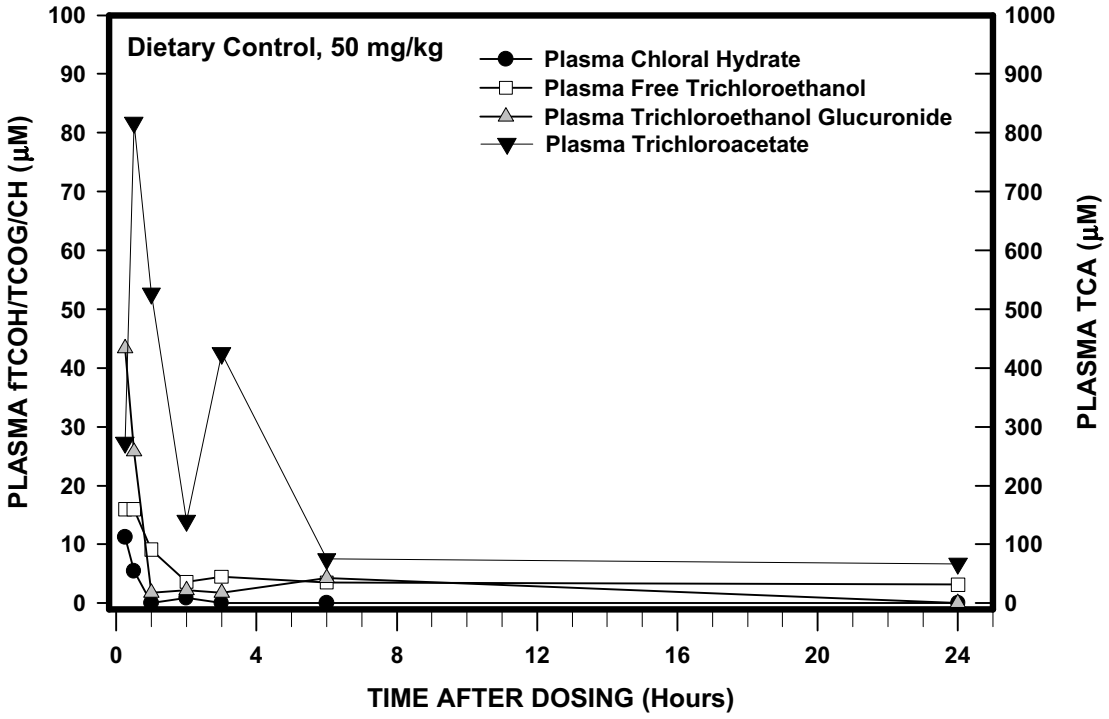


FIGURE J5
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Dietary-Controlled 50 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate

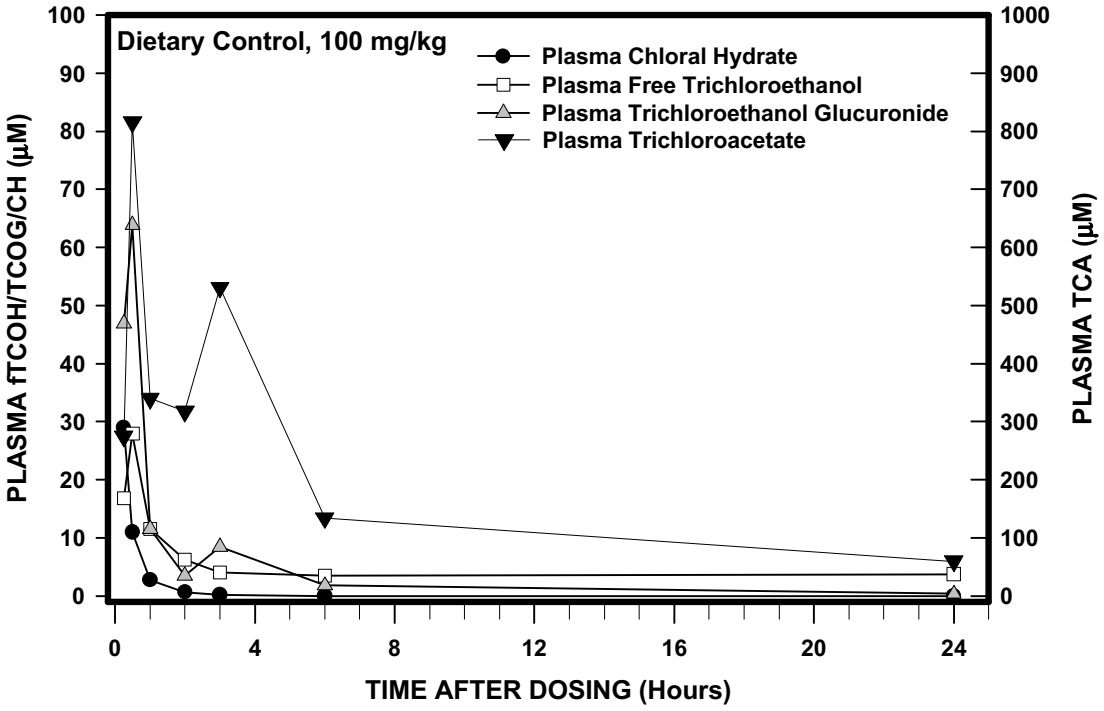


FIGURE J6
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Dietary-Controlled 100 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate

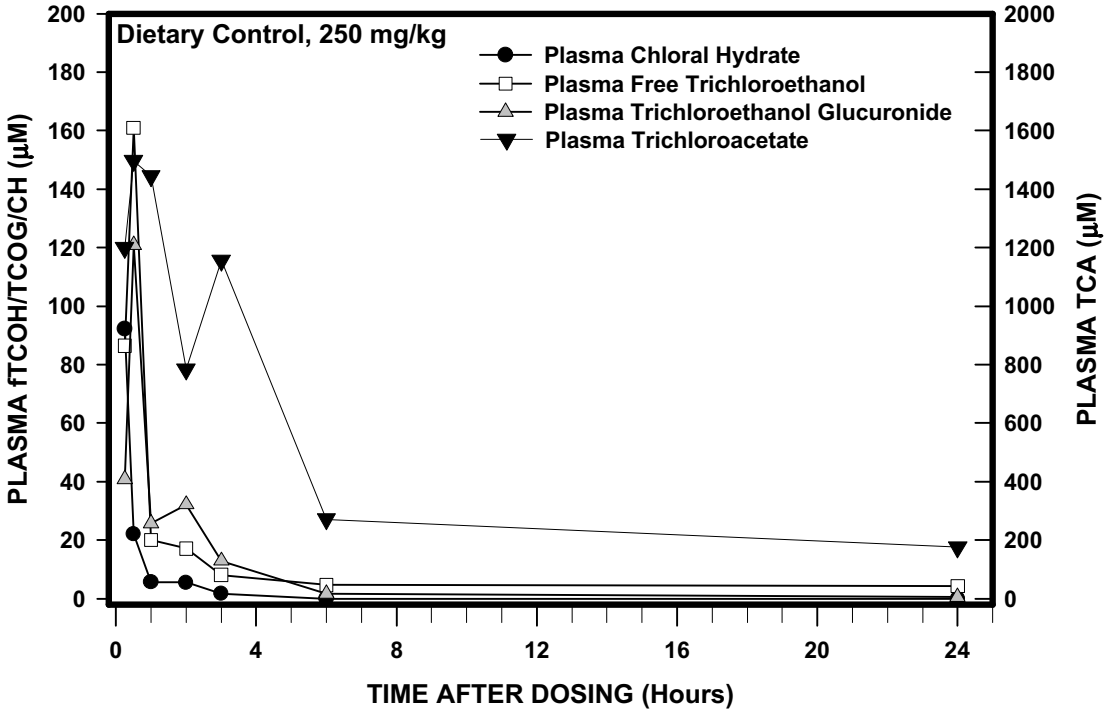


FIGURE J7
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Dietary-Controlled 250 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate

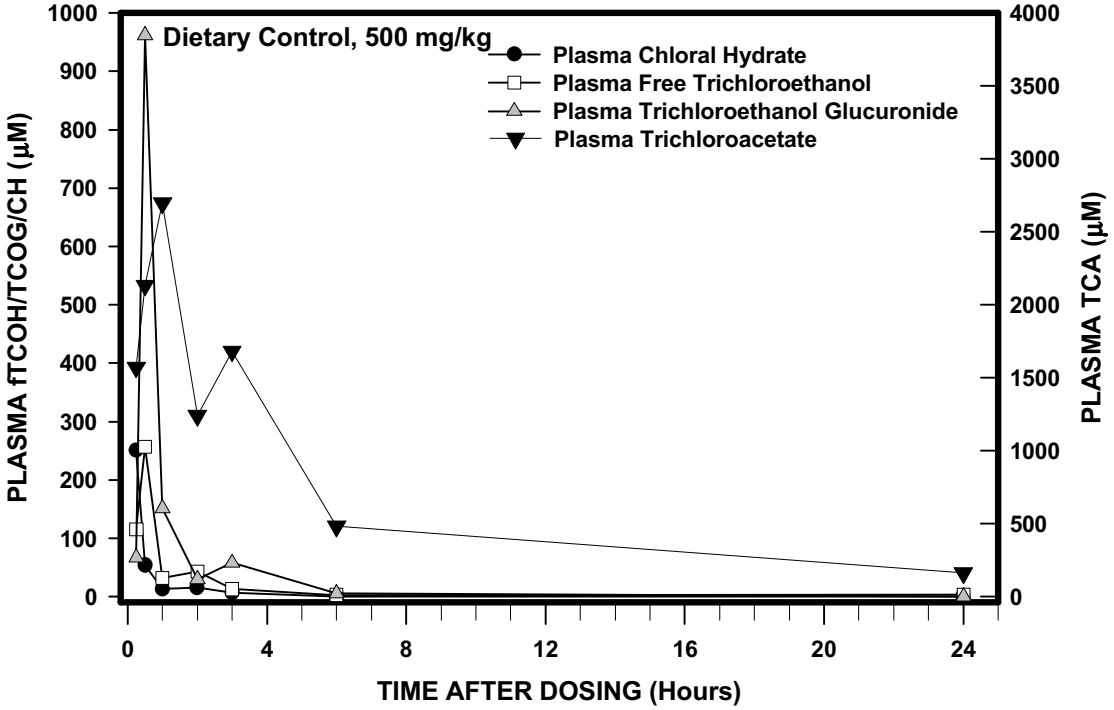


FIGURE J8
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Dietary-Controlled 500 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate

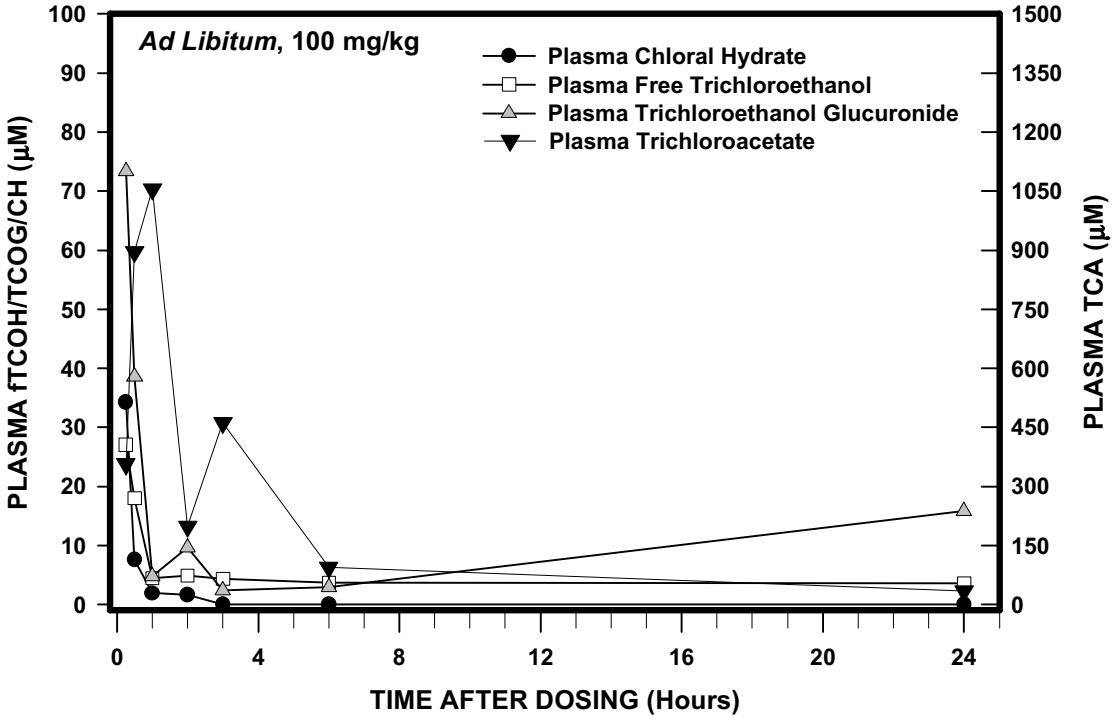


FIGURE J9
Plasma Concentrations of Chloral Hydrate and Its Metabolites in *Ad Libitum*-Fed 100 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate

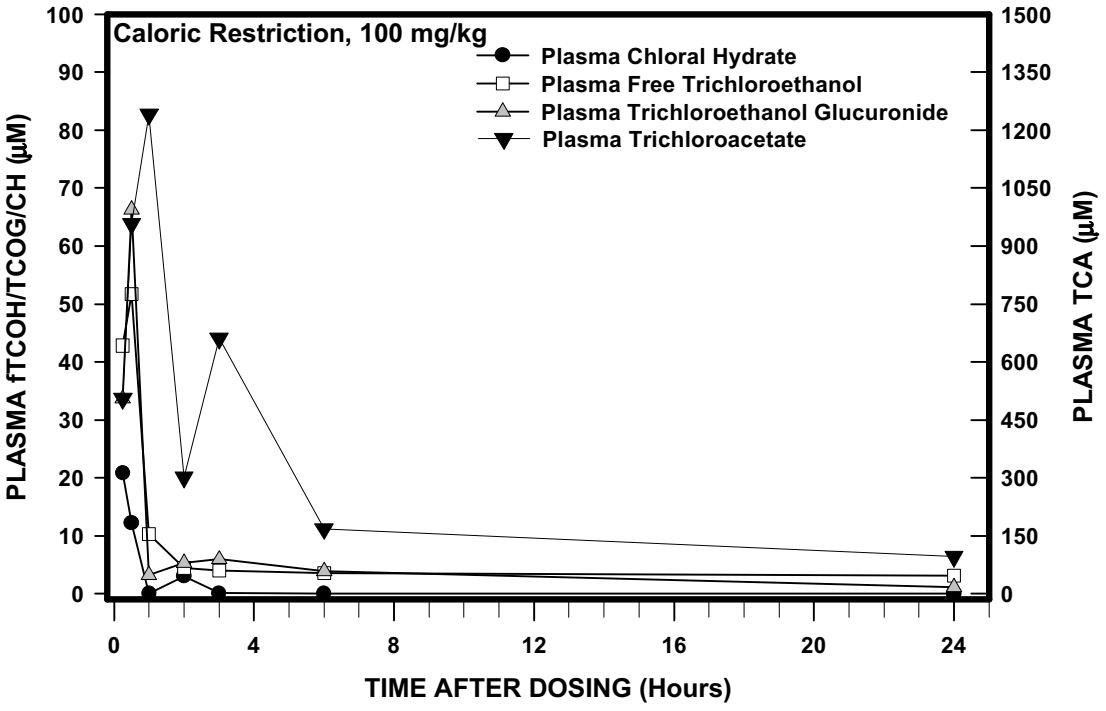


FIGURE J10
Plasma Concentrations of Chloral Hydrate and Its Metabolites in Calorically Restricted 100 mg/kg Male Mice in the Supplemental Study of Chloral Hydrate

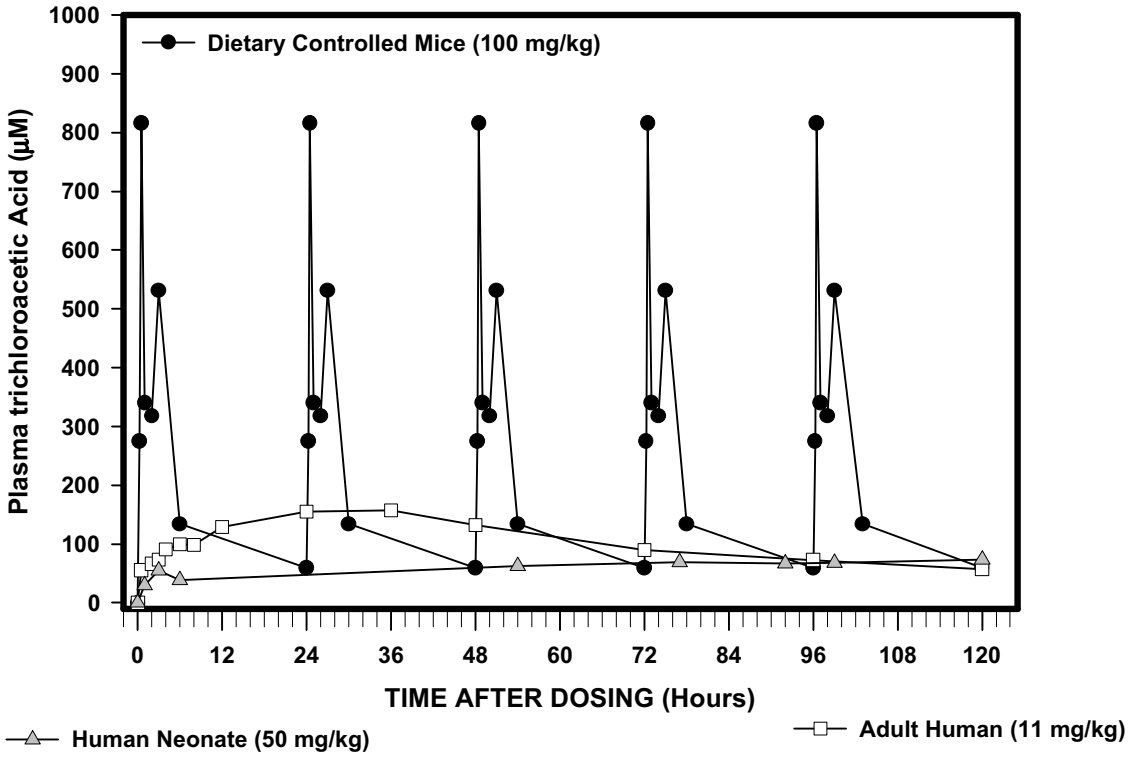


FIGURE J11
Comparison of Plasma Trichloroacetate Concentrations in Mice and Human Subjects Orally Administered Chloral Hydrate
The mouse data were extrapolated to five daily doses, assuming similar toxicokinetic profiles on each day. Human data were obtained from Gorecki *et al.* (1990). The AUC values are 20.0, 12.6, and 7.2 mM•hour for the mouse, human adult and neonate, respectively.

TABLE J7
Plasma Trichloroacetate Area-under-the-Curve Values for Mice Administered Chloral Hydrate by Gavage over 14 Days[a]

Dose (mg/kg)	*Ad Libitum*-Fed		Dietary-Controlled		Calorically Restricted	
50	2.67 ± 0.63	A	3.13 ± 0.77	A	3.08 ± 0.48	A
100	3.50 ± 1.04	A	4.00 ± 0.80	AB	5.66 ± 1.61	A
250	6.43 ± 1.86[b]	AB	9.07 ± 1.72	B	10.16 ± 2.21[b]	A
500	13.78 ± 3.35[b]	B	16.11 ± 2.18[c]	C	19.12 ± 3.51[b]	B

[a] The AUC values were calculated from plasma trichloroacetate profiles of individual mice by means of a SigmaPlot transform program. Values (n=6) are expressed as mM•hour (mean ± standard error of the mean). Statistical analysis was performed by an SAS GLM-Tukey's test. Each diet group was compared by dose, and each dose group was compared by diet. Means not sharing the same letter are significantly different from each other with respect to dose ($P<0.05$). There were no significant effects due to diet. n=6 unless otherwise noted.

[b] n=4

[c] n=5

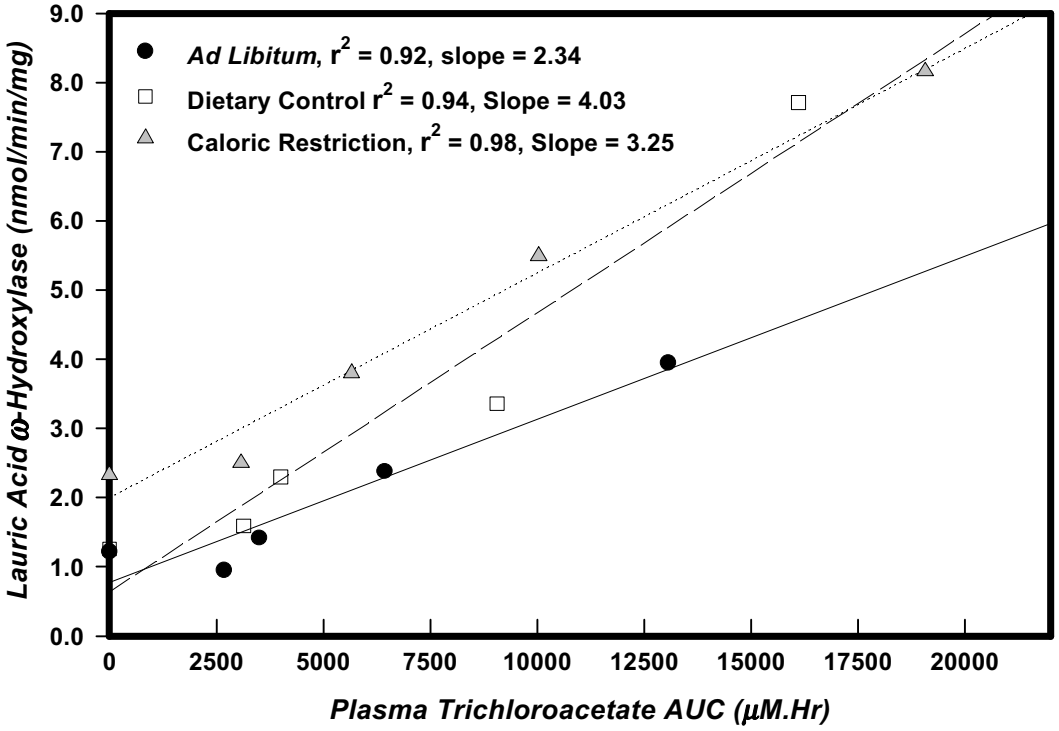


FIGURE J12
Relationship Between Plasma Trichloroacetate Concentrations and Hepatic Microsomal Lauric Acid ω-Hydroxylase Activity in Male Mice in the Supplemental Study of Chloral Hydrate
The AUC values from Table J7 were plotted against the corresponding lauric acid ω-hydroxylase activities from Figure J3. Although the trichloroacetate AUC values are higher in the 250 and 500 mg/kg dietary-controlled and calorically restricted groups than in the *ad libitum*-fed groups, it does not appear that the increased trichloroacetate can account for all of the increased induction response to chloral hydrate exhibited by lauric acid ω-hydroxylase in the dietary control or caloric restriction groups.